UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali

Dipartimento di Biotecnologie e Bioscienze

Dottorato in Biotecnologie Industriali XXIV ciclo

Glucose and osmotic stress-dependent calcium signalling in *Saccharomyces cerevisiae*: evidences for novel transporter systems and calcineurin involvement

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Anno accademico 2010/2011

Ringrazio il Professor Martegani per avermi dato la possibilità di lavorare nel suo laboratorio.

Ringrazio infinitamente Renata per avermi sempre seguito con disponibilità e attenzione.

Grazie Fiorella e Lory (mi mancherete…) per i preziosissimi consigli tecnici, ma soprattutto perché persone migliori in questo percorso non avrei potuto incontrare: avete reso speciale ogni giorno qui dentro!

Grazie Chiara per l'aiuto fondamentale in quest'ultimo anno, ma soprattutto per avermi sopportata! Non sai quanto sei stata preziosa per me e per la mia salute mentale…

Grazie Cindy e Marco, semplicemente perché non sarebbe stato lo stesso senza di voi e la vostra allegra compagnia.

Grazie a Michela e a tutto il resto del gruppo, Serena, Sonia, Francy, Francy per tutti i momenti piacevoli che mi avete regalato.

Infine grazie a tutte le persone che mi hanno accompagnato in questo percorso, a quelle che ci sono sempre state e sempre ci saranno, a quelle che sono arrivate ad un certo punto e anche a quelle che purtroppo ho perso per strada…

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Aim of the thesis

The major goal of this work is to understand function and regulation mechanisms of calcium transport systems in *Saccharomyces cerevisiae*, in response to nutrients and hypotonic shock.
Calcium represents, in fact, one of the most important second messengers in all eukaryotic cells, and particularly in budding yeast, where it plays essential roles in regulating many fundamental cellular processes, such as cell cycle, mating, sensing of glucose and glucose starvation, resistance to salt stress and cell survival.
Yeast cells actively maintain cytosolic free Ca^{2+} concentration at extremely low levels, in a range of 50-200 nM, through a finely regulated homeostasis maintaining mechanism.
Glucose addition to nutrient-deprived cells triggers a rapid and transient increase in cytosolic Ca^{2+} level, mainly due to an influx of calcium from the extracellular environment (Eilam *et al*., 1990). Conversely, hypotonic shock induces an increase in cytosolic Ca^{2+} level, mainly mediated by calcium release from intracellular stores (Batiza *et al*., 1996), though the intracellular transporters involved in this signalling are not yet identified.
Glucose-triggered calcium influx from extracellular environment is mediated by a high affinity calcium transporter (HACS), composed by Mid1p/Cch1p subunits, during growth in minimal medium, whereas in rich media cultured cells it seems to be mediated by a still unidentified transporter, named GIC (for Glucose Induced Calcium Channel).
By taking advantage of a bioluminescent assay *in vivo* allowing us to monitor cytosolic Ca^{2+} level changes, based on aequorin bioluminescent protein, the role of the known calcium channels and of the still unidentified putative transporters in glucose and hypotonic shock-dependent Ca^{2+} signalling, was here investigated.
In order to better characterize GIC and the unknown hypotonic shock-responsive intracellular transporters, a pharmacological approach was applied, testing their sensitivity to common blockers of mammalian voltage-gated calcium channels.
In addition, the effects of glucose-induced Ca^{2+} signalling on calcineurin, the major effector for intracellular calcium, were here investigated, demonstrating for the first time that calcineurin activation, normally recognized as being essential for survival under diverse stress conditions, can be also responsive to nutrients. The emergent role of calcineurin in regulating functionality of calcium transporters depending on nutrient

availability and the crosstalk between calcineurin pathway and nutrient sensing were also investigated in this work.

Introduction

The yeast Saccharomyces cerevisiae as model organism

The budding yeast *Saccharomyces cerevisiae* is one of the most widely studied eukaryotic model organisms in molecular and cellular biology. It is classified as a GRAS organism (Generally Regarded As Safe), since it has been extensively used for many years in food industry, i. e. for the production of beer and wine, as well as in pharmaceutical industry for the production of recombinant proteins.

The genome of the budding yeast had been completely sequenced in 1996 through a worldwide collaboration. The sequence of 12068 kilobases, organized in 16 linear chromosomes, defines 5885 potential protein-encoding genes, approximately 140 genes specifying ribosomal RNA, 40 genes for small nuclear RNA molecules and 275 transfer RNA genes (Goffeau *et al.,* 1996). The whole yeast genome sequence is contained in the *Saccharomyces* Genome Database (SGD), that provides both basic information about genes and their products, and tools such as sequence similarity searching (Cherry *et al*., 1998).

The position of *S. cerevisiae* as a model eukaryote owes much to its intrinsic advantages as an experimental system. It is a unicellular organism that (unlike many more complex eukaryotes) can be grown on defined media, which gives the experimenter complete control over its chemical and physical environment. It can grow both on rich media, containing amino acids, nucleotidic precursors, vitamins and other metabolites normally synthesized *ex novo* by cells themselves, and on minimal media, containing only the essential elements for its growth. Culturing yeast is simple, economical and rapid, being characterized by a doubling time of about 90 minutes in rich media.

Yeast can exist stable in either haploid or diploid state. Haploid cells can be of two different mating types, *a* and α, and can conjugate generating a diploid cell. Diploid a/α cells can replicate indefinitely through mitosis, while under carbon and nitrogen sources starvation, can undergo meiosis generating four haploid spores that can be isolated and individually analysed.

The availability of many simple molecular biology techniques to manipulate budding yeast allows to perform classical mutation analysis in order to investigate genes function. In contrast to many other eukaryotic organisms,

exogenous DNA integration proceeds exclusively through homologous recombination in yeast. These aspects have led to development of many techniques to replace wild-type genes with mutated alleles, in order to study the function of many genes *in vivo.*
All these aspects confer a great applicability of *S. cerevisiae* within different fields of biotechnology.

Saccharomyces cerevisiae cell cycle

The eukaryotic cell cycle is the repeated sequence of events that enable the division of a cell into two daughter cells each containing the information and machinery necessary to repeat the process. It is divided into four phases G_1, S, G_2 and M, and another phase named G_0 can also exist, representing a quiescent state with a seriously impaired metabolic activity that cell undergoes in adverse environmental conditions such as nutrients starvation (Fig. 1).
The cell cycle of *S. cerevisiae* is controlled mainly in G_1 phase at a point called *Start*. At *Start* environmental signals such as nutrients availability are monitored: the cell at this point "decides" if to proceed in the cell cycle or if to block it by entering in G_0 quiescent phase or by conjugating (Pringle and Hartwell, 1981). *Start* point passing is an irreversible process: once cell cycle is began, it has to be concluded until the next G_1 phase.

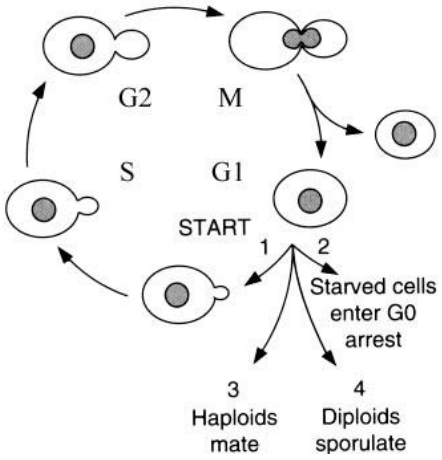


Figure 1. The *Saccharomyces cerevisiae* cell cycle.

The progression through the cell cycle is controlled at the molecular level by cyclin-dependent kinases (Cdks). Cdks are serine and threonine kinases whose action depends on association with their activating subunits, cyclins. Cyclin abundance is regulated by protein synthesis and degradation; the activity of Cdks is therefore regulated to a large degree by the presence of different cyclins (Bloom and Cross, 2007).
In *S. cerevisiae* a single Cdk, Cdc28, associates to multiple cyclins to regulate the cell cycle. Nine different cyclins associate with Cdc28 and thereby confer stage-specific functions: six different B-type cyclins (*CLB1-6*) are involved in different aspects of S phase and mitosis, and three G_1-specific cyclins (*CLN1-3*) are necessary for *Start* (Fig. 2). Mechanisms that contribute to cyclins specificity include the differential transcriptional activation of cyclins, the degradation of cyclins, the association of cyclin-Cdk complexes with different Cdk inhibitors, the localization of cyclins and the inhibitory phosphorylation of Cdk (Dirick *et al*., 1995).

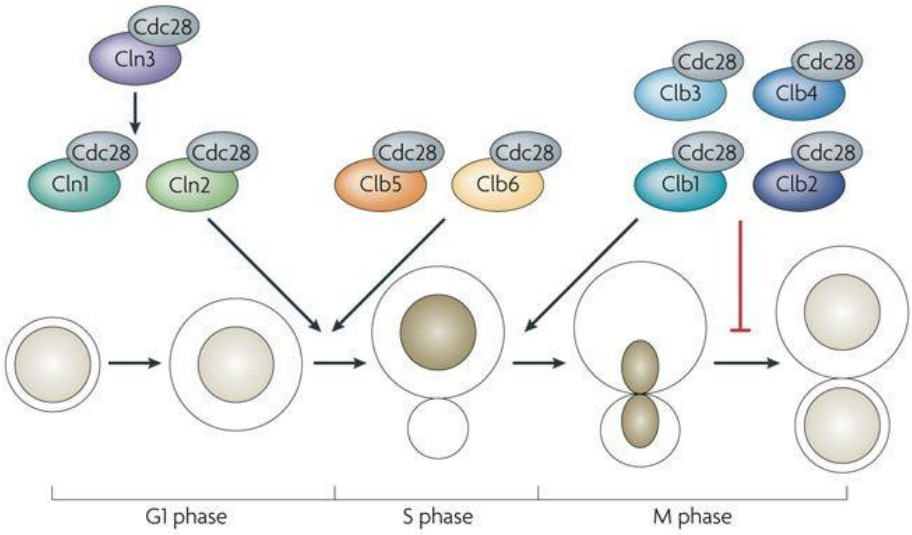


Figure 2. Cyclins in the budding yeast cell cycle (Bloom and Cross, 2007).

In order to maintain a constant size during cell proliferation, cells need to grow and double in mass during each round of the cell cycle. The coordination of mass accumulation with cell cycle progression relies on a size mechanism, so that DNA replication and/or cell division start only when cells have reached a critical cell size (Wells, 2002). In this way, tiny newborn cells will have to grow more than mother cells before being able to

overcome the cell size checkpoint; conversely, a larger cell will overcome the cell size checkpoint earlier than the "normal, average" cell. As a result, both small and large cells will stabilize cell size to the "normal, average" value. The critical cell size required for the G_1/S transition in budding yeast is usually indicated as P_s, which represents the protein content per cell at the onset of DNA replication. Ploidy, nutrients and growth rate modulate P_s, that is smaller in slow-growing cells than in fast-growing cells (Barberis *et al*., 2007).
G_1 phase *CLN3* cyclin plays an essential roles in P_s regulation. Cln3-Cdc28 complex promotes the downstream cell cycle regulators *CLN1*,*2* and *CLB 5,6* transcription through the activation of SBF and MBF transcription factors. Cln3p concentration is low if compared with other cyclins and remains roughly constant during G_1 phase. Cln3p levels are modulated by growth conditions being higher in fast-growing cells and lower in slow-growing cells, and its increase during G_1 phase seems to reflect the increase in cellular mass: experimental data suggest that Cln3p amount increase promotes S phase entry, considering that Cln3p level alteration affects P_s (Tyers *et al*., 1993).

Glucose signalling in Saccharomyces cerevisiae

In the budding yeast glucose not only serves as a carbon and energy source, but it also has a strong regulatory effect on a range of physiological properties. Actually, addition of glucose to yeast cells grown on other carbon sources triggers a wide variety of processes directed towards the sole and optimal utilization of this sugar and affects other seemingly unrelated properties such as stress resistance and the level of storage carbohydrates. Furthermore glucose influx and the flow through glycolysis are stimulated, while gluconeogenesis is inhibited; there is also a drastic increase in growth rate which is preceded by a characteristic upshift in ribosomal RNA and protein synthesis (Rolland *et al*., 2000).
Glucose-uptake capacity is increased through the induction of several glucose-transporter-encoding *HXT* genes, whose products are responsible for the glucose uptake by the cell from the extracellular environment.
S. cerevisiae contains a whole series of hexose transporters homologues (Hxt 1-17p, Gal2p, Snf3p and Rgt2p), all displaying different substrate affinities and expression patterns. Depending on the amount of glucose present in the medium, specific transporters are expressed: high affinity transporters like Hxt6p and Hxt7p are highly expressed on non-fermentable carbon sources and repressed by high levels of glucose, whereas low

affinity transporters such as Hxt1p and Hxt3p are induced by the presence of a high glucose concentration. The transporters with intermediate affinity for glucose like Hxt2p and Hxt4p, on the other hand, are induced by low levels of glucose and repressed by high levels of glucose (Rolland *et al*., 2002).

Two transporter homologues, Rgt2p and Snf3p, have been proposed to function as sensors of extracellular glucose for regulation of *HXT* genes expression. Rgt2p and Snf3p are two unusual members of the hexose transporter family, having only limited sequence similarity to the other hexose transporter homologues differing especially in the long C-terminal cytoplasmic tails. *RGT2* encodes a putative glucose transporter that is required for maximal expression of the high glucose-induced *HXT1* gene, while *SNF3* is required for low glucose-induced expression of *HXT2* and *HXT4*, suggesting that they play regulatory roles in glucose-induced gene expression (Fig. 3). Actually a dominant mutation in *RGT2* was identified that causes constitutive glucose-independent expression of the *HXT1* gene and, when introduced in *SNF3*, this mutation causes similar effects resulting in constitutive expression of *HXT2*. Furthermore, *RGT2* is expressed constitutively at a low level while *SNF3* is glucose-repressed: these data suggest that Rgt2p senses high extracellular glucose concentrations, while Snf3p senses low glucose concentrations.

Rgt2p and Snf3p however can't act as glucose transporters: *RGT2* or *SNF3* overexpression in *hxt* mutant strains isn't able to restore their ability to grow on glucose; on the other hand *HXT* genes overexpression in *snf3Δ* and *rgt2Δ* mutant strains, though restoring their ability to grow on glucose, can't restore glucose signalling, indicating that these mutant strains are not only impaired in glucose transport (Özcan *et al*, 1996 and 1998).

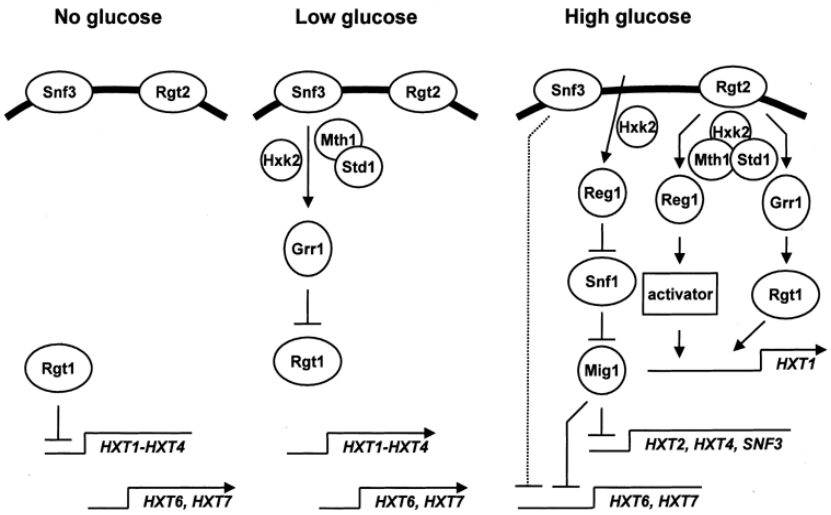


Figure 3. Regulation of *HXT* transporter genes expression in response to glucose (Rolland *et al*., 2002).

Ras/cAMP/PKA pathway

Signal transduction pathways that regulate growth in response to nutrients are essential for cell viability. In budding yeast, one aspect of nutrient-induced growth control is mediated by the Ras/cAMP/PKA pathway.

In *S. cerevisiae* there are two genes, *RAS1* and *RAS2*, encoding for two small monomeric G-proteins that switch from an inactive form, bound to GDP, to an active form, bound to GTP. The activation of Ras proteins requires GDP replacement with GTP and is regulated by guanine nucleotide exchange factors (GEFs), encoded by *CDC25* and *SDC25* in budding yeast; while the switch from the active to the inactive form involves hydrolysis of bound GTP by the intrinsic GTPase activity of Ras proteins and is stimulated by GTPase-activating proteins (GAPs), encoded by *IRA1* and *IRA2* in budding yeast (Santangelo, 2006).

Ras proteins activation in turns stimulates production of cAMP by the essential product of the adenylate cyclase gene *CYR1*. cAMP is one of the most important second messengers in yeast and other eukaryotic cells, activating the cAMP-dependent protein kinase A (PKA) by binding to its regulatory subunits (encoded by *BCY1*), thereby releasing and activating the catalytic protein kinase subunits (encoded by *TPK1*, *TPK2* and *TPK3*). In derepressed yeast cells, growing on non-fermentable carbon source or in stationary phase, glucose addition triggers a rapid and transient increase in

the cAMP level, initiating a PKA phosphorylation cascade (Rolland *et al.*, 2002).
The transient cAMP signal has been proposed to trigger a fast resetting from gluconeogenic to fermentative metabolism; actually, PKA is involved in post-translational regulation of a variety of proteins, for instance of key enzymes of gluconeogenesis and glycolysis. For example the gluconeogenic enzyme, fructose-1,6-bisphosphatase, is rapidly inactivated after glucose-induced activation of PKA, whereas phosphofructokinase 2, an important regulator of the major glycolytic enzyme phosphofructokinase 1, is rapidly activated. Also, neutral trehalase and glycogen phosphorylase are activated upon glucose addition, resulting in mobilization of threalose and glycogen.
In addition, PKA exerts control at the transcriptional level by repressing STRE-controlled genes and inducing ribosomal protein genes (Rolland *et al.*, 2000).
The cAMP pathway is essential for viability in budding yeast and depletion of cAMP causes a response similar to nutrient starvation: the cells arrest at *Start* in the G_1 phase of the cell cycle and accumulate permanently in the stationary phase G_0. In addition, mutants with an overactive cAMP pathway show a major sensitivity to heat shock and nutrient starvation, low levels of the storage carbohydrates glycogen and threalose, failure to arrest properly in G_1 phase of the cell cycle upon nutrient limitation, poor growth on non-fermentable and weakly fermentable carbon sources and failure to sporulate in diploid cells. Conversely, reduced activity of the pathway causes enhanced heat resistance, enhanced levels of glycogen and threalose, constitutive expression of heat shock genes and other genes which are only expressed in stationary phase in wild-type cells and in diploid cells causes sporulation in rich nutrient media (Colombo *et al.*, 1998).
PKA activity is counteracted by phosphodiesterase activity, encoded by *PDE1* and *PDE2* genes, respectively with a low and high affinity for cAMP, that downregulate Ras signalling by hydrolyzing cAMP. In addition, a very potent system down-regulating cAMP levels in yeast is feedback inhibition of cAMP synthesis by PKA.

GPCR complex role in glucose signalling

In addition to Ras proteins, the heterotrimeric G proteins are also key regulators of growth and development in eukaryotic cells. In *S. cerevisiae*, two distinct G protein α subunits (G_α) genes (*GPA1* and *GPA2*) have been

found: *GPA1* is involved in the negative regulation of the mitogen-activated protein kinase (MAPK) pathway induced by pheromone stimulation; *GPA2* is involved in cAMP regulation, considering that its overexpression results in an increase in glucose-induced cAMP level and that Gpa2p is required for glucose-induced cAMP synthesis.

Gpa2p and Ras2p seem to have partially redundant functions in cAMP pathway (Fig. 4), acting in parallel, and cells with defects in both pathways are impaired for growth. *GPA2* overexpression suppressed the growth defect phenotype of a temperature-sensitive *ras2* mutant strain, even if glucose-dependent cAMP signal was not affected in a *gpa2* mutant strain; furthermore the *gpa2 ras2* double mutant strain showed a severe growth defect even in a nutrient-rich medium (Tamaki, 2007). However, the constitutive *GPA2* allele did not suppress the inviability of a *ras1Δ ras2Δ* strain, confirming that Gpa2p and Ras pathways act independently.

Screening a yeast genomic library, using Gpa2p as a bait, resulted in the isolation of plasmids containing short fragments of *GPR1*. The full-lenght *GPR1* encodes a protein of 961 amino acids containing seven membrane-spanning domains, a feature typical of G protein-coupled receptors superfamily. The putative structure of this protein indicates that it would contain a very large third cytoplasmic loop of about 346 amino acids, and a large cytoplasmic tail of about 281 amino acids responsible for the interaction with Gpa2p (Xue *et al*., 1998).

Furthermore a mutant allele of *GPR1* was independently isolated in a screening for mutants that are specifically deficient in the rapids loss of high stress resistance in stationary-phase cells upon initiation of fermentation. This identified mutant was deficient in glucose-induced, but not in intracellular acidification-induced, stimulation of cAMP synthesis (Kraakman *et al*., 1999).

By studying *gpr1* and *gpa2* mutant strains, the functional interaction between these two components of GPCR system was investigated, revealing a role in glucose-sensing for activation of the cAMP pathway.

Glucose-triggered cAMP signal was largely absent in a *gpr1* mutant strain; the *gpr1Δ gpa2Δ* double mutant behaved as the single *gpr1Δ* and *gpa2Δ* strains, indicating that both proteins act in the same pathway. Moreover, the lack of cAMP signal in a *gpr1Δ* strain was rescued by the constitutively activated $GPA2^{val132}$ allele.

As was found for the *gpa2 ras2* double mutant strains, also *gpr1 ras2* mutant strains show severe growth defects confirming that GPCR system regulates cAMP level in response to glucose in a pathway parallel to the Ras signalling pathway.

In addition to GPCR system, glucose activation of cAMP synthesis also requires uptake and phosphorylation of the sugar. In *hxt*-null strains, inactivated in all *HXT* glucose transporters activity, addition of glucose to cells growing on non-fermentable carbon sources doesn't trigger significant changes in cAMP level; furthermore this cAMP signal can be restored by the constitutive expression of the galactose permease, able to transport also glucose with high affinity kinetics. These data suggest that the extracellular glucose detection process is an essential requirement for glucose-induced cAMP signal, but that glucose transporters do not seem to have a regulatory function but are only required for glucose uptake.

Gpr1 is responsible for glucose sensing, displaying a very low affinity for this sugar (apparent K_m = 75 mM) and responds specifically to extracellular α and β D-glucose and sucrose, but not to fructose, mannose or any glucose analogues.

cAMP signal induced by low glucose concentrations isn't affected by *GPR1* or *GPA2* deletion. Pre-addition of low glucose concentrations (5 mM) seriously affects consequent high glucose-induced cAMP signal in *gpr1*Δ or *gpa2*Δ strains, actually such low glucose concentration isn't able to activate GPCR complex but stimulates glucose phosphorylation by hexokinases.

In fact, another essential requirement for glucose-induced cAMP synthesis is glucose phopshorylation by hexose kinases, encoded by *HXK1*, *HXK2* and *GLK1* in yeast: in mutant strains, impaired in hexokinase activity, cAMP signal in response to low glucose concentrations is entirely eliminated (Rolland *et al*., 2000).

Glucose-induced cAMP signal seems to be mainly mediated by GPCR system, whose inactivation confers typical features normally associated with a reduced cAMP level in the cell.

Further studies suggest that GPCR complex regulates growth by maintaining the cell size. It is known that cAMP pathway regulates cell size, considering that mutant strains with reduced activity in this pathway are small in size while its hyperactivation results in an increased cell mass. The *gpr1* and *gpa2* mutant strains show smaller size than the wild-type strain during growth on glucose and the protein synthesis rate is reduced, resulting in the reduced growth rate. GPCR complex components are also required for a rapid increase in cell size in response to glucose: when glucose is added to cells growing on non-fermentable carbon sources, wild-type cells quickly increase in size, whereas little change is observed in the *gpr1* and *gpa2* mutant strains (Tamaki *et al*., 2005).

GPCR system represents one of the most important mechanisms by which yeast cells sense extracellular stimuli and convert them into intracellular

signals. Nutrient signalling pathways involving GPCR complex are conserved in a wide variety of eukaryotic organisms.

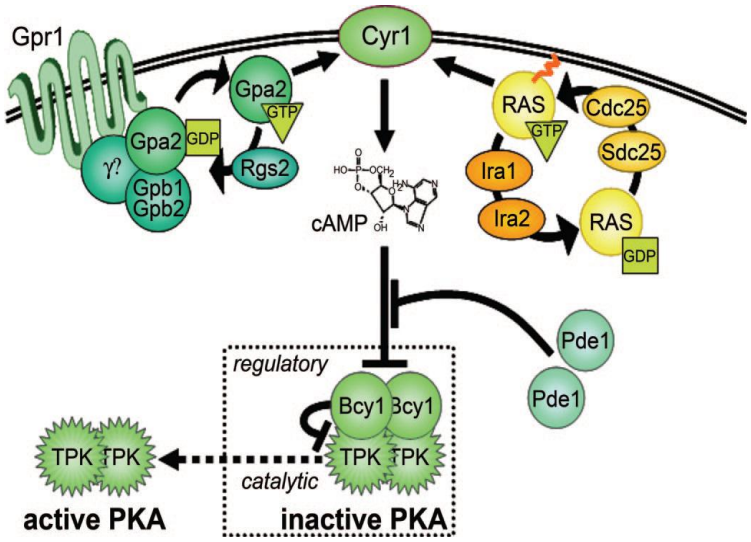


Figure 4. Cytoplasmic events in PKA signalling (Santangelo, 2006).

The phosphatidylinositols pathway

Phosphoinositide-specific phospholipase C (PLC) isozymes found in eukaryotic cells comprise a related group of proteins that cleave the polar head group from inositol phospholipids. In mammalian cells this pathway has been largely investigated: it regulates several cellular processes such as cellular proliferation, neuronal signalling, hormones secretion and smooth muscle contraction in response to many extracellular stimuli as neurotransmitters, hormones or growth factors.
PLC cleave phosphatidylinositol 4,5-bisphosphate (PIP_2), a minor membrane phospholipid, in response to the binding of various ligands to their cell surface receptors, generating two different second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 is released as a soluble structure into the cytosol and it diffuses to bind IP_3 receptors, mainly located on endoplasmic reticulum (ER) membranes: this signal is responsible from calcium release from ER stores increasing cytosolic

calcium concentration and causing a cascade of intracellular changes and activity. In addition, calcium signal and the membrane-resident product DAG together, are responsible for the activation of protein kinase C (PKC), which is involved in different cellular processes as MAPK pathway activation. Furthermore, PLC substrate PIP_2 itself is an important signal in the cell modulating the activity of a number of actin-binding proteins and as a site needed for the membrane attachment of many signalling proteins (Lyu *et al.*, 1996).

The need to stringently regulate the cellular concentration of PIP_2 and its conversion to DAG and IP_3 is reflected in part by the fact that there are at least ten distinct mammalian PLC isoforms and multiple mechanisms linking these subtypes to various receptors. The 10 known mammalian PLC isozymes can be divided into three subtypes, β, γ and δ, differing in their structural organization, in size and in mechanisms of their receptor-mediated activation.

Sequence homology among the three isotypes is limited to two domains, designed X and Y (of ~ 150 and 160 amino acids respectively), which are thought to constitute the catalytic core of each isozyme. A pleckstrin homology (PH) domain is located in the NH_2-terminal region, preceding the X domain, in all three types of PLC. Whereas PLC-β and PLC-δ isozymes contain a short sequence of 50-70 amino acids that separates X and Y domains, PLC-γ isozyme have a long sequence of ~ 400 amino acids that contains Src homology (SH) domains. PLC-γ isozymes also contain an additional PH domain that is split by the SH domains.

PH and SH domains are protein modules shared by many signalling proteins; whereas PH domains mediate interaction with the membrane surface by binding to PIP_2, SH domains mediate interaction with other proteins by binding to phosphorylated tyrosine residues (SH2) or proline-rich sequences (SH3).

The three-dimensional structure of PLC isozymes also revealed two accessory modules: an EF-hand domain which would serve as a flexible link between the PH domain and the rest of the enzyme, that binds Ca^{2+} ions, and a C2 domain which would mediate the Ca^{2+}-dependent binding to lipid vesicles (Fig. 5). The non-conserved domains of each isoform confer its mode of regulation and, in some cases, its subcellular location.

All eukaryotic PLC isozymes require calcium for activity: Ca^{2+} ions are required both for the function of C2 domain and for the catalysis being located at the active site (Rebecchi and Pentyala, 2000; Rhee and Bae, 1997).

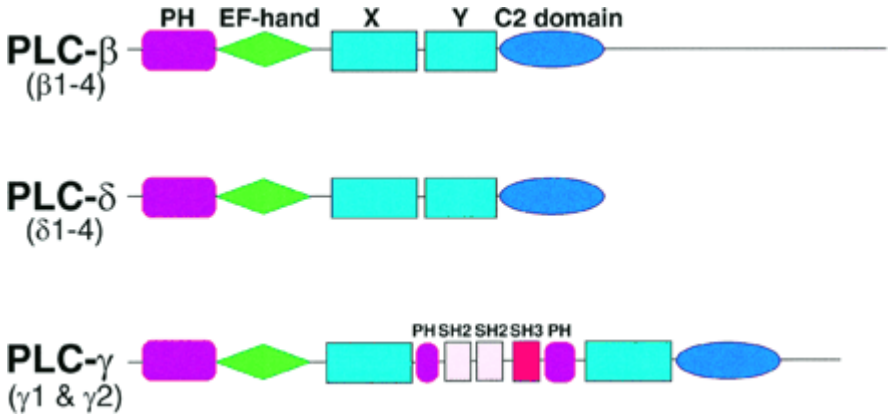


Figure 5. Linear representation of the various domains identified in the three types of PLC isozymes (Rhee *et al*., 1997).

There are many human diseases and disorders related to PLC isozymes mutations; the mice homozygous mutants for PLC-β1 or PLC-β4 are born normal but subsequently manifest postnatal dwarfism, the PLC-β4 mutants also show a defect in motor coordination and aberrant cerebral development, PLC-γ1 mutation is lethal at early mid-gestation. Finally, platelets from a patient with a mild inherited bleeding disorder as well as abnormal platelets aggregation and secretion were shown to have one-third the amount of PLC-β2 compared with normal platelets.

The phosphoinositide-specific phospholipase C in Saccharomyces cerevisiae

The budding yeast contains a single phospholipase C gene (*PLC1*) encoding a δ type PIP_2-specific enzyme. The *PLC1* gene, identified by several research groups through different approaches, encodes for a 869 amino acids polypeptide with a molecular mass of ~ 100.6 kDa. Sequence identity between the yeast protein Plc1p and the PI-PLCs from other species is 43 to 53% in the X domain and 18 to 29% in the Y domain. Yeast Plc1p seems to be most closely related to the δ isoforms in several aspects: the X and Y domains, situated near the C terminus rather than internally as in the β and γ isotypes, display higher sequence identity with the δ isoforms than with the other subclasses; furthermore the overall size of Plc1p is more similar to that of the δ isotypes (~ 760 amino acids) than to those of the much larger

β (~ 1220 amino acids) and γ (~ 1300 amino acids). Finally the amino-terminal region of Plc1p has 21% sequence identity with the corresponding amino-terminal segment of the rat and bovine δ isotypes, and also contains a EF-hand like Ca^{2+}-binding motif. Differently from mammalian PI-PLCs, yeast Plc1p contains an additional amino-terminal extension and is predicted to be an extremely basic protein revealing any potential membrane-spanning segments (Flick and Torner, 1993).

Though *PLC1* is not an essential gene in *Saccharomyces cerevisiae*, cells carrying a null allele display severe growth defects depending on the strain background and growth medium. *plc1Δ* cells observed by microscopy showed no morphological aberration and their growth was not arrested at any specific phase of the cell cycle suggesting that *PLC1* may be required during various stages of the cell cycle (Yoko-o *et al*., 1993). However, under ideal conditions yeast cells with *PLC1* disrupted grow about three times slower than wild-type cells in liquid cultures at 30°C; furthermore, these mutant cells are temperature-sensitive being completely unable to grow at 37°C, exhibit aberrant chromosome segregation and a major sensitivity to hypertonic stress. Both growth and chromosome segregation defects can be suppressed by calcium addition to cultural medium (Payne and Fitzgerald-Hayes, 1993).

Like the loss of *PLC1*, also its overexpression results in growth defects that are more pronounced at higher growth temperature, suggesting that an imbalance in Plc1p activity perturbs cell function.

It is likely that Plc1p might be involved in nutrient-induced signal transduction in yeast, considering that *plc1* mutant strains are defective in the utilization of non-fermentable carbon sources. By performing a two-hybrid screen with Plc1p as a bait, a physical interaction with Gpr1 and Gpa2 components of GPCR complex was found. In particular it has been demonstrated that both Plc1p and Gpa2p interact strongly with the two carboxyl-terminal Gpr1p regions and, to a much lesser extent, with the third intracellular loop; furthermore there is also a weak but significant interaction between Plc1p and Gpa2p. It turned out that the Gpr1p/Plc1p complex is formed either in the presence or absence of Gpa2p, and at the same way Plc1p/Gpa2p complex is formed either in the presence or absence of Gpr1p, whereas Gpa2p associates with Gpr1p only in the presence of Plc1p but not in its absence. Plc1p seems to act in the same pathway as Gpr1p and Gpa2p, since *plc1Δ gpa2Δ* and *plc1Δ gpr1Δ* double mutants show the growth phenotype of *plc1Δ,* but in parallel with the Ras2p-controlled pathway, considering that *plc1Δ ras2Δ* double mutants show a synthetic growth defect (Ansari *et al*., 1999).

In addition, a further evidence that Plc1p acts together with GPCR complex, in a pathway separated from Ras1p/Ras2p and converging on PKA, has emerged from a genome-wide expression analysis: it was found that Plc1p contributes to the regulation at transcriptional level of approximately 2% of yeast genes in cells grown in rich media, and this set of genes showed a strong correlation with genes controlled by Msn2/Msn4 transcription factors, which are in turn negatively regulated by PKA. PKA directly phosphorylates Msn2p/Msn4p, keeping them into the cytosol. Actually, in *plc1Δ* cells, the increased expression of stress-responsive genes seems to be mediated by decreased PKA-mediated phosphorylation of Msn2p and increased binding of Msn2p to stress-responsive promoters. In addition, *plc1Δ* cells display other phenotypes characteristic of cells with decreased PKA activity, such as a major accumulation of glycogen than a wild-type strain, and also a decreased basal and glucose-induced cAMP level (Demczuk *et al*., 2008).
Thus, these findings together suggest that Plc1p acts together with Gpr1p and Gpa2p in a pathway separated from the Ras1p/Ras2p pathway and that the two pathways converge on cAMP synthesis and PKA activity. However, whether the catalytic activity of Plc1p is required for normal regulation of cAMP synthesis and PKA signalling, or whether Plc1p serves only as a scaffold protein that mediates the interaction between Gpr1p and Gpa2p independently of its catalytic activity, remains to be elucidated.

Phospholipase C involvement in glucose-induced calcium signalling

It is still in debate if yeast Plc1p is involved in any signal transduction system like the phosphatidylinositols pathway in mammalian cells. Yeast lacks any genes resembling IP_3-activated calcium channels, suggesting that probably IP_3 produced by Plc1p could regulate cellular Ca^{2+} homeostasis in a different manner.
It is known that ammonium sulphate addition to nitrogen-depleted yeast cells resulted in a transient increase in IP_3, but surprisingly it did not trigger any increase in intracellular calcium concentration. In addition similar IP_3 signals were also observed in wild-type cells treated with the phospholipase C inhibitor 3-nitrocumarin and in the cells deleted in *PLC1* gene. These findings suggest that IP_3 can also be generated independently from Plc1p-dependent PIP_2 cleavage (Bergsma *et al*., 2001).
However, it has been largely demonstrated that Plc1p is required for glucose-dependent calcium signalling in budding yeast. It is known that glucose addition to nutrient-starved cells induces an increase in free

intracellular calcium concentration, mainly due to an influx from extracellular environment. This signal, as previously described for glucose-induced cAMP signal, requires GPCR complex and glucose hexokinases activity. Glucose-induced Ca^{2+} peak was completely abolished in *plc1Δ* strains and in the isogenic wild-type strain treated with 3-nitrocumarin, suggesting that Plc1p is essential for glucose-induced calcium increase. Plc1p however seems to act downstream GPCR complex, considering that in *gpa2Δ* and *gpr1Δ* strains low glucose-induced Ca^{2+} signal isn't abolished whereas it is totally absent in the *plc1Δ* strain (Tisi *et al.*, 2002).

Anyway, a general requirement of Plc1p for calcium channel activity is excluded, but Plc1p involvement seems to be very specific for glucose-induced calcium flux. Actually, it is known that hypotonic shock also induces an increase in intracellular calcium concentration in budding yeast, but this signal is completely independent from Plc1p activity.

Differing from mammalian cells, calcium accumulation in yeast is most likely due to calcium influx from the external medium; furthermore no homologues of the mammalian IP_3 receptors have been found in the yeast genome raising the question whether IP_3 was involved in Plc1p-dependent calcium signalling.

In budding yeast cells two inositol polyphosphate kinases (Arg82/ ArgRIII/ Ipk2p and Ipk1p) rapidly phosphorylate IP_3 produced by Plc1p to IP_6, needed for some cellular processes such as mRNA export from the nucleus (Michell *et al*., 2003).

Actually, it was found that mutations in IP biosynthetic pathway components, such as Plc1p, Ipk2p and Ipk1p, were synthetically lethal with a conditional allele of the gene encoding for the nuclear-pore protein Gle1, essential for a late step in mRNA export and able to directly bind IP_6. Really, a role for IP_6, but for any of the pyrophospahate derivatives of IP_6, in mRNA export was demonstrated (Weirich *et al*., 2006).

In order to clarify IP_3 role as a second messenger responsible for calcium signalling, glucose-induced calcium signalling was monitored in mutant strains impaired in Arg82p and Ipk1p activity, that significantly accumulate IP_3. All these mutant strains, as Plc1p-overexpressing strains, display a higher glucose-induced Ca^{2+} peak, suggesting that IP_3 could be involved in generating a calcium pulse and also that further phosphorylation of IP_3 is not required to elicit the calcium signal (Tisi *et al*., 2004).

Differing from mammalian cells, there is no evidence in budding yeast that could link the other product of Plc1p activation, diacylglycerol, to calcium signalling; whereas IP_3 and Plc1p glucose-dependent activation seem to be indispensable for cytosolic calcium concentration increase.

Calcium in Saccharomyces cerevisiae: an essential second messenger for the cell

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.
Also in budding yeast, calcium plays a very important role as second messenger being involved in the regulation of many fundamental cellular processes, such as cell cycle, mating, sensing of glucose and glucose starvation, resistance to salt stress and cell survival.
Subsequently, there exists a variety of cellular systems in eukaryotic cells for the regulation of calcium homeostasis, including influx and efflux from the cell, as well as sequestration and release from cellular organelles.
The budding yeast actively maintains cytosolic free Ca^{2+} 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. It is generally believed that this asymmetric distribution avoids aggregation of Ca^{2+} with phosphate-containing molecules in the cytosol while still providing various organelles with sufficient Ca^{2+} for their proper function. Actually calcium becomes an intracellular signal when its cytosolic concentration is transiently increased (Wolfe and Pearce, 2006).

Calcium homeostasis regulation

The regulation of intracellular Ca^{2+} homeostasis in all eukaryotic cells is a remarkably intricate process finely regulated by a variety of transporters, pumps and exchangers mainly located on plasma and internal cellular membranes (Fig. 6).
While in mammalian cells the endoplasmic reticulum (ER) serves as a major storage compartment that provides a readily mobilizable source of Ca^{2+} for use in Ca^{2+} signalling, this role is recovered in budding yeast by vacuole. The vacuole in yeast cells contains more than 95% of the total cellular Ca^{2+}: in rich medium-growing cells free vacuolar Ca^{2+} concentration is estimated of about 30 μM, while the total Ca^{2+} concentration in this organelle is

estimated to be 2-4 mM, suggesting that most of the vacuolar Ca^{2+} is buffered by inorganic polyphosphate (Dunn *et al*., 1994).

This large store of Ca^{2+} is maintained through the action of two transporters located on vacuole membrane, the high affinity Ca^{2+}-ATPase Pmc1p and the Ca^{2+}/H^{+} exchanger Vcx1p.

Although clearly related in sequence to the mammalian PMCA (Plasma Membrane Ca^{2+}-ATPases) family, yeast Pmc1p lacks domains involved in regulation by calmodulin and acidic phospholipids typical of the mammalian members and localizes to the vacuolar membrane instead of the cell surface.

Yeast mutant cells impaired in Pmc1p activity accumulate Ca^{2+} in the vacuole at less than 20% 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).

Pmc1p works in concert with the vacuolar Ca^{2+}/H^{+} antiporter Vcx1p, driven by the proton electrochemical gradient set up by the vacuolar H^{+}/ATPase, to remove excess Ca^{2+} from the cytosol. Vcx1p was genetically identified based on its ability to confer tolerance to high Ca^{2+} and Mn^{2+} salts in the culture medium when overexpressed. Although it was predicted that Vcx1p was responsible for most Ca^{2+} transport into the vacuole *in vivo* in normal growth conditions, *vcx1* mutant cells exhibited wild-type levels of vacuolar Ca^{2+} and wild-type tolerance to supplemental Ca^{2+} salts in the culture medium (Cunningham, 2011).

It turned out more recently that vacuolar Ca^{2+} can be released in a regulated manner through the action of vacuolar channel Yvc1p (also named TRPY1), the only TRP channel homolog identified in budding yeast. TRP (Transient Receptor Potential) channels comprise a super-family of selective and non-selective cation-permeable ion channels, identified in various vertebrates, flies and worms. All TRP members have six predicted trans-membrane domains (TMs) with cytosolic N- and C-termini and a pore region located between TM5 and TM6. TRP proteins are mainly located in mammalian cells on the plasma membrane of both neuronal and non-neuronal cells.

Yvc1p was first found by electrophysiological studies of isolated *S. cerevisiae* vacuolar membrane vesicles. Channel gating of Yvc1p is voltage-dependent and its open probability strongly depends on the cytosolic Ca^{2+} concentration, in a process commonly known as *Ca^{2+}-induced Ca^{2+} release*: Yvc1p opening *in vitro* requires un-physiological Ca^{2+} concentrations (in a range of millimolar), whereas the presence of reducing agents to the cytosolic side, that could reproduce the strongly reducing cellular environment, lowers the rather un-physiological high Ca^{2+} concentrations

required for channel activation and increases the channel's open probability (Palmer *et al*., 2001). Furthermore, Yvc1p-dependent currents are mechanosensitive: applied pressure, stretch force or raising the bath osmolarity directly activate the channel, regardless of the cytosolic Ca^{2+} concentration, even if tens of micromolar Ca^{2+} greatly enhance the force-induced activity.

Yvc1p is the major responsible for Ca^{2+} release into the cytosol in response to hypertonic shock, such as high salinity and high sugar; actually *yvc1Δ* strains display no significant increase in cytosolic Ca^{2+} concentration after such treatment (Chang *et al*., 2010).

In addition to the vacuole, the Golgi apparatus and ER are also important for maintaining proper intracellular Ca^{2+} homeostasis in yeast to ensure the proper folding and processing of proteins transported through the secretory pathway.

The Golgi localized pump Pmr1p (for Plasma membrane ATPase related), the first identified member of the family of secretory pathway Ca^{2+} ATPases (SPCA), plays an essential role in maintaining the luminal Ca^{2+} concentration required for the proper glycosylation and processing of proteins in this compartment. The loss of Pmr1p results in a number of alterations in Ca^{2+} homeostasis, including an increased rate of cellular Ca^{2+} uptake from the extracellular environment and a greater sensitivity to elevated extracellular Ca^{2+} levels. The elevated Ca^{2+} uptake observed in the *pmr1Δ* mutant, mediated by different calcium transporter systems located on the plasma membrane, is reminiscent of the mammalian capacitative Ca^{2+} entry (CCE) response, triggered by release of ER Ca^{2+} that lead to the generation of a store depleted signal (Aiello *et al*., 2004).

Pmr1p also seems to play a role in controlling, at least in part, the luminal Ca^{2+} concentration of the yeast ER, being ER Ca^{2+} severely reduced in *pmr1Δ* strains. However, yeast ER appears to play a lesser role in cellular Ca^{2+} storage: the physiological steady-state level of free Ca^{2+} in this compartment was estimated ~ 10 μM, a value significantly lower than free Ca^{2+} in the mammalian ER (estimated from 1 to 400 μM) (Strayle *et al*., 1999). It is most likely that the lack of specialized SERCA-type Ca^{2+} pumps, responsible for the Ca^{2+} storage into the ER in mammalian cells, from the yeast ER and the moderate level of free Ca^{2+} in this compartment might well limit the use of this compartment for intracellular Ca^{2+} signalling and thus implicate other organelles (Golgi, vacuole) as dynamic Ca^{2+} stores in yeast.

Despite its minor role in intracellular Ca^{2+} storage, on ER membrane a putative calcium transporter has been identified, not yet functionally characterized, encoded by *CLS2/CSG1* gene. This protein was identified through a genetic screening performed in order to isolate mutants impaired

in growing in medium containing high Ca^{2+} concentrations, a common approach toward identifying genes and proteins involved in regulating cellular Ca^{2+} levels. Actually, *csg1* (Calcium Sensitive Growth) mutants, in the presence of high Ca^{2+} concentrations (10-50 mM $CaCl_2$), accumulated a much higher amount of Ca^{2+} relative to a wild-type strain. Moreover, these mutants had normal vacuolar Ca^{2+} accumulation, but the large increase was observed in another exchangeable Ca^{2+} pool (Beeler *et al.*, 1994). Cls2p/Csg1p was later revealed to be predominantly localized on the ER membrane: it is a hydrophobic protein carrying a consensus Ca^{2+}-binding loop of the EF-hand type in a cytoplasmic loop. Although it is not a homologue of Ca^{2+}-ATPase or calcium channels such as the ryanodine receptor or the IP_3-receptor in mammalian cells, Cls2p/Csg1p likely possesses an important function in Ca^{2+} homeostasis of the ER (Tanida *et al.*, 1996). However Cls2p/Csg1p role as calcium channel is still in debate.

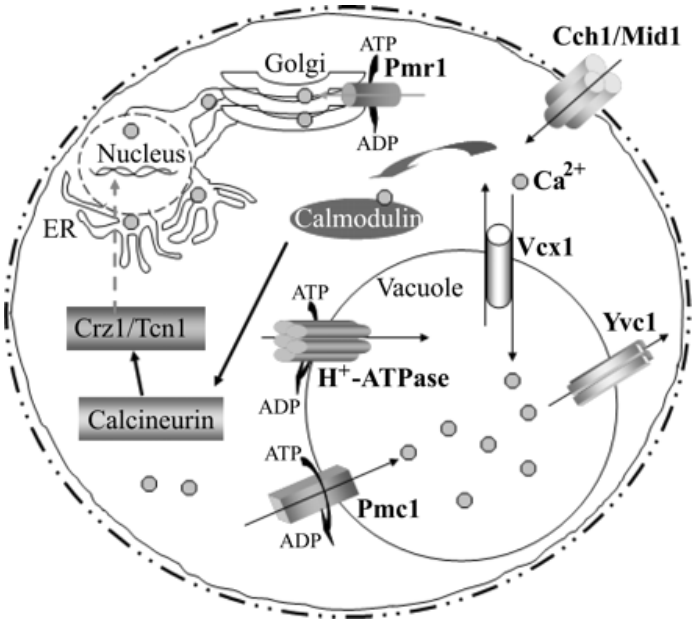


Figure 6. Ca^{2+} signalling and transport pathways in *S. cerevisiae* (Ton and Rao, 2004).

Mutations in many calcium channels and transporters result in a large number of human diseases, collectively termed channelopaties. Possessing a fully annotated genome, *S. cerevisiae* would appear to be an ideal organism in which to study this class of proteins associated to diseases. For

example mucolipidosis type IV (MLIV) is a lysosomal storage disorder characterized by psychomotor retardation and visual abnormalities. The gene responsible for this disease was found to encode a putative calcium transporter homologous to the T-type calcium TRP class of transporters, which yeast Yvc1p belongs to.
Another quite diffused disorder related to calcium transport alteration is the neuromuscular disease Brody Myopathy, which clinically manifests as a cramping of skeletal muscles post activity and which is caused by a mutation in the Pmc1p homolog ATP21p, a subunit of the calcium ATPase.
The presence of observable phenotypes, such as enhanced sensitivity to high Ca^{2+} concentration in the growth medium, in yeast mutant strains impaired in calcium channels activity provides an excellent opportunity to study this class of proteins using yeast as a model in order to understand the pathogenesis of a channelopathy. Another significant utilization of yeast would be to screen for drugs that can compensate for ion channel dysfunction (Wolfe and Pearce, 2006).
Moreover, calcium homeostasis and signalling are crucial also for the intricate working of the mammalian heart: most of the factors involved in this process in budding yeast are conserved and operate similarly in cardiac myocytes. Thus, exploring and understanding the calcium homeostasis/signalling system in yeast, could provide a shortcut to help treat relevant human diseases such as pathological cardiac hypertrophy and heart failure (Cui *et al*., 2009).

Calcium transport across the plasma membrane: HACS and LACS systems

Differing from mammalian cells, Ca^{2+} signalling in yeast cells is often dependent on Ca^{2+} influx from the extracellular environment, in response to different stimuli such as pheromones and nutrients exposure or hypotonic stress, rather than on Ca^{2+} release from intracellular stores. This Ca^{2+} influx is mediated by different transporters located on the plasma membrane.
In budding yeast the best characterized membrane calcium transporter system is represented by HACS (High Affinity Calcium System), composed by Mid1 and Cch1 subunits.
This transporter system was identified by studying Ca^{2+} signalling dependent on pheromone exposure.
In *S. cerevisiae* the mating process of haploid cells, controlled by the mating pheromones *a* and α factors which are synthesized and secreted by *a* and α

mating type cells respectively, induces several responses in cells of the opposite mating type: in the early stage cells rapidly alter the pattern of gene expression, expose cell surface agglutinins that facilitate mating and arrest mitotic cell division in the G_1 phase. In the late stage, cells induce morphological changes (so-called shmoos) with one or more projections on their surface that act as a point of contact between mating cells and lead to cell and nuclear fusion.

It is largely known that mating pheromone also elicits in yeast cells a rise in cytosolic Ca^{2+} concentration in the late stage of the mating pheromone response pathway and that this signal is essential to maintain the viability of shmoos. This Ca^{2+} signal is generated once in the late stage of the yeast pheromone response (Nakajima-Shimada *et al*., 2000).

Pheromone-triggered Ca^{2+} signal is dependent on Ca^{2+} influx from the extracellular environment: the Ca^{2+} rise was not detected when cells were treated with α-factor in a Ca^{2+}-deficient medium (Iida *et al*., 1990).

MID1 (Mating pheromone-Induced Cell Death) gene was identified in a screening for mutants defective in the late stages of conjugation and Ca^{2+} signalling: in absence of extracellular Ca^{2+}, *MID1* mutations are lethal after pheromone exposure and differentiation of the cells into shmoos. In addition, in a Ca^{2+}-depleted medium, *mid1* mutant strains exhibit remarkably reduced Ca^{2+} influx after exposure to pheromone, and their viability in the presence of pheromone is completely restored by adding $CaCl_2$ to the culture medium (Iida *et al*., 1994).

Mid1p is a transmembrane protein with some homology to regulatory $\alpha 2\delta$-subunits of mammalian voltage-gated calcium channels (VGCCs), that is thought to affect the timecourse and/or voltage dependence of activation and inactivation.

Yeast genome sequencing project has led to identification of *CCH1* gene, encoding for a homologue of $\alpha 1$ subunit VGCCs of mammals. Although overall similarity between Cch1p and the VGCC $\alpha 1$ subunit is low (24% identity over the length of the proteins), several key features such as size, topology and domain structure of the $\alpha 1$ subunits are retained in Cch1p: Cch1p contains four repeat units (I to IV) of six transmembrane domains that tetramerize to form the core of the Ca^{2+} channel (Fig. 7). Indeed, most of the sequence identity between Cch1p and the mammalian calcium channel subunits is present within regions thought to play key roles in defining channel specificity (domain P) and voltage dependence (transmembrane domain S4). All four hydrophobic domains (I, II, III and IV) contain amino acids residues indicative of the Ca^{2+}-selective P segment, and three (II, III and IV) of the four domains contain a highly conserved

glutamate residue that is thought to play a critical role in Ca^{2+} ions coordination. Moreover, each of the S4 segments of I, II and III domains contains repeated motifs of a positively charged residues followed by two hydrophobic residues, similar to segment that have been shown to act as voltage sensors in ion channels of higher eukaryotes (Paidhungat and Garrett, 1997).

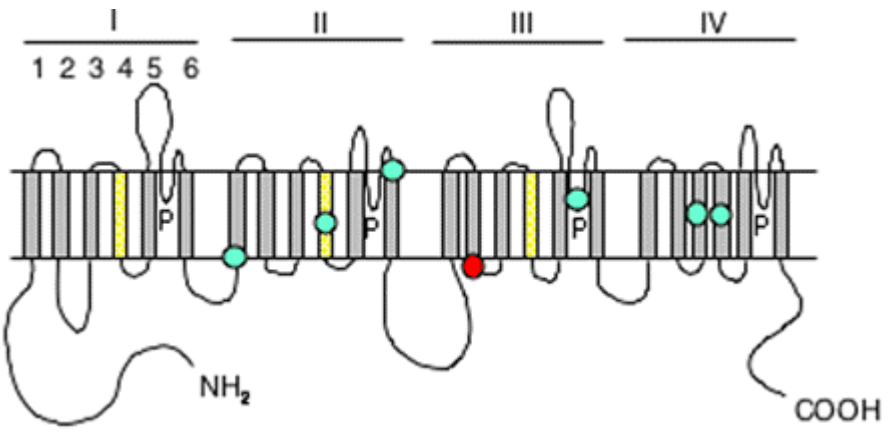


Figure 7. Structure of the Cch1 protein (I-IV represent putative domains and 1-6 putative transmembrane segments; in yellow are indicated S4 segments containing repeated motifs of a positively charged residue followed by two hydrophobic residues and circles indicate strain-dependent amino acids variations) (Iida *et al.*, 2007).

CCH1 gene was also identified in a genetic screening as a suppressor of *cdc1* (Ts) temperature-sensitive mutation. *CDC1* is an essential gene whose product biochemical activity and physiological function is unknown; however *cdc1-1* (Ts) mutants are Ca^{2+} sensitive and displayed an increase in intracellular Ca^{2+}. *CCH1* deletion blocked the increase in intracellular Ca^{2+} content elicited by *cdc1-1* (Ts).

As demonstrated for the pheromone-induced Ca^{2+} signal, also in these experiments Mid1p and Cch1p seem to act in the same Ca^{2+} uptake process: *mid1Δ cch1Δ* double mutant strains make no difference with the single mutant strains, being affected to the same extent (Paidhungat and Garrett, 1997). Mid1p and Cch1p interaction was demonstrated; in addition Mid1p was found to localize also on ER membrane but up to now any its involvement in Ca^{2+} release from this compartment to the cytosol is known (Yoshimura *et* al., 2004).

Recently a novel probable new subunit of HACS has been identified, encoded by *ECM7* gene. *ECM7* was identified in a screening of a collection of yeast mutants lacking non-essential genes for defects in Ca^{2+} uptake during response to mating pheromones and tunicamycin, a blocker of proteins glycosylation.

ecm7 mutant strains exhibited striking defects in Ca^{2+} uptake that were quantitatively similar to those of *mid1* and *cch1* mutants; in particular HACS activity was largely abolished in these mutants suggesting that Ecm7p may function as a regulatory subunit of HACS. Curiously, *ecm7* mutants exhibited weaker phenotypes than *mid1* and *cch1* mutants, and were not recovered in the previous genetic screening that yielded *mid1* and *cch1* mutants, possibly because residual HACS activity may exist in *ecm7* mutants.

Ecm7p was found to be homologous to γ- subunits of VGCCs and other members of the PMP-22/EMP/MP20/Claudin superfamily of four-spanner membrane proteins. Ecm7p contains four transmembrane helices with its N- and C-termini located in the cytoplasm; in addition, it contains a highly conserved motif in the first extracellular loop strongly resembling to the signature sequence of the PMP-22/EMP/MP20/Claudin superfamily. In *S. cerevisiae* proteome eleven other members of this superfamily were identified, in addition to Ecm7p, but none of the single mutants exhibited abnormal Ca^{2+} accumulation in these screening conditions.

A physical interaction between Ecm7p and Mid1p/Cch1p was found by performing co-immunoprecipitation experiments. Interestingly, the abundance of Ecm7p was slightly diminished in *mid1* and *mid1 cch1* mutant strains as well as Mid1p levels were reduced in *cch1* and *cch1 ecm7* mutant strains, suggesting that Cch1p stabilizes Mid1p and subsequently Mid1p stabilizes Ecm7p. On the other hand, Ecm7p seems not to stabilize HACS subunits. These findings suggest that Ecm7p directly or indirectly interacts with HACS subunits and directly or indirectly regulates HACS through unknown mechanisms (Martin *et al*., 2011).

Primarily, HACS system was found to be responsible for a CCE-like mechanism activation in budding yeast: the depletion of Ca^{2+} from secretory compartments, caused for example by Pmr1 pump inactivation, stimulated an influx of Ca^{2+}, then revealed to be mediated by HACS system (Locke *et al*., 2000).

Peiter and co-workers, by performing experiments in *mid1Δ cch1Δ* mutant strains in order to search for other environmental stimuli inducing a Ca^{2+}-dependent stress response, demonstrated that HACS system is also involved in the response to cold stress, that was been shown previously to induce a continuous raise in cytosolic Ca^{2+} concentration, and iron toxicity.

Growth of mutant strains impaired in HACS activity on low Ca^{2+}-containing media was severely affected at 10°C, and this cold-sensitive phenotype was partially suppressed by Ca^{2+} addition to the culture medium, suggesting that probably an alternative unidentified Ca^{2+} influx pathway independent on HACS activity could mediate cold-stress response. In addition, *mid1Δ cch1Δ* strains growth was almost completely abolished by the exposure to 10 mM Fe^{2+}, and their iron ipersensitivity was almost completely rescued by Ca^{2+} addition to the culture medium (Peiter *et al*., 2005).

Another typical stress that yeast suffers derives from ethanol produced during fermentation, and Ca^{2+} ions seem to be protective for high concentrations of ethanol. It was previously demonstrated that moderate amounts of Ca^{2+} (1 mM) suppress the growth inhibitory effect of moderate levels of ethanol (10%). Actually, Ca^{2+} fluxes in yeast have been observed after cells exposure to ethanol: the increase in cytosolic Ca^{2+} level is rapid, peaking about 4 seconds after ethanol exposure, returning immediately to near baseline and continuing to decline slowly over the next 3 minutes. Moreover, this Ca^{2+} signal is dose- dependent and increases in magnitude in high-osmolarity media. *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%. These findings suggest a role of HACS as a major ethanol-responsive Ca^{2+} transporter. Actually Cch1p seems to have a protective effect for cells growing in the presence of ethanol (Courchesne *et al*., 2011).

A major role of HACS system in mediating Ca^{2+}influx in yeast cells, in response to different environmental stimuli, has been coming to light.

A distinct low affinity Ca^{2+} influx system, LACS (for Low Affinity Calcium System), was identified being stimulated by pheromone signalling during growth in rich medium.

MID1 and *CCH1* were identified and found to be essential for survival in the presence of pheromones in experiments performed in a synthetic minimal growth medium containing 100 μM Ca^{2+}.

Surprisingly, when pheromone-triggered Ca^{2+} signal was observed in rich (YPD) medium-growing cells, mutant strains impaired in HACS activity showed no decrease in survival relative to wild-type. In these growth conditions, a HACS-independent Ca^{2+} signal in response to pheromone exposure was observed, but it disappeared in minimal medium-growing cells. In mutant strains impaired in HACS activity, the apparent K_m for Ca^{2+} was estimated to be ~ 3 mM, suggesting that Ca^{2+} transport was mediated by a low affinity transporter (subsequently named LACS), in these growth conditions (Muller *et al*., 2001).

In order to identify components and regulators of the LACS system, Muller and co-workers screened a collection of pheromone-responsive genes that when deleted lead to defects in LACS, but not in HACS, activity. In addition to many factors involved in polarity establishment, projection formation and cell fusion, Fig1 protein was identified in this study. Unlike the other identified mutants, *fig1* mutants displayed a cell fusion defect that can be suppressed by high Ca^{2+} conditions. Moreover, *fig1* mutants mated at rates similar to wild-type strains suggesting that Fig1p does not seem to be required for cell cycle arrest and for induction of mating-specific genes.
Fig1p expression is strongly induced upon activation of the pheromone signalling pathway, and the kinetics of its appearance are similar to that of LACS stimulation (Muller *et al*., 2003).
Really *FIG1* was already identified in another genetic screening as a gene specifically regulated during mating process. As many genes involved in the mating response, *FIG1* revealed the presence of upstream sites similar to the PRE consensus sequence, representing the binding sites for Ste12 transcription factor that mediates pheromone-induced transcription (Erdman *et al*., 1998).
Although Fig1p does not resemble any known Ca^{2+} channels, it seems to function as a subunit or close regulator of LACS system. Fig1p is a N-glycosylated protein that localizes to the plasma membrane via its four predicted transmembrane helices (TM). Fig1p 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.
However, to date LACS system remains to be largely investigated at the molecular level.

Calcium channels inhibitors

Most of the studies on Ca^{2+} channels inhibitors have been performed in mammalian cells, in order to block the activity of VGCCs.
VGCCs constitute a large family of channels whose functional conformations are sensitive to changes in the electric potential across the cell membrane. Several types of VGCCs have been identified electrophysiologically and genetically, in different cellular types, and have

been designated L, N, P/Q, R and T. L-type Ca^{2+} channels have a crucial role in the initiation of cardiac and smooth muscle contraction, thus the most studied L-type Ca^{2+} channels blockers are employed in the treatment of many cardiovascular diseases. In addition to its similarity sequence to the pore-forming $\alpha 1$ subunits of mammalian L-type VGCCs, Cch1p HACS subunit of budding yeast also displays pharmacological traits similar to those of L-type VGCCs.

The most common inhibitors of L-type Ca^{2+} channels in mammalian cells are represented by the class of phenylalkylamines (PAAs), benzothiazepines (BTZs) and dihydropyridines (DHPs).

In particular, Cch1p was revealed to be sensitive to nifedipine and verapamil, belonging respectively to DHPs and PAAs classes; conversely diltiazepam, another common L-type VGCCs blocker belonging to BTZs class, seemed to increase Cch1p activity. The three described class of drugs are chemically unrelated and display different mechanisms of action by binding to different sites on L-type Ca^{2+} channels. PAAs are tertiary amines, existing as predominantly positively charged species at physiological pH, that are considered to be pore-blocking drugs that block Ca^{2+} channels directly by occluding the transmembrane pore through which Ca^{2+} ions move. In contrast, DHPs are lipophilic compounds that appear to block the pore indirectly by stabilizing a channel state with a single Ca^{2+} ion bound in a blocking position in the pore (Hockerman *et al*., 1997).

In *MID1/CCH1* overexpressing yeast strains, grown in SD.Ca100 medium (a low Ca^{2+} medium containing 100 μM $CaCl_2$ as the sole Ca^{2+} source), nifedipine and verapamil were able to inhibit Ca^{2+} accumulation: Ca^{2+} accumulation measurement in these strains, incubated for 2 hours in the presence of one of the Ca^{2+} channel blockers and $^{45}CaCl_2$, revealed that nifedipine and verapamil were able to block Ca^{2+} accumulation to levels of 55% and 71% respectively. K_i values of nifedipine and verapamil were estimated to be 248 μM and 1.750 mM respectively; these values are much higher than those calculated for nifedipine and verapamil on mammalian VGCCs $\alpha 1$ mammalian subunits, probably because of the necessity of higher concentrations of the blockers for the presence of cell wall in yeast cells, or maybe for some differences in the binding sites of these L-type blockers. Actually, the three classes of drugs bind to two or three of the transmembrane segments IIIS5, IIIS6 and IVS6 in mammalian L-type VGCCs, and the binding of each class is unique in each segment. Alignment of the corresponding transmembrane segments of Cch1p with a mammalian VGCC shows that the binding sites of Cch1p for the three classes are limited, probably justifying the low affinities of verapamil and nifedipine to Cch1p (Teng *et al*., 2008).

However, the best known competitive inhibitor of Ca^{2+} fluxes in many organisms is magnesium. Mg^{2+} is the most abundant divalent cation in yeast cells and it predominantly serves as a counterion for solutes, particularly ATP and other nucleotides, RNA and DNA. By binding to RNAs and many proteins, Mg^{2+} also contributes to establishing and maintaining physiological structures and acts as an important cofactor in catalytic processes. Mg^{2+} also stabilizes membranes and active conformations of macromolecules. Cellular Mg^{2+} concentrations are maintained in the millimolar range (~ 15 to 20 mM), some three orders of magnitude higher than those of Ca^{2+}. The vast majority of Mg^{2+} is bound to ligands, leaving a small fraction of up to 5% in a free ionized state. Cellular Mg^{2+} homeostasis involves systems facilitating influx and others that mediate extrusion of the ion.

Mg^{2+} mechanism of action as Ca^{2+} channels inhibitor has been largely investigated in mammalian cells. For example in mammalian L/T-type Ca^{2+} channels, Mg^{2+} acts at an extracellular site to regulate Ca^{2+} influx. There is evidence that this modulatory effect of Mg^{2+} involves the EF-hand motif in the COOH-terminus of Ca^{2+} channels in a competitive inhibition mechanism (Scheuer *et al*., 2005). In pancreatic acinar cells, an increase in intracellular Mg^{2+} results in a decrease of Ca^{2+} influx, whereas intracellular Ca^{2+} mobilization is associated with a reduction in Mg^{2+}; in yeast cells, vacuolar Ca^{2+} accumulation is blocked by increased Mg^{2+} in the medium.

Recently, a whole-genome experiment in *S. cerevisiae* revealed a set of genes up-regulated upon Mg^{2+} starvation, that were also found to be up-regulated by high Ca^{2+} levels. In addition, an increase in cytosolic Ca^{2+} level was observed immediately after cells were transferred to low-Mg^{2+} medium, with the consequent activation of calcineurin pathway, which represents the major effector of intracellular Ca^{2+}. Thus, cellular Mg^{2+} and Ca^{2+} levels appear linked in many circumstances, such as that high Mg^{2+} results in low Ca^{2+} and vice versa. Actually, an effect of Mg^{2+} withdrawal on the opening of Ca^{2+} channels and influx of Ca^{2+} appears to be likely (Wiesenberger *et al*., 2007).

Also metallic divalent ions, such as Ni^{2+}, have been found to inhibit Ca^{2+} permeation via voltage-dependent Ca^{2+} channels in mammalian cells. Ni^{2+} is a strong blocker of T-type calcium channels and it seems to act in part by binding to a site on the extracellular side of the channel, affecting current through the channel allosterically, and also by directly blocking the pore (Obejero-Paz *et al*., 2008). However any studies in budding yeast have yet demonstrated Ni^{2+} action as Ca^{2+} transporters inhibitor.

Finally, also Gd^{3+} trivalent cations, derived from gadolinium lanthanide, are particularly effective in blocking plasma membrane SOCs (Store-Operated calcium Channels) channels in mammalian cells, activated by a fall in ER Ca^{2+}

content in order to maintain ER Ca^{2+} levels necessary for the proper protein synthesis and folding. Gd^{3+} is able to fully inhibit the current through open SOCs Ca^{2+} channels in the low micromolar concentration range (at concentrations <10 μM), by competitively binding within the selectivity filter in the pore of Ca^{2+} channels (Parekh and Putney Jr., 2005).
Gd^{3+} was revealed to be able to inhibit Ca^{2+} transport also in budding yeast.
Batiza and co-workers demonstrated that Ca^{2+} signal triggered by hypotonic shock in yeast cells, generated primarily from a release of Ca^{2+} from the intracellular stores and then sustained from an influx of Ca^{2+} from the extracellular environment, could be eliminated by gadolinium suggesting that the Ca^{2+} rise upon hypotonic shock is dependent upon SACs-like Ca^{2+} channels. In these experiments, at lowest concentrations Gd^{3+} decreased the kinetics of the hypotonically induced Ca^{2+} flux in a concentration-dependent manner without affecting the time of the maximum increase; but only 10 mM Gd^{3+} completely eliminated the hypotonic shock response. The lower sensitivity to gadolinium in yeast cells, compared to what observed in mammalian cells, may simply result from the presence of the cell wall in yeast cells (Batiza *et al*., 1996).
Moreover, Gd^{3+} was revealed to inhibit HACS-mediated Ca^{2+} influx from the extracellular environment in Chinese hamster ovary cells expressing functional yeast Mid1p HACS subunit (Kanzaki *et al.,* 1999).
Considering that Cch1p homologous proteins were found for over 30 fungal species, including several pathogenic fungi, investigating the pharmacological features and the consequent modulation of fungal Cch1p and other Ca^{2+} transporters function could be useful in the development of cures for fungal diseases in humans, besides in the discovering of novel class of Ca^{2+} homeostasis/signalling players.

Evidences for novel calcium transporters

In *S. cerevisiae* genome no sequence resembling mammalian calcium channels, besides the previously described HACS and LACS components and the intracellular calcium transporters Yvc1p and Cls2p, has been identified to date.
However, Cui and co-workers, basing on a mathematical model which reproduced Ca^{2+} fluxes in *yvc1Δ cch1Δ* yeast cells treated with different concentrations of $CaCl_2$ and $MgCl_2$ and subjected to hyperosmotic stress with 800 mM $CaCl_2$, demonstrated that two different Mg^{2+}-sensitive Ca^{2+} influx pathways, mediated by two different transporter systems named X and M, exist in yeast cells, with different cation affinities. They monitored

cytosolic Ca^{2+} level in these cells expressing aequorin protein, a bioluminescent protein that in the presence of its synthetic cofactor and Ca^{2+} ions, emits light at a specific wavelength correlated to the intracellular Ca^{2+} level.
They observed that very little differences in extracellular $MgCl_2$ concentrations resulted in great difference in cytosolic Ca^{2+} level monitored after the stimulus.
The experimental data fitted with a model comprising two different Ca^{2+} transporters, one of which (transporter X) with an extremely low inhibition constant for Mg^{2+} functioning only in the presence of extremely low extracellular Mg^{2+}, and the other (transporter M) with a high Mg^{2+} inhibition constant whose influx is much less sensitive to the change of extracellular Mg^{2+} than that of transporter X. Both transporters X and M make considerable contribution to the total Ca^{2+} influx in absence of extracellular Mg^{2+}, whereas for relatively high extracellular Mg^{2+} concentrations the flux contribution of transporter X becomes negligible. Previously published data demonstrated the existence of a constitutive Mg^{2+}-sensitive Ca^{2+} influx pathway, sustained by HACS system, that could remind to the transporter X described by Cui and co-workers (Locke *et al*., 2000). The novel transporter M, emerging from this model, appears to be located on the plasma membrane and displays very low affinity for Ca^{2+} (apparent K_m = 505.43 mM), competitive inhibition by extracellular Mg^{2+} (K_i = 149.18 mM) and rapid feedback inhibition by intracellular Ca^{2+} (Cui *et al*., 2009).
Basing on these displayed features, transporter M seems not to share the characteristics described for LACS system as far as pheromone-induced Ca^{2+} signalling, suggesting the existence in budding yeast of a novel Ca^{2+} transporter not yet identified at the molecular level. The relatively low affinity for Ca^{2+} relative to Mg^{2+} also suggests that transporter M may function primarily as a Mg^{2+} transporter in physiological conditions.

Calcineurin: the major effector of intracellular calcium

Calcineurin is an eukaryotic Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase type 2B (PP2B), representing the major player in Ca^{2+}-dependent signal transduction pathways, involved in the regulation of a wide variety of cellular processes. The serine/threonine phosphatase family members are classified into four different subtypes, PP1, PP2A (2A), PP2B (2B) and PP2C (2C), differing for their substrate specificity, sensitivity to various inhibitors and also in divalent metal ions

requirement for their activity. PP2B is the only class of phosphatase that requires Ca^{2+} and calmodulin for activity.

Calcineurin has been purified from several mammalian tissues, and the holoenzyme has been shown to consist of two polypeptides: the active site is located on the A subunit which, in mammals, is 57-59 kDa depending on the isoform, whereas the regulatory B subunit is a 19 kDa peptide containing four Ca^{2+}-binding EF-hand motifs. Calcineurin A subunit consists of a catalytic domain homologous to other serine/threonine phosphatases and three regulatory domains at the COOH terminus that distinguish calcineurin from other family members. These regulatory domains have been identified as the calcineurin B binding domain, the calmodulin-binding domain and the "autoihibitory" domain, which binds in the active site cleft in the absence of Ca^{2+}/calmodulin inhibiting the enzyme. It has been shown that when Ca^{2+}/calmodulin bind to the enzyme, inhibition ceases and likely involves a conformational change that exposes the active site. The calcineurin B subunit, highly conserved throughout evolution, is encoded in mammals by two different genes, one which is ubiquitously expressed while mRNA for the second gene is found only in testes. This subunit is responsible for Ca^{2+} binding: it was demonstrated that four Ca^{2+} ions bind with high affinity (revealing a dissociation constant $K_d \leq 10^{-6}$ M). In particular, one high affinity (K_d = 0.024 μM) and three lower affinity sites (K_d = 15 μM) were identified (Rusnak and Mertz, 2000).

Some of the most thorough work investigating biological roles for calcineurin have used the yeast *S. cerevisiae* as a model system. Two yeast genes, *CNA1* and *CNA2*, encode for proteins highly related to the catalytic A subunit of mammalian calcineurin, which were isolated for their ability to bind ^{125}I-labeled yeast calmodulin: the predicted Cna1 protein was shown to contain many regions of near identity to mammalian calcineurin A over its entire length. Mutant strains carrying a null allele of *CNA1* had no readily identifiable phenotypes, suggesting that probably a second related gene could exist whose product could also perform the essential functions of *CNA1*. Actually, *CNA2* gene was afterwards isolated and the predicted Cna2 protein revealed to be very similar both to Cna1p and to mammalian calcineurin A. Again, *CNA2* deletion was not lethal, even in a strain containing a *cna1* null mutation.

Most of the structural features of the mammalian calcineurin catalytic subunit are conserved in Cna1p and Cna2p, even if yeast subunits are larger than in mammals, displaying a molecular mass of 63 and 69 kDa respectively.

Single or double mutants in *CNA1* and *CNA2* were identical to wild-type parental strain with respect to morphology and growth on different media;

moreover, the mutants were able to mate and diploid strains homozygous for either the *cna1* or the *cna2* null mutation, or for both, were able to sporulate. However, the mutant and wild-type strains differed in their response to the pheromones: mutant strains were more sensitive than wild-type to α factor-induced growth arrest, and once arrested *cna1 cna2* strains failed to resume growth in the continued presence of the pheromone (Cyert *et al*., 1991). These findings suggest that calcineurin is involved in the recovery of yeast cells from mating pheromone-induced cell cycle arrest, in agreement with the previously described role for Ca^{2+} influx in the late stages of the mating pathway.

By using an assay specific for detection of calcineurin activity in *S. cerevisiae*, a third prominent component was found in the purified fraction, encoding for a 16 kDa polypeptide displaying several properties that were characteristic of the B regulatory subunit of mammalian calcineurin. *CNB1* gene was isolated form a yeast expression library in the vector λgt11 by using an antiserum raised against bovine calcineurin.

Cnb1p is highly related in amino acid sequence to the mammalian calcineurin B subunit (56% identity) and, like its mammalian counterpart, is a Ca^{2+}-binding protein carrying four Ca^{2+}-binding EF-motifs.

Like mutant strains impaired in calcineurin catalytic subunit, also *cnb1* mutant strains were viable; furthermore, a *cna1 cna2 cnb1* triple mutant was able to grow normally in standard laboratory conditions. Anyway, a *cnb1* mutant, like *cna1 cna2* mutants, failed to recover and to resume growth after the cell cycle arrest imposed by long-term exposure to pheromones, confirming that Cnb1p, as well as Cna1p and Cna2p, seems to be required for the execution of calcineurin function during adaptation but not during initial pheromone response. In keeping with a role for calcineurin in pheromone response, 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).

A number of natural products have been isolated that are potent inhibitors of calcineurin. The most potent, 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. A number of other compounds have demonstrated inhibitory activity against calcineurin, with a lower affinity, such as okadaic acid or the fungal metabolite dibefurin. Since the discovery of these natural product inhibitors, several new synthetic compounds have been found to be reasonable inhibitors of calcineurin activity (Rusnak and Mertz, 2000).

Calcineurin-regulated transcription is dependent on Crz1 transcription factor

Calcineurin is dispensable for growth under standard culture conditions, but it becomes essential under specific environmental stress conditions such as exposure to ions (OH^-, Mn^{2+}, Na^+/Li^+) and prolonged incubation with mating pheromone, or in mutants with cell wall defect. Under standard laboratory growth conditions, cytosolic Ca^{2+} levels are low, calcineurin is inactive and cells lacking the enzyme display no growth defects; however, in response to stress, cytosolic Ca^{2+} levels raise, calcineurin becomes active and it dephosphorylates key substrates.

The most critical function of calcineurin-dependent signalling, in response to such cellular stresses, is to control gene expression: calcineurin activation leads to increased expression of more than 160 genes, fallen into several classes, encoding for integral membrane proteins, components of the cell wall, proteins participating in vesicle trafficking, lipid/sterol synthesis and protein degradation.

Stathopoulos and Cyert identified in *FKS2* promoter, a gene encoding for a β-1,3-glucan synthase whose mRNA level increased in response to Ca^{2+} and mating pheromone in a calcineurin-dependent fashion, a short region (24 bp in length) that was necessary and sufficient for calcineurin-dependent transcriptional induction and that was subsequently named CDRE (for Calcineurin-Dependent Responsive Element). In a genetic screening they identified a novel transcription factor, Crz1p, that when overexpressed was able to bypass a requirement for calcineurin activation of a CDRE-containing reporter gene. Crz1p was found to be required for transcriptional induction of both CDRE-driven reporter genes and genomic *FKS2* in response to Ca^{2+} and physically to bind to the CDRE sequence. All of other genes up-regulated by calcineurin activation, identified using DNA microarrays, showed reduced activation in a *crz1Δ* mutant and most of them contain the Crz1p consensus CDRE motif in their promoter regions, suggesting that they also are directly regulated by Crz1p.

Crz1p appears to be the major mediator of calcineurin-regulated gene expression in yeast: *crz1Δ* phenotypes traits are similar to those of calcineurin mutants, even if *crz1Δ* strains are a quite less sensitive to ions or pheromone prolonged exposure than *cnb1Δ* strains. Moreover, *crz1Δ* and *cnb1Δ crz1Δ* double mutant strains display the same sensitivity to these described treatments. These findings suggest that Crz1p and calcineurin act in the same pathway. However, calcineurin mutants have other defects that are not mimicked in *crz1Δ* mutants, such as sensitivity to high pH and

alterations in cell cycle control. In addition, *crz1Δ* mutants exhibit Ca^{2+}-sensitive growth, whereas calcineurin deficient cells are Ca^{2+} tolerant. These findings together demonstrate that calcineurin carries additional functions that are not mediated by Crz1p (Stathopoulos and Cyert, 1997).

Calcineurin modulates Crz1p activity by regulating its cellular localization. Under resting conditions, Crz1p is distributed throughout the cell; however, following a rise in intracellular Ca^{2+} concentration, it rapidly accumulates in the nucleus in a calcineurin-dependent manner. This nuclear accumulation is readily reversible, as inhibition of calcineurin by FK506 leads to rapid re-distribution of Crz1p to the cytosol. Crz1p is transported into the nucleus through the Nmd5p importin, that is sensitive to Crz1p phosphorylation state: *in vitro* only the dephosphorylated form of Crz1p binds Nmd5p, as well as *in vivo* only the dephosphorylated form of Crz1p is efficiently imported into the nucleus. Exit of Crz1p from the nucleus is also regulated by its phosphorylation state: Crz1p nuclear export requires the Msn5p exportin, which has been reported to export only the phosphorylated form of its substrates from the nucleus. In addition, a nuclear localization signal (NLS), whose phosphorylation at the level of specific residues leads to a conformational change of Crz1p determining the accessibility of the NLS for Nmd5p binding, and a nuclear export sequence (NES), interacting with Msn5p exportin when phosphorylated on specific residues, have been identified in Crz1p sequence (Polizotto and Cyert, 2001; Boustany and Cyert, 2002).

Thus, calcineurin dephosphorylates Crz1p regulating its localization: when calcineurin activity is low, Crz1p is phosphorylated and accumulates in the cytosol due to a low rate of nuclear import and a high rate of nuclear export, conversely active calcineurin dephosphorylates Crz1p which rapidly accumulates in the nucleus due to increased nuclear import and decreased nuclear export.

In addition, a physical interaction between calcineurin and Crz1p has been observed, at a docking site on Crz1p different from the NES and NLS regions. This site was revealed a small motif with the sequence PIISIQ, required for calcineurin to dephosphorylate Crz1p and to regulate its nuclear export, mediating direct binding to calcineurin. Actually, a *crz1* mutant lacking this site shows severely impaired regulation by calcineurin *in vivo*, and fails to interact with calcineurin both *in vivo* and *in vitro* (Cyert, 2003).

In mammals, calcineurin modulates transcription through the family of nuclear factor of activated T-cells (NFAT), regulating 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 Crz1p in yeast. NFAT is composed of a cytosolic protein which, when dephosphorylated, rapidly accumulates in the nucleus and, combining with various components, activates transcription of a specific set of genes. NFAT dephosphorylation results in conformational changes that expose nuclear localization sequences and mask a nuclear export sequence. Thus, as observed for Crz1p, calcineurin causes rapid changes in NFAT localization by concerted regulation of nuclear import and export. Also NFAT physically interacts with calcineurin, through a conserved docking site.
However, although Crz1p and NFAT have remarkably similar modes of regulation, they display very little sequence similarity, except for the PxIxIT-related calcineurin docking domain, suggesting that Crz1p and NFAT clearly evolved independently (Fig. 8).

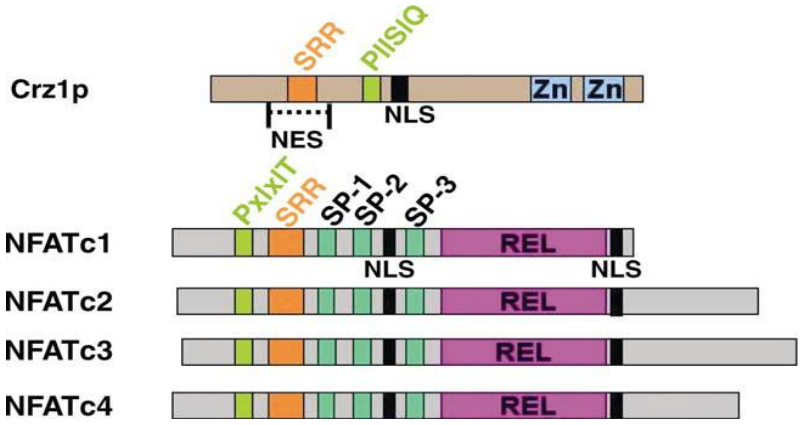


Figure 8. Schematic representation of sequence motifs in Crz1p and NFATc family members (SRR indicate serine-rich regions, SP-1,3 indicate Serine-Proline repeats, ZN indicate Zn^{2+} finger motifs, REL indicate REL-related DNA-binding domains) (Cyert, 2003).

Calcineurin-dependent regulation of calcium homeostasis

Calcineurin has a prominent role in regulating the activity of Ca^{2+} pumps and exchangers responsible for Ca^{2+} homeostasis in yeast (Fig. 9).
It turned out that the growth defect showed by mutant strains, lacking either vacuolar pump Pmc1p or Golgi-located pump Pmr1p, in high Ca^{2+} containing media, were restored by calcineurin inactivation, both by

mutation and by addition of FK506 inhibitor: these findings suggest that calcineurin activation can have a negative effect on these mutant strains growth. Also growth of these mutants in high Ca^{2+} media is restored by recessive mutations that inactivate the high-affinity Ca^{2+}-binding sites in calmodulin.

The mechanism by which external Ca^{2+} inhibits the growth of *pmc1* and *pmr1* mutants was in fact investigated by characterizing spontaneous revertants carrying secondary mutations that restore the ability of these mutants to proliferate in high Ca^{2+} media: the identified revertants carried additional mutations in *CNB1*, *CNA1* and *CNA2* genes (Cunningham and Fink, 1994).

Calcineurin appears from these experiments to inhibit a factor required for growth of *pmc1 pmr1* mutants in media containing high Ca^{2+}. Among the genes identified from a high-dosage library which, when overexpressed, restored *pmc1* mutant growth on high Ca^{2+} media, besides *PMC1* and *PMR1*, also *VCX1*, encoding for the vacuolar H^+/Ca^{2+} exchanger, was identified. Really, it was observed that the large increase in Ca^{2+} sequestration observed in *pmc1 cnb1* double mutants, relative to *pmc1* mutants, was completely dependent on *VCX1* function, since *vcx1 pmc1 cnb1* triple mutants accumulated only low levels of non-exchangeable Ca^{2+}. Furthermore, *vcx1 pmc1 cnb1* triple mutants were extremely sensitive to added Ca^{2+}. However, the additional *vcx1* null mutation in a *pmc1* strain only slightly affected Ca^{2+} accumulation.

Therefore, *VCX1* was thought to play a much larger role in Ca^{2+} sequestration in *cnb1* mutants than in strains in which calcineurin was functional (Cunningham and Fink, 1995).

These findings together seem to suggest a calcineurin-dependent positive regulation on Pmc1p and Pmr1p activity, and a negative regulation on Vcx1p activity.

Actually, calcineurin has been found to induce *PMC1* and *PMR1* genes at transcriptional level via Crz1p. *CRZ1* was revealed to be essential for the calcineurin-dependent induction of *PMR1* and *PMC1* reporter genes in response to Ca^{2+}.

As far as calcineurin-dependent Vcx1p regulation, it is likely that calcineurin inactivates Vcx1p by a post-translational mechanism because the repression of Vcx1p expression seems insufficient to account for the dramatic effects of calcineurin on *VCX1*-dependent Ca^{2+} tolerance and sequestration.

In addition, in the presence of high saline concentrations, signalling through calcineurin regulates the expression at the transcriptional level of *ENA1/PMR2A*, which encodes the P-type ATPase that is primarily

responsible for Na^+ efflux across the plasma membrane; calcineurin also post-transcriptionally activates the Trk1-Trk2 K^+ transport system to the high affinity state resulting in higher K^+/Na^+ selective uptake (Matsumoto *et al*., 2002).

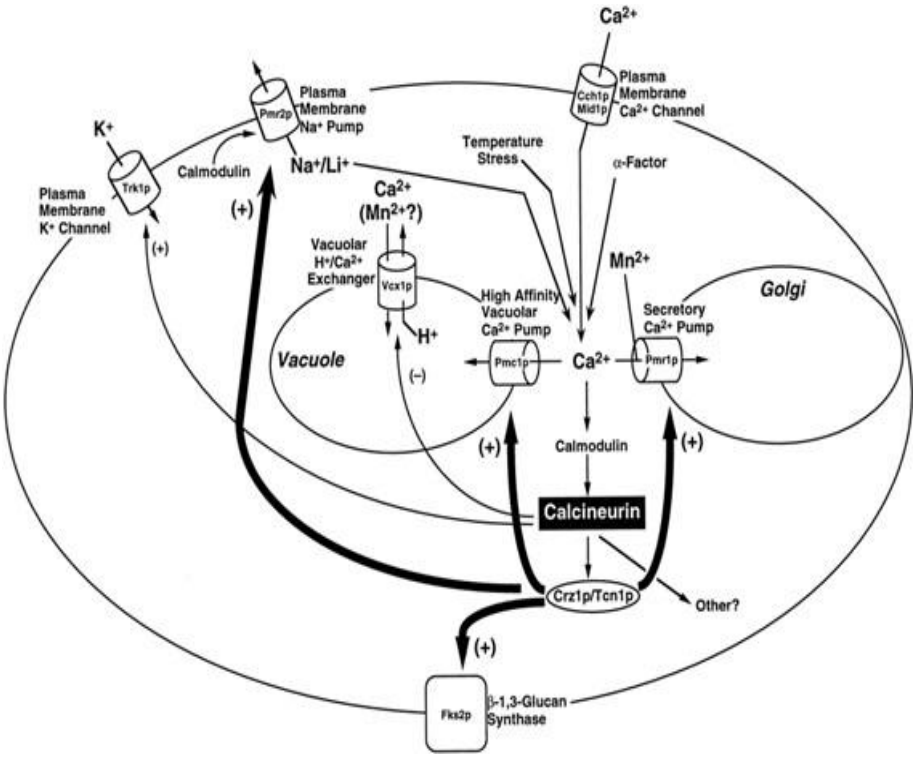


Figure 9. Role of calcineurin in regulating ion, and in particular Ca^{2+}, homeostasis in *S. cerevisiae* (Rusnak and Mertz, 2000).

Calcineurin-dependent regulation of the cell cycle

While Ca^{2+} has essential roles in mediating G_1 events in mammals, in budding yeast it seems to be required at all stages of the cell cycle, with a prominent role in regulating G_2/M transition (Iida *et al*., 1990).
The Ca^{2+}-dependent cell cycle regulation in budding yeast was identified by genetic investigations of the physiological effects of exogenous $CaCl_2$ on mutant strains lacking the *ZDS1* gene, an enigmatic gene identified in many genetic screenings for its ability to suppress the phenotypes caused by defects in various genes when it is overexpressed on a high-copy vector.

The function of Zds1p is required for transcriptional repression of the *SWE1* gene, encoding a negative regulator of the Cdc28/Clb complex, and of the *CLN2* gene, encoding the G_2 cyclin.

In the presence of high external $CaCl_2$, *zds1* mutants exhibited a characteristic phenotype, such as the formation of an elongated bud and the inhibition of the cell cycle in the G_2 phase, that was bypassed by inactivation of calcineurin or of Swe1p individually. These findings suggested that the effect of Ca^{2+} on the cell cycle progression might be exerted through the activation of calcineurin and Swe1p. Thus, calcineurin-dependent pathways seem to be required to sustain a high *SWE1* mRNA level in the G_2 phase, when its level normally in the absence of external Ca^{2+} declines to a low level, surprisingly in a Crz1p-independent manner.

However, calcineurin appears to up-regulate Swe1p multilaterally, not only at the transcriptional level. Actually, calcineurin was also found to dephosphorylate Hsl1p, an inhibitory kinase of Swe1p, delocalizing it from the bud neck and subsequently addressing it to the proteasome-dependent degradation. In addition, calcineurin promotes Swe1p accumulation by down-regulating the proteasome activity through the action on Yap1 transcription factor. *YAP1* gene, encoding a member of the YAP-family of transcriptional regulators, was in fact isolated in a genetic screening for its ability to suppress the Ca^{2+} sensitivity of *zds1* mutant strains, when overexpressed. Yap1p level really was diminished by the presence of external $CaCl_2$ in a calcineurin-dependent manner, being dephosphorylated directly by calcineurin. Yap1p is responsible for the activation of the *RPN4* gene, which encodes a transcription factor that modulates the expression levels of the genes for the ubiquitin-proteasome system. These findings together, suggest that Yap1p is down-regulated by the activation of calcineurin pathway, resulting, in collaboration with the other mechanisms for Swe1p up-regulation, in the arrest in G_2 phase of the cell cycle (Miyakawa and Mizunuma, 2007).

An overview of multilateral calcineurin-dependent regulation of the G_2/M transition inhibitor Swe1p is represented in figure 10.

Calcineurin seems to act in concert with Mpk1 pathway in regulating Swe1p kinase and therefore G_2/M transition.

Mpk1 pathway is the major responsible for the maintenance of cell wall integrity in yeast cells, in response to different stimuli such as hypotonic stress, pheromone exposure and oxidative stress. The signalling cascade is comprised of a family of cell surface sensors coupled to a small G-protein called Rho1p, which activates a set of effectors involved in the regulation of a wide variety of processes including gene expression related to cell wall biogenesis and remodelling and organization of the actin cytoskeleton.

Briefly, Mpk1 cascade is a linear pathway comprised of Pkc1p, the MEKK Bck1p, two redundant MEKs Mkk1/2 and the Mpk1p.
In addition to calcineurin, also Mpk1p seems to up-regulate at post-translational level Swe1p and Cln2p, playing a redundant function in controlling G_2/M transition. Actually, although the defect of either calcineurin or the Mpk1 MAPK cascade alone does not lead to lethality under normal conditions, simultaneous defects of these pathways are synthetically lethal.

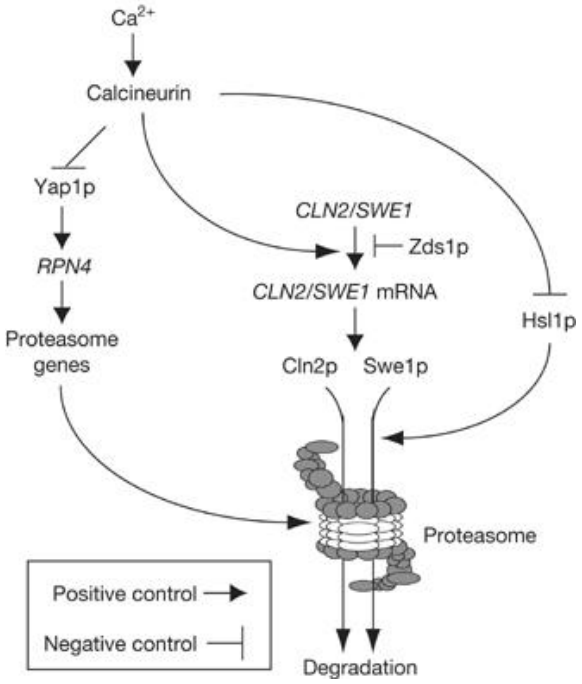


Figure 10. Model of multilateral calcineurin-dependent up-regulation of Swe1p in budding yeast (Yokoyama *et al.*, 2006).

Calcineurin also seems to play an important role in regulating bud emergence in concert with HOG (High Osmolarity Glycerol) pathway, which mediates adaptation to high osmolarity, coupling the high osmolarity signal to glycerol accumulation. This pathway regulates several stress genes for osmoprotection including *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, an enzyme involved in glycerol synthesis. HOG pathway is also involved in the regulation of the cell cycle, budding pattern, and cell division after osmotic stress. It is counteracted by Pkc1-Mpk1/Slt2 MAPK

cascade, which responds to low osmolarity and high temperature conditions maintaining cell wall integrity.
Calcineurin and HOG pathway seem to be antagonistic in the regulation of bud emergence.
The additional deletion of *HOG1* gene suppressed the lethality in a *cnb1Δ mpk1Δ* double mutant strain; in contrast, the *CNB1 MPK1* double deletion had no significant effect in the osmosensitivity of a *hog1Δ* strain suggesting that the Ca^{2+} signalling and the HOG pathways are antagonistic in the growth regulation, but not in the high osmolarity response.
In the presence of high Ca^{2+} concentrations in the medium (50 mM $CaCl_2$), bud emergence and actin polarization at the presumptive bud site were delayed in a wild-type strain, but in a *cnb1Δ* strain this delay was abrogated indicating that it occurred in a calcineurin-dependent manner. Moreover, in a *hog1Δ* strain bud emergence was significantly longer if compared with the wild-type strain, but actin polarization was only slightly affected. Thus, HOG activation seems to stimulate bud emergence at a step after actin had correctly become localized at the bud site. The Ca^{2+}-induced delay of bud emergence in the *hog1Δ* strain was only partially suppressed by *CNB1* deletion, suggesting the calcineurin inactivation can suppress the defect in actin polarization, but not in the bud emergence.
Thus, calcineurin and HOG pathways seem to regulate the process of the bud emergence at different steps and in different directions: while calcineurin negatively regulates actin polarization at the bud site, HOG pathway positively regulates bud formation at a later step after actin has polarized.
It was shown that the Ca^{2+}-induced inhibition of actin polarization in a *hog1Δ* strain was not suppressed by *CRZ1* deletion, suggesting that other calcineurin targets, which still remain to be identified, are involved in this process. It is likely that these two pathways regulate each other (Shitamukai *et al*., 2004).

Calcineurin and nutrients

Calcineurin, normally associated to the response to a wide variety of cellular stresses, has more recently been associated to the response to nutrient also.
Actually, in a genome wide-transcriptional analysis of the response of budding yeast to severe alkaline pH stress, a common stimulus able to activate calcineurin pathway, it has been observed that a large number of

genes involved in hexose transport and carbohydrate metabolism are induced after such stimulus. The *HXT2* gene, encoding the high affinity hexose transporter, accounts for one of the most potent responses to high pH and its expression is significantly reduced in calcineurin-deficient mutants.

As previously described in figure 3, *HXT2* is induced by low levels of glucose in a Snf1p-dependent manner; however, it was found that while Snf1p lack fully blocked expression of *HXT2* on low glucose, induction of *HXT2* by high pH was only partially abolished in the *snf1* mutant strain, indicating the presence of additional regulatory events not triggered by external glucose.

It was observed that the alkaline pH-dependent expression of *HXT2* was diminished by ~ 50% in wild-type cells treated with the calcineurin inhibitor FK506 or in *cnb1Δ* mutants. In addition, a role for calcineurin in *HXT2* induction was further underscored by the fact that the exposure of cells to 0.2 M $CaCl_2$ resulted in potent activation of its promoter. Remarkably, while the high pH-induced *HXT2* expression is calcineurin-dependent and isn't affected by *SNF1* deletion, induction of *HXT2* by low glucose is unaffected in the *cnb1Δ* strain.

However, it was observed that shifting cells to low glucose, in the presence of 0.2 M extracellular $CaCl_2$, resulted in additive induction of *HXT2* : it is likely that the activation of *HXT2* upon alkaline stress depends on parallel inputs mediated by Snf1p and calcineurin.

Moreover, it was found that calcineurin induces *HXT2* transcriptionally through Crz1p: Crz1p appears in the nucleus shortly after alkaline treatment (5 minutes), similarly to what is observed in calcium-treated cells, and also a CDRE consensus element was found in *HXT2* promoter. Many other genes related to glucose metabolism, and in particular genes induced by glucose limitation, 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. This novel role of calcineurin is supported also by the finding that activation of the calcineurin pathway by extracellular Ca^{2+}overrides the growth defect of a *snf1* mutant on low glucose (Ruiz *et al*., 2008).

Endogenous modulators of calcineurin activity

All known calcineurin-dependent transcriptional changes are believed to be mediated through Crz1p, thus it is likely that the major antagonistic of calcineurin activity could act on this transcription factor.

It was previously suggested that calcineurin and PKA pathways could function antagonistically in yeast, since overexpression of phoshodiesterase-encoding gene *PDE1* suppresses the Na^+ sensitivity of calcineurin-deficient cells (Hirata *et al*., 1995). Actually, it was then demonstrated that Crz1p is a direct target of PKA, that by phosphorylating it and preventing its nuclear localization, opposes to calcineurin signalling.

A physical interaction between Crz1p and PKA was demonstrated both *in vivo* and *in vitro*, and the region containing the consensus site for phosphorylation by PKA was identified within the NLS sequence of Crz1p, revealing three essential serine residues (409, 410 and 423) for PKA-dependent phosphorylation.

Actually, a fragment of Crz1p containing the NLS fused to three copies of GFP was cytosolic; however, mutations of these serine residues to alanine resulted in strong nuclear localization of the Crz1p NLS. In addition, whereas wild-type Crz1p binds the importin Nmd5p only upon dephosphorylation by calcineurin as previously described, $Crz1p^{S409,410,423A}$ mutant form interacts with Nmd5p regardless of calcineurin activity.

Thus, PKA and calcineurin pathways are connected through their regulation of a common effector, Crz1p. It is likely that PKA, whose activity is elevated when cells are grown in optimal conditions, functions to repress the stress response counteracting calcineurin pathway, thereby integrating cell growth and metabolism with environmental stimuli (Kafadar and Cyert, 2004).

Interestingly, calcineurin and PKA function antagonistically in multiple processes in mammals also. In particular, NFAT nuclear factor, which as well as Crz1p translocates from the cytosol to the nucleus upon dephosphorylation by calcineurin, when it is re-phosphorylated by PKA and other kinases, returns to the cytosol terminating the signalling.

In a screening on proteomic scale, performed in order to identify putative kinases targeting Crz1p, a novel antagonist of calcineurin signalling was identified: Hrr25p, a homolog of casein kinase I, a conserved family of kinases that regulate processes as diverse as membrane trafficking and circadian rhythm and DNA repair in yeast, was isolated for its ability to phosphorylate Crz1p *in vitro*.

As demonstrated for PKA, it has been observed that *HRR25* overexpression prevents Crz1p from efficiently accumulating into the nucleus, and

stimulates its rapid export from the nucleus, thus decreasing its ability to activate gene expression. In addition, *HRR25* overexpression results in hyperphosphorylation of Crz1p. Conversely, as expected, loss of Hrr25p activity results in increased basal and Ca^{2+}-induced Crz1p-dependent CDRE activation, as well as increased nuclear accumulation in response to low Ca^{2+} concentrations.

A stable interaction between Crz1p and Hrr25p, independently of Hrr25p catalytic activity, was found suggesting that the two proteins might form a long-lived complex.

Although significant, the effects of Hrr25p on Crz1p activity, localization and phosphorylation are partial, suggesting that additional kinases could be involved in Crz1p regulation. In fact, Crz1p phosphorylated *in vitro* by Hrr25p, is not phosphorylated to the same extent as *in vivo* phosphorylated Crz1p, as determined by observing the electrophoretic mobility. Also, in the absence of Hrr25p, Crz1p is phosphorylated, albeit to a lesser degree.

Furthermore it turned out that Crz1p activity is not regulated only through its localization: when tethered permanently in the nucleus, Crz1p is still activated by extracellular Ca^{2+} in a calcineurin-dependent manner, although the activation is less than that of wild-type Crz1p, and this activation is unaffected by Hrr25p (Kafadar *et al*., 2003).

The addition of Hrr25p to the calcineurin pathway, as well as PKA role, together suggest new possible mechanisms for Crz1p regulation in response to a changing environment.

Generally growth and stress are incompatible states: stressed cells adapt to an insult by restraining growth, and conversely growing cells keep stress responses at bay. In budding yeast a remarkable connection between growth and stress emerged from the finding that TORC2-SLM pathway, a central activator of cell growth, negatively regulates calcineurin signalling and vice versa, providing a molecular basis for the mutual antagonism of growth and stress.

TOR, a serine/threonine kinase representing a central controller of cell growth, is found in two structurally and functionally distinct protein complexes, TORC1 and TORC2. TORC2 is involved in particular in the control of polarization of the actin cytoskeleton, essentially through its two main homologous effectors, Slm1p and Slm2p.

A functional interaction between calcineurin and TOR has been suggested by a two-hybrid interaction between Cna1p or Cna2p and the TORC2 substrates Slm1p and Slm2p.

TORC2 controls transcription in budding yeast, mainly negatively, and it was found that ~ 50% of genes inhibited by TORC2 overlapped with a set of genes, whose expression was dependent on calcineurin and Crz1p. Actually,

by examining Crz1p-dependent transcription in a *tor2* mutant on different known Crz1p target genes, it was observed that these genes were induced even in the absence of any other stress, indicating that TORC2 inhibits Crz1p-dependent transcription under normal growth conditions. Furthermore, in *tor2* mutant strains, Crz1p appeared exclusively (in 15-20% of cells) or mainly (45-50% of cells) nuclear, whereas in wild-type cells it is almost exclusively cytoplasmic. Thus, TORC2 complex has been suggested to negatively regulate Crz1p activity by inhibiting its nuclear localization. In particular, in *slm1,2* mutants Crz1p accumulates in the nucleus in a calcineurin-dependent manner activating transcription of the target genes even in the absence of stress. Moreover, calcineurin inactivation suppressed the growth and actin defects of a *slm1,2* mutant, and calcineurin appeared to be constitutively active in *slm1,2* mutants, as well as in *tor2* mutants. These findings, together with the demonstrated physical interaction between calcineurin catalytic subunit and Slm1,2p, suggest that TORC2 counteracts calcineurin signalling via Slm1,2p.

Again, the logical of this mutual antagonism may be the need to cope with the conflicting states of growth and stress: TORC2 may prevent the calcineurin-activated stress response during favourable conditions, and conversely, calcineurin may prevent TOR-mediated growth during stress conditions. Thus, the inhibition of calcineurin by TORC2 could allow cells to resume growth after a stress has been overcome. The mechanism by which TORC2 complex inhibits calcineurin remains to be elucidated, also considering that any change in the cytosolic Ca^{2+} level was detected in *tor2* mutants, suggesting that TORC2 probably inhibits calcineurin by a mechanism other than the modulation of cytoplasmic calcium (Mulet *et al.*, 2006).

An analogous mechanism was found also in mammalian cells, where the mTOR-triggered skeletal myotube hypertrophy in response to insulin-like growth factor-1, can be enhanced by the calcineurin inhibitor cyclosporin A.

Materials and Methods

Strains

Escherichia coli

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

Saccharomyces cerevisiae

Table 1. Yeast strains used on this study.

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::Sp his5⁺*	This work
RT961	K601 *mid1::LEU2 yvc1::Sp his5⁺*	This work
RT962	K601 *cch1::TRP1 yvc1::Sp his5⁺*	This work
RT963	K601 *mid1::LEU2 cch1::TRP1 yvc1::Sp his5⁺*	This work

RT970	K601 *fig1::Sp his5^{+}*	This work
RT971	K601 *mid1::LEU2 fig1::Sp his5^{+}*	This work
RT972	K601 *cch1::TRP1 fig1::Sp his5^{+}*	This work
RT973	K601 *mid1::LEU2 cch1::TRP1 fig1::Sp his5^{+}*	This work
RT974	K601 *mid1::LEU2 cch1::TRP1 fig1::Sp his5^{+} yvc1::KanMX4*	This work
RT1000	K601 *fig1::Sp his5^{+} yvc1::KanMX4*	This work
RT990	*MATα ade 2-1 can 1-11 his 3-11,15 leu 2-3,112 trp 1-1 ura 3-1 cdc35::KanMX pde2::TRP1 yak1::LEU2*	Görner *et al*., 2002
RT1170	K601 *cnb1::Sp his5^{+}*	This work
RT1174	K601 *mid1::LEU2 cch1::TRP1 fig1::Sp his5^{+} cnb1::KanMX4*	This work
RT1251	K601 *ecm7::Sp his5^{+}*	This work
RT1250	K601 *mid1::LEU2 cch1::TRP1 fig1::Sp his5^{+} ecm7::KanMX4*	This work
RT1252	K601 *cls2::Sp his5^{+} yvc1::KanMX4*	This work
RT1253	K601 *cls2::Sp his5^{+}*	This work
RT1254	K601 *mid1::LEU2 cch1::TRP1 yvc1::Sp his5^{+} cls2::KanMX4*	This work
RT1255	K601 *yor365c::Sp his5^{+}*	This work
RT1256	K601 *mid1::LEU2 cch::TRP1 fig1::Sp his5^{+} yor365c::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).

Growth conditions and cultural media 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 Amp^R 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) whith 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 (OD_{600}) or by Coulter Counter (Coulter Electronics Z2).

Mutant strains creation

YVC1, *FIG1*, *CNB1*, *ECM7*, *CLS2* and *YOR365c* genes were deleted in wild-type and 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*$^{+}$ from *Schizosaccharomyces pombe*, as a template (Wach *et al*., 1997). The primers are listed in the Table 2.
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.
YVC1, *CNB1*, *ECM7*, *CLS2* and *YOR365* genes deletion in the *mid1::LEU2 cch1::TRP1 fig1::Sp his5*$^{+}$ mutant strain, as well as *CLS2* and *FIG1* deletion in the *yvc1::Sp his5*$^{+}$ mutant strain, 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 used for the previously described PCR, listed in the Table 2. 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.

Table 2. Primers used in this study to create mutant strains.

Gene	Primer	Sequence
YVC1	Yvc1pFA6aFOR	ATTCAGTTATAAAATATAATATTACTAGAAC AGGAGCATT**CGGATCCCCGGGTTAATTAA**
YVC1	Yvc1pFA6aREV	TTCTGAGAAATTAATTAAGCAGTATTTGAAC ACATGTCGT**TGGATCTGATATCATCGATG**
FIG1	Fig1pFA6aFOR	GTAAACAAACAAACAAACAAACAAAAAAA AAAAAAAAAAA**CGGATCCCCGGGTTAATTAA**
FIG1	Fig1pFA6aREV	TTTTATCCTCAAATAAACATATAAGTTTTGAG CGGATATT**TGGATCTGATATCATCGATG**
CNB1	Cnb1pFA6aFOR	TTAAAAATCACTAGTTTCTTTTTTAGCGGAAT GCAATAAA**CGGATCCCCGGGTTAATTAA**
CNB1	Cnb1pFA6aREV	CGTATTATTCTTCTTTTCTTAAAAATATTGGC ATACCATA**TGGATCTGATATCATCGATG**
ECM7	Ecm7pFA6aFOR	TTAGTTTCATTTACACCATTTCTTTGTGTATC AGTCATTG**CGGATCCCCGGGTTAATTAA**
ECM7	Ecm7pFA6aREV	TTTTCTCTTCTTCGTTGCTTAATGATGTTTAG TTCTGAAA**TGGATCTGATATCATCGATG**
CLS2	Cls2pFA6aFOR	TGAAAGAAATGTTTGTAGGCCATTTTCTTCC AGAACAGAT**CGGATCCCCGGGTTAATTAA**
CLS2	Cls2pFA6aREV	GAAGAACATCCTAGTAATTGTCTTGAAAATA ATCAAGATA**TGGATCTGATATCATCGATG**
YOR365c	YOR365pFA6aFOR	ATATTTTGGAAGGATGATGAAGCAAAGTCG TAATGCTATT**CGGATCCCCGGGTTAATTAA**
YOR365c	YOR365pFA6aREV	ATTTAGTTCCGAGATGGTCTTACAACCTTTC AAATTCCAA**TGGATCTGATATCATCGATG**

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 H_2O.
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):

TAE buffer	Tris-HCl	242 g
	glacial acetic acid	57.1 ml
	EDTA	18.61 g
		dH_2O 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 10^7 cells /ml).
2. Centrifuge cells at 4000 rpm for 15 min.
3. Discard the supernatant and resuspend cells in 200 µl of SZB buffer:

SZB buffer	sorbitol	1 M
	Na citrate	0.1 M
	EDTA	0.06 M, pH 8
	2β-mercaptoethanol	115 mM
	zymoliase	100000 U/g (0.2 g ml)

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 and mix by inverting.

SDS/TE	SDS	2%
	Tris-HCl	100 mM, pH 9
	EDTA	10 mM

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 dH_2O.

Trasformation 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 OD_{600} 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 H_2O. 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 10^9 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 H_2O 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 H_2O.
12. Resuspend the pellet in 50 µl of sterile H_2O, spread the cells on the appropriate selective medium plates and incubate for 2-3 days at 30°C.

Growth curves during shift-up from ethanol to glucose

The growth curves presented in this work are referred to cell populations subjected to a shift-up from ethanol to glucose.
Briefly, cells were grown in YP medium containing 2% ethanol as sole carbon source, until exponential phase (~ 6-7 x 10^6 cells/ml). In this phase 2 or 3 samples were collected every 1.5 hours in order to determine cellular density and the percentage of budded cells within the population. At time zero (t_0), 2% glucose was added to the culture: at fixed times samples were collected in order to determine cellular density and budded cells percentage.

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 glucose

1. Inoculate yeast strains in YPD or minimal medium (3 OD/treatment in YPD medium, 6 OD/treatment in minimal medium) from a culture grown overnight in SD (-ura) medium and grown overnight at 30°C.
2. Harvest exponentially growing cells (5-6 x 10^6 cells/ml) by filtration on nitrocellulose filters (Millipore, pore sizes 22 µm) and wash twice with cold water (1 volume/wash).
3. Recover the filter and resuspend the cells in 100 mM 2-(N-morpholino)-ethanesulphonic acid (MES)/Tris, pH 6.5 (MES/Tris buffer) at a density of about 10^8 cells/ml.
4. Incubate the cells at room temperature for 1 h, with shaking (starvation).
5. Centrifuge at 4000 rpm for 10 min. Discard the supernatant.
6. Resuspend the cells in 100 mM MES/Tris buffer, pH 6.5, at a densitiy of 2.5 x 10^9 cells/ml. Transfer the cellular suspension in a 1.5 ml microcentrifuge tube.
7. Centrifuge at 7500 rpm for 2 min. Discard the supernatant.
8. Resuspend the cells in 10 µl of 100 mM MES/Tris buffer, pH 6.5, for each treatment.
9. Add 50 µM coelenterazine (stock solution 1 µg/µl dissolved in 99.5% methanol, conserved in the dark at -20°C, Molecular Probes).
10. Incubate at room temperature in the dark for 20 min.
11. Centrifuge at 7000 for 2 min. Discard the supernartant in order to eliminate the excess coelenterazine.
12. Wash three times with 100 mM MES/Tris buffer, pH 6.5, (200 µl/wash) and by centrifugation at 7000 rpm for 1-2 min.
13. Resuspend the cells in the adequate volume of 100 mM MES/Tris buffer, pH 6.5, (200 µl/treatment).
14. Transfer the cellular suspension into the luminometer tubes (200 µl each), in the presence of solution of $CaCl_2$, of the calcium chelator ethylene glycol tetraacetic acid (EGTA) and, when indicated, of the metals (stock solutions prepared in 100 mM MES/Tris buffer, pH 6.5) or inhibitors (gadolinium chloryde hexahydrate, Nifedipine dissolved in DMSO, Verapamil hydrochloryde, Sigma-Aldrich), at the final concentrations indicated in the legends of the figures.
15. Monitor light emission with the luminometer at intervals of 10 s for 1 min. before (until the signal is stable) and for at least 6 min. after the addition of glucose to a final concentration of 100 mM (stock solution 1 M in 100 mM MES/Tris buffer, pH 6.5). Light emission is reported in relative luminescence units (RLU/s).

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 $CaCl_2$ in 100 mM MES/Tris buffer, pH 6.5), monitoring light emission for 18 min. This maximum intensity was used to normalize light emission according to the amount of aequorin expressed.

Free Ca^{2+} concentration in the presence of EGTA was determined using an on-line available Max-Chelator program (Patton *et al*., 2004).
Dose-response curves were constructed by calculating the initial shape of the peak in light emission curve, and plotting it in a semi-log plot as a function of external calcium concentration. Apparent K_m was calculated by fitting the curve with a Hill function in the form $y = y_0 + (y_{max} - y_0)[Ca^{2+}]/(K_m + [Ca^{2+}])$.
Competition curves were constructed by plotting the percentage of inhibition of the maximal response as a function of the concentration of the added competitive metal or inhibitor. Apparent IC_{50} was calculated by fitting the curve with a Hill function in the form $I(Me) = [1 + (IC_{50}/[Me])^n]^{-1}$, where IC_{50} is the concentration at half maximal block, and *n* is the Hill coefficient. K_i was calculated by plotting apparent K_m values, calculated in presence of different concentrations of metal on Lineweaver-Burk plots, on inhibitor concentration, and fitting them to the linear equation: apparent $K_m = K_m (1+ [I]/K_i)$.

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 10^6 cells/ml) by centrifugation at 4000 rpm for 10 min.
3. Resuspend the cells in YPD medium at a density of about 10^8 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 solution of $CaCl_2$, of the calcium chelator ethylene glycol tetraacetic acid (EGTA), (stock solutions prepared in H_2O) and, when indicated, of the inhibitors (gadolinium chloryde hexahydrate, Nifedipine dissolved in DMSO, Verapamil hydrochloryde, Sigma-Aldrich), 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 H_2O. Light emission is reported in relative luminescence units (RLU/s).

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.

Calcineurin reporter assay

1. Inoculate yeast strains, transformed with pAMS366 plasmid, in YPD or minimal medium, with or without addition of either 3 mM cAMP, 1 mM $CaCl_2$ or 1 mM EGTA when indicated, from a culture grown overnight in SD (-ura) medium and grown overnight at 30°C.
2. Harvest exponentially growing cells (5-6 x 10^6 cells/ml), (2.5 x 10^6 cells/treatment needed), by filtration on nitrocellulose filters (Millipore, pore sizes 22 µm) and wash twice with cold water (1 volume/wash).
3. Recover the filter and resuspend the cells in 100 mM 2-(N-morpholino)-ethanesulphonic acid (MES)/Tris, pH 6.5 (MES/Tris buffer, (200 µl/treatment).
4. Take immediately after filtration 200 µl of cellular suspension ("exponentially growing" treatment), collect cells by centrifguation

at 13000 rpm for 2 min., resuspend the cells in 550 µl of pre-cold Z-buffer and quickly freeze in dry ice and conserve at -80°C

Z-buffer	NaH_2PO_4	50 mM
	Na_2HPO_4	75 mM
	KCl	10 mM
	$MgSO_4$	1 mM

5. Incubate the remaining cellular suspension at room temperatur for 1.5 h, with shaking (starvation).
6. Separate 200 µl-aliquots in 1.5 ml microcentrifuge tubes, and collect cells either immediately after starvation ("90-min starved" treatment), or after the induction with 100 mM glucose for 1 h (stock solution 1 M glucose in 100 mM MES/Tris buffer, pH 6.5) at room temperature in the presence of 1 mM $CaCl_2$ ("$CaCl_2$ + glc" treatment) or 1 mM ethylene glycol tetraacetic acid (EGTA) ("EGTA + glc" treatment), (stock solutions 10X in 100 mM MES/Tris buffer, pH 6.5), and process as described in passage 4.

Dosage of β-galactosidase activity

This assay takes advantage of β-galactosidase ability to hydrolize its chromogenic substrate CPRG (chlorophenol-red-β-D-galactopyranoside), consisiting of a galactose unit bound through a β(1,4) link to a chlorophenol-red molecule. When this compound is hydrolized, released chlorophenol-red becomes quantifiable at 574 nm. By measuring the speed formation of chlorophenol-red, it is possible to determine β-galactosidase activity present in solution.

1. Thaw simultaneously all the sample, conserved at -80°C.
2. Remove from each sample a 100 µl-aliquot and use it to determine the OD_{600} (diluted in cuvette 1:10).
3. Add to the remaining cells 100 µl of zymolyase solution (zymolyase T20, ICN, USA; 0.5 mg/ml in Z-buffer) and incubate at 37°C for 1 h, with occasional shaking.
4. Maintaining the cells at 37°C, add 100 µl of CPRG (chlorophenol-red-β-D-galactopyranoside, Sigma-Aldrich; 4

mg/ml in Z-buffer) and, after incubation for suitable time, stop the reaction by adding 200 µl of 1 M Na_2CO_3.

5. Centrifuge at 13000 rpm for 15 min.
6. Transfer the superanatant in cuvette and read at 574 nm.

β-galactosidase activity, expressed in Miller Units (MU), is calculated as follows: $MU = [(OD_{574} – OD_{574}\ ctrl) \times 1000]/(OD_{600} \times t_{inc})$.
Each value is the average of two independent extracts of the same strain.
The experiments were repeated at least three times giving similar results.

Results

Characterization of glucose-triggered Ca^{2+} signal

Glucose addition to nutrient-starved yeast cells induces a rapid and transient rise in cytosolic Ca^{2+} level, reaching its maximum within 100-120 s in presence of extracellular Ca^{2+}. The timing of this response is peculiar in W303 background, where two peaks are observed, a first and generally higher at 60 s and a second one at 180 s.

This Ca^{2+} signal appears to be mediated almost exclusively by an influx of Ca^{2+} from the extracellular environment, as demonstrated by the very faint Ca^{2+} peak observed in presence of the extracellular Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) (Tisi *et al*., 2002).

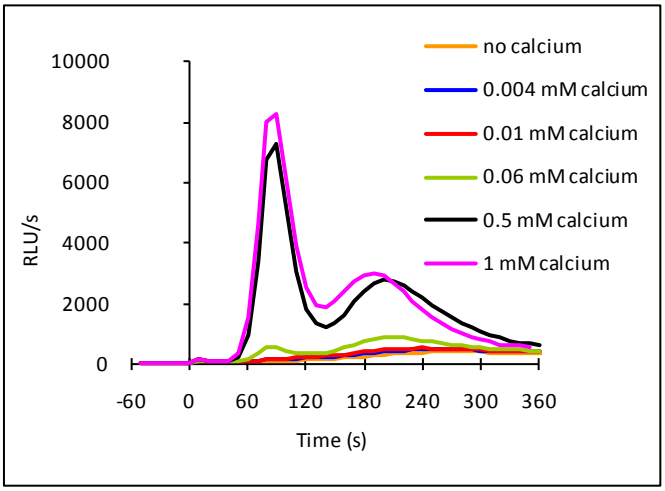
Glucose-induced Ca^{2+} (GIC) signalling is modulated by nutrients: it is much higher in YPD-growing cells than that observed in minimal medium-growing cells, confirming the literature data about pheromone-induced Ca^{2+} signalling, where the signal observed in YPD medium is more than 10 times higher than that observed in minimal medium (Muller *et al*., 2001).

In the wild-type strain, as well as in all the mutant strains inactivated in known Ca^{2+} transporters (data not shown), grown either in YPD or minimal medium, GIC signalling is dependent on extracellular Ca^{2+} concentration (Fig. 11A and B).

HACS system was largely characterized, in cells overexpressing both Mid1 and Cch1 subunits, revealing a K_m of 12 µM (Teng *et al*., 2008), whereas LACS system was reported with a K_m of ~ 3 mM in response to pheromone exposure (Muller *et al*., 2001).

Interestingly, glucose-induced calcium response seems not to involve such low affinity system: though the large standard deviation intrinsically related to the bioluminescent technique used in our reported experiments, we estimated, as described in Materials and Methods section, the apparent K_m in the YPD-growing wild-type strain as 56.2 ± 4.2 µM, suggesting that this response is mediated by high affinity calcium transporter systems.

A



B

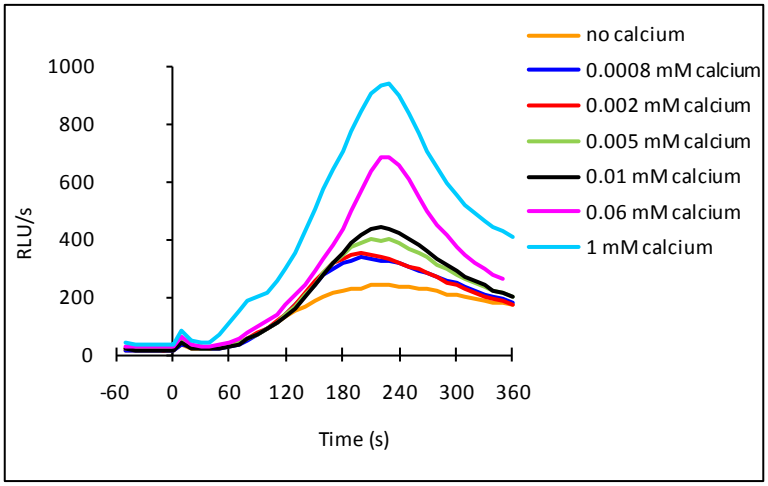


Figure 11. GIC signalling is modulated by the cultural medium. Aequorin-expressing wild-type cells grown in YPD (panel A) or in minimal medium (panel B), were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of the indicated extracellular free calcium concentrations, calculated as described in Materials and Methods section.

A novel high affinity calcium channel mediates GIC response

In order to investigate the calcium transporters involved in GIC response, we monitored Ca^{2+} signal in mutant strains carrying the deletion of *FIG1* gene, encoding the only component of LACS system identified up to now, alone or together with *MID1* and *CCH1* encoding HACS subunits.
None of the mutants analysed, *mid1Δ cch1Δ*, *fig1Δ* and *mid1Δ cch1Δ fig1Δ*, showed any seriously impaired response to glucose during growth in YPD medium, suggesting that the known transporter systems play only a minor role in mediating Ca^{2+} influx in these growth conditions (Fig. 12).

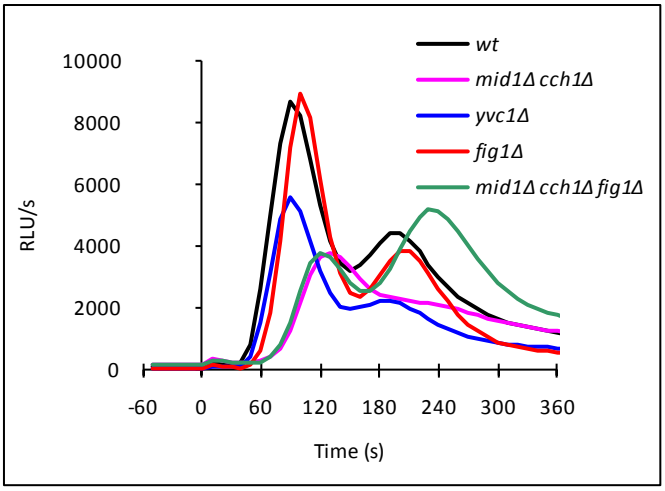


Figure 12. Deletion of known transporters only marginally affects GIC response in YPD medium. Wild-type and *mid1Δ cch1Δ*, *yvc1Δ*, *fig1Δ*, *mid1Δ cch1Δ fig1Δ* mutant cells expressing the bioluminescent aequorin protein, grown in YPD medium, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of 1 mM extracellular $CaCl_2$.

As we can observe in the Fig. 12, although Fig1p was reported to be the major responsible for pheromone-induced Ca^{2+} influx during growth in YPD medium, its deletion has a negligible effect on GIC response. Conversely, HACS inactivation affects more seriously GIC response, whereas it is reported to be fully functional during growth in minimal medium and down-regulated by calcineurin in rich media (Muller *et al*., 2001). Basing on our experiments, however, HACS and LACS systems seem not to play an essential role in mediating GIC response during growth in rich media. Furthermore, *yvc1Δ* mutant strain, inactivated in the vacuolar Yvc1p TRP-

homolog Ca^{2+} channel, displayed only a slight reduction in GIC signal, suggesting that it could be involved in the amplification of this signal: this is likely, considering that Yvc1p is known to mediate Ca^{2+} release into the cytosol from the vacuole by mechanical activation and a Ca^{2+}-induced Ca^{2+} release mechanism (Palmer *et al*., 2001). In addition, it was recently demonstrated that Yvc1p, in a combined action with the vacuolar Ca^{2+}/ATPase pump, plays an essential role in the control of intracellular Ca^{2+} availability and activation of the plasma membrane H^{+}/ATPase in conditions of external calcium shortage. Moreover, Yvc1p seems to participate in glucose-induced intracellular calcium signalling in IP_3-dependent manner, although there are no evidence suggesting that IP_3 would act directly on Yvc1p leading to the opening of this channel and then increasing cytosolic calcium level in the glucose-induced process (Bouillet *et al*., 2011) and although this signal requires the presence of at least some traces of external calcium.

Surprisingly, in the *mid1Δ cch1Δ fig1Δ* triple mutant strain GIC signal is still evident, even if the biphasic signature typical of W303 background is affected: in this mutant, in fact, the later peak at 180 s becomes the major component of this response.

These findings together suggest that in YPD-growing cells a novel Ca^{2+} transporter, still unidentified at the molecular level, mediates the main part of Ca^{2+} influx in response to glucose after nutrient starvation. Moreover, in the *mid1Δ cch1Δ fig1Δ* mutant strain also, as it was found for the wild-type strain, GIC response is dependent on phospholipase C activity, since it was completely abolished in presence of the Plc1p inhibitor 3-nitrocumarin (Fig. 13).

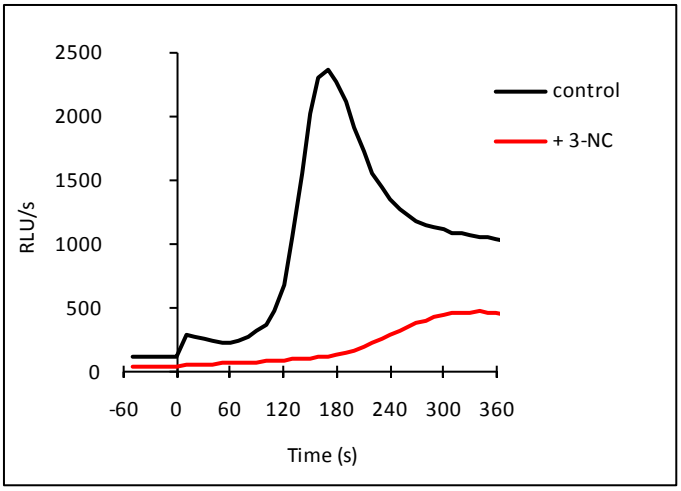
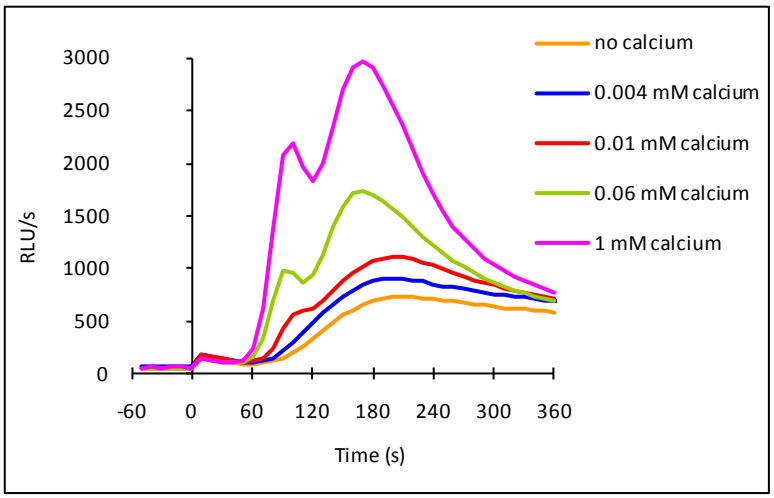


Figure 13. GIC signalling is dependent on Plc1p activity. *mid1Δ cch1Δ fig1Δ* cells expressing the bioluminescent aequorin protein, grown in YPD medium, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of extracellular 1 mM $CaCl_2$ and, when indicated, of 50 µg/ml 3-nitrocumarin, added 15 min before glucose stimulus.

In order to better characterize the Ca^{2+} signal mediated by this unknown transporter, which hereafter will be called GIC (for Glucose-Induced Calcium) channel, GIC response was analysed in detail in YPD-growing *mid1Δ cch1Δ fig1Δ* cells (Fig. 14A).

A



B

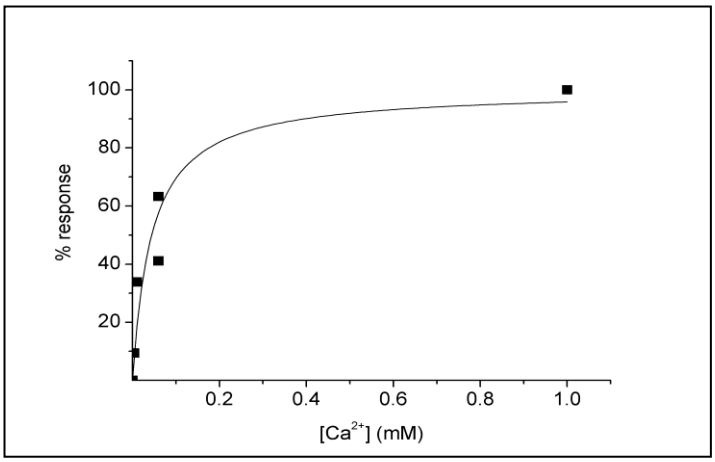


Figure 14. *mid1Δ cch1Δ fig1Δ* cells, expressing the bioluminescent aequorin protein and grown in YPD medium, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of the indicated extracellular free calcium concentrations (panel A). The apparent K_m for calcium of the GIC response in YPD medium grown *mid1Δ cch1Δ fig1Δ* cells, evaluated as described in Materials and Methods section, is 43.8 ± 10.3 µM (panel B).

The response was still appreciable at low-submillimolar Ca^{2+} concentrations, suggesting that GIC transport could rely on a high affinity Ca^{2+} channel. Actually, by creating a dose-response curve, the apparent K_m for calcium in the *mid1Δ cch1Δ fig1Δ* strain was estimated 43.8 ± 10.3 µM, not so far from

that calculated for a wild-type strain in the same growth conditions (Fig. 14B).
Having observed such a difference in GIC response between YPD and minimal medium growing cells, GIC signalling was also monitored in the same previously described mutant strains grown in minimal medium (Fig. 15).

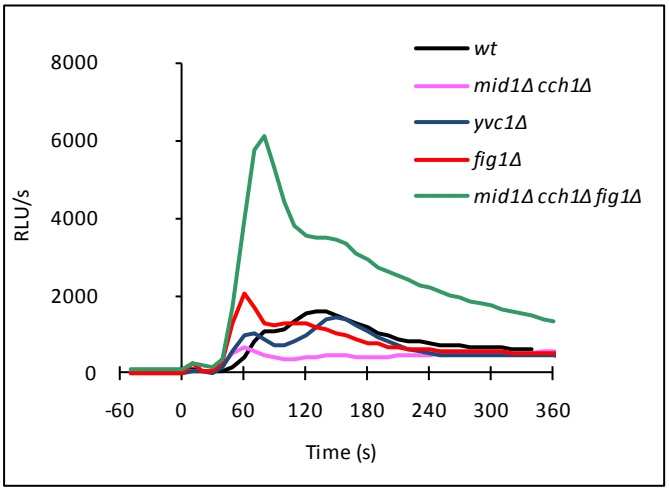


Figure 15. Deletion of known transporters seriously affects GIC response in minimal medium. Wild-type and *mid1Δ cch1Δ*, *yvc1Δ*, *fig1Δ*, *mid1Δ cch1Δ fig1Δ* mutant cells expressing the bioluminescent aequorin protein, grown in minimal medium, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of 1 mM extracellular $CaCl_2$.

As it can be observed in Fig. 15, HACS inactivation affects GIC response more seriously in minimal medium-growing cells than in YPD-growing cells, consistently with its prominent role in mediating pheromone-triggered Ca^{2+} influx in these growth conditions reported in literature (Muller *et al*., 2001). Surprisingly, the *mid1Δ cch1Δ fig1Δ* mutant strain makes no differences as far as GIC signal is concerned, either if it is grown in YPD or minimal medium, showing a significant increase in the response in comparison to the wild-type strain in the last condition. Thus, GIC transporter, which is fully functional during growth in rich media, driving the major part of glucose-triggered Ca^{2+} influx, appears to be inhibited by Fig1 protein during growth in minimal medium, since it becomes fully functional in these growth conditions only in the absence of the LACS component.

By analysing in detail GIC response in *mid1Δ cch1Δ fig1Δ* cells, grown in minimal medium, it appears that Ca^{2+} signal timing and signature is comparable with that obtained for the same mutant strain grown in YPD medium, though the large variability intrinsically related to the bioluminescent technique used in these experiments (Fig. 16 and 14A).

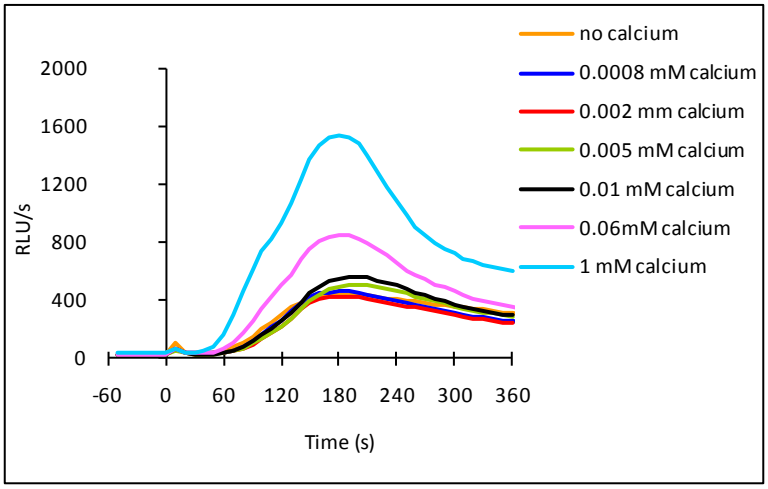


Figure 16. *mid1Δ cch1Δ fig1Δ* cells, expressing the bioluminescent aequorin protein and grown in minimal medium, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of the indicated extracellular free calcium concentrations.

Pharmacological and functional characterization of the unknown GIC transporter

No sequence resembling mammalian Ca^{2+} channels and transporters, besides the known HACS and LACS components, was identified in the whole yeast genome sequence. In order to better characterize the putative unknown GIC transporter, we tested the sensitivity of GIC response to various inhibitors of Ca^{2+} transport.
In particular, we chose to analyse the effects on GIC response of different divalent cations and of common Ca^{2+} channel blockers in current use for the inhibition of L-type voltage-gated Ca^{2+} channels in mammals.

Mg^{2+} acts as a competitive inhibitor on GIC transporter

Mg^{2+} is the most abundant divalent cation in cells, where it plays essential roles as cofactor in many catalytic processes. Cellular Mg^{2+} concentration is maintained in the millimolar range (~ 15 to 20 mM), some three orders of magnitude higher than those of Ca^{2+}.

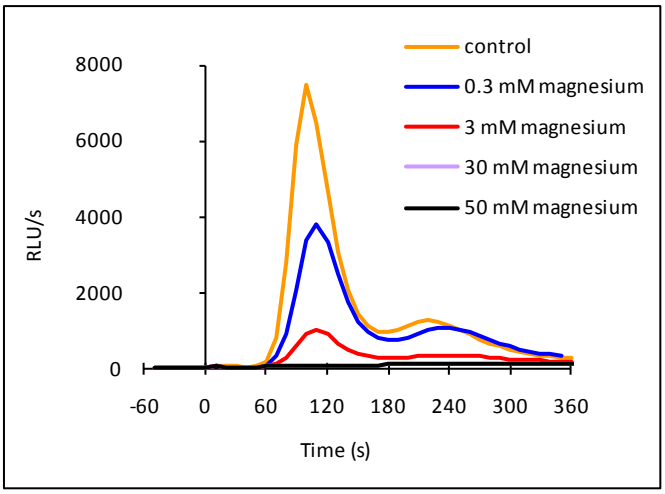
Mg^{2+} and Ca^{2+} ions are known to affect each other in a competitive way such as that high Mg^{2+} results in low Ca^{2+} and vice versa. Actually, the impaired growth of yeast cells in high Ca^{2+} conditions is ameliorated by the presence of Mg^{2+} salts in the medium and an effect of Mg^{2+} withdrawal on the opening of Ca^{2+} channels and influx of Ca^{2+} appears to be likely (Wiesenberger *et al.*, 2007).

Glucose-triggered Ca^{2+} signalling appears to be very sensitive to the presence of $MgCl_2$ in the culture medium, being significantly reduced by Mg^{2+} concentrations lower than 1 mM and completely abolished at concentrations higher than 30 mM (Fig. 17A).

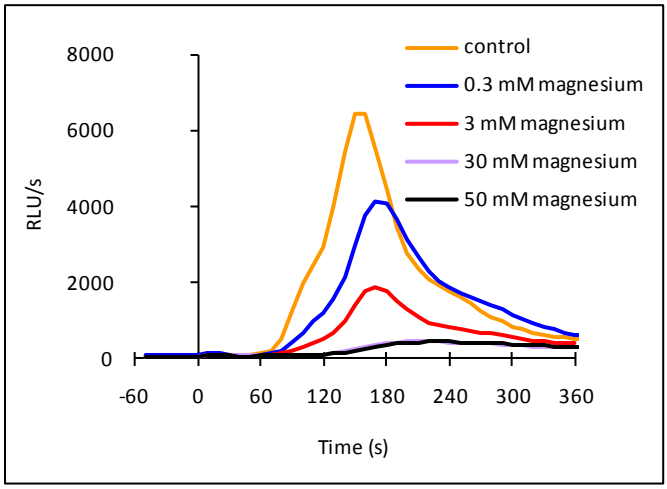
By creating a dose-response curve the apparent IC_{50} for Mg^{2+} was estimated, in the YPD-growing wild-type strain, as 0.52 ± 0.03 mM.

The sensitivity to Mg^{2+} is only slightly relieved in mutant strains impaired in HACS system; however, the *mid1Δ cch1Δ fig1Δ* mutant strain is still sensitive to Mg^{2+}, showing an apparent IC_{50} of 1.04 ± 0.05 mM, revealing a lower sensitivity if compared with the wild-type strain (Fig. 17B and C).

A



B



C

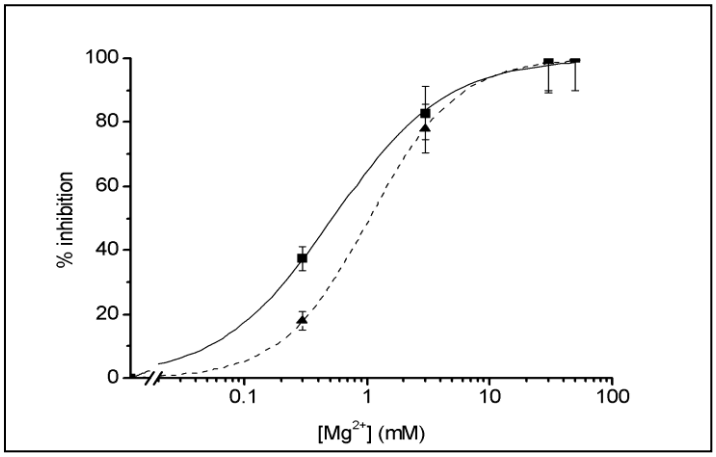


Figure 17. GIC signalling is sensitive to Mg^{2+}. Wild-type (panel A) and *mid1Δ cch1Δ fig1Δ* (panel B) aequorin expressing cells, grown in YPD, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of 1 mM $CaCl_2$ and the indicated concentrations of $MgCl_2$. The percentage of inhibition of total calcium response was reported in panel C (squares and solid line for wild-type strain, triangles and dashed line for *mid1Δ cch1Δ fig1Δ* mutant strain).

The difference observed in sensitivity to Mg^{2+} is reliable, confirming that the target of inhibition is calcium transport and not some other components of the pathway involved in the signal transduction.

Mg^{2+} is reported binding to an extracellular site to regulate Ca^{2+} influx in mammalian L/T-type Ca^{2+} channels, acting as a competitive inhibitor; thus, we investigated its mechanism of action also in budding yeast cells. Fitting of the curves of inhibition, obtained from the experiments reported in figures 17A and B to Hill function, as explained in Materials and Methods section, gave *n* values not far from 1, suggesting a simple competitive inhibition to the same ligand site. Actually, by determining the initial slope of GIC response in the *mid1Δ cch1Δ fig1Δ* strain while co-varying the Ca^{2+} and Mg^{2+} concentrations, a kinetic typical of a competitive inhibition was found, giving a K_i of 0.33 ± 0.05 mM and a constant V_{max} (Fig. 18).

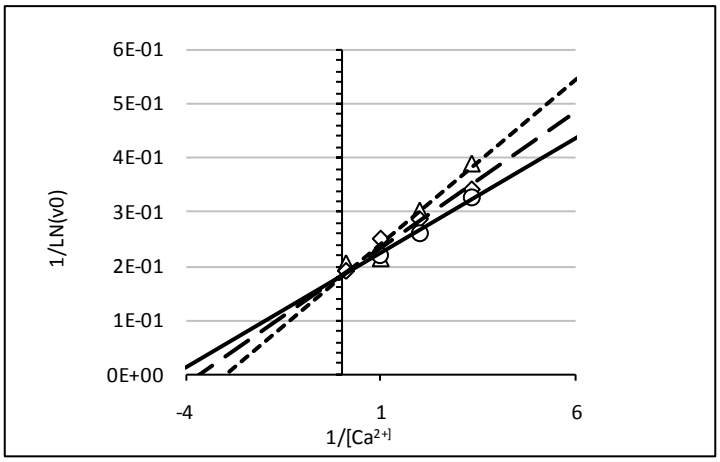


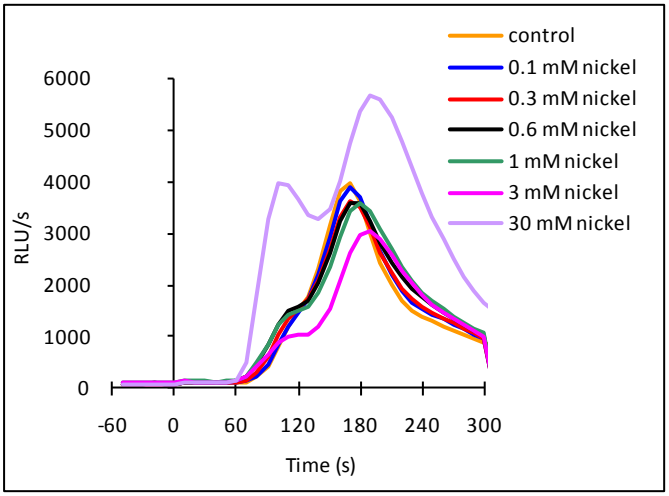
Figure 18. Mg^{2+} displays a competitive inhibition mechanism on GIC-mediated response. The Lineweaver-Burk plot of calcium response initial slope (v_0) in the *mid1Δ cch1Δ fig1Δ* strain was here reported in the presence of the following Mg^{2+} concentrations: circles and solid line, 0.3 mM; diamonds and dashed line, 0.5 mM; triangles and dotted line, 1.5 mM.

GIC transporter displays some atypical pharmacological features

GIC response was also analysed concerning its sensitivity to several bivalent cations in the wild-type and *mid1Δ cch1Δ fig1Δ* mutant strains, in order to characterize the unknown channel sensitivity to metal ions.
Wild-type and mutant strains displayed no difference in sensitivity to Zn^{2+} and Mn^{2+}, showing only half inhibition of the response in presence of millimolar concentrations of metals (data not shown).

Interestingly, the sensitivity to Ni^{2+}, a common strong blocker of mammalian T/L type of voltage-gated Ca^{2+} channels, was different.
GIC response in wild-type YPD-growing cells was not very sensitive to Ni^{2+}, reaching a maximum inhibition of less than 20% of the total response in presence of Ni^{2+} concentrations higher than 1 mM (data not shown). Conversely, GIC response in *mid1Δ cch1Δ fig1Δ* YPD-growing cells was completely resistant to Ni^{2+}, even in presence of submillimolar concentrations. Furthermore, at the highest Ni^{2+} concentration tested GIC response seemed even to be stimulated (Fig. 19A). This effect was revealed being dependent on Yvc1p-dependent amplification signal: by testing GIC response sensitivity to Ni^{2+} in a *mid1Δ cch1Δ fig1Δ yvc1Δ*, in order to get rid of any interference caused by Yvc1p activity, it was found that Yvc1p is actually responsible for signal stimulation at higher concentration of Ni^{2+}; anyway, even in the absence of Yvc1p, Ni^{2+} is unable to inhibit GIC-mediated response over 20% (data not shown).

A



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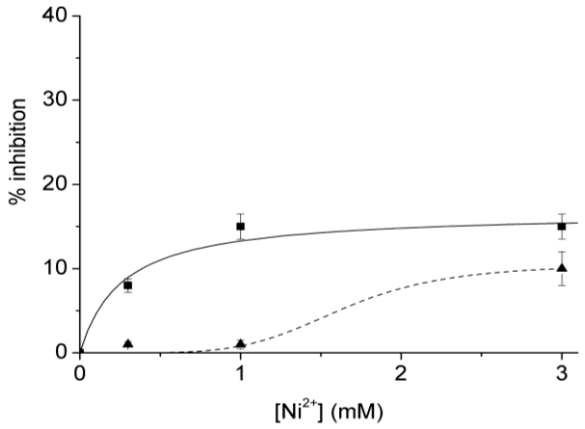
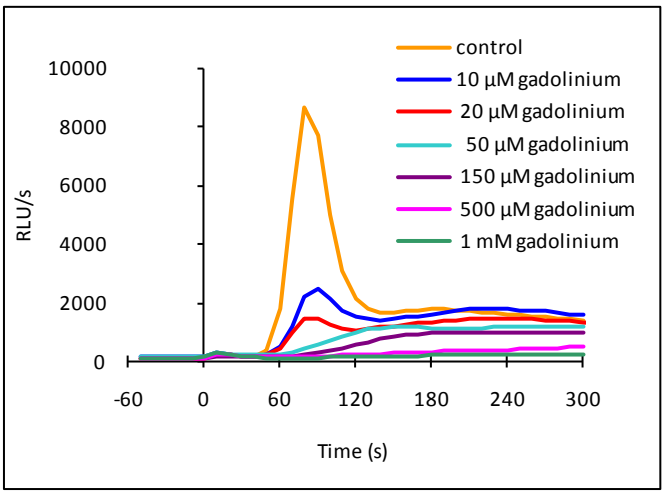


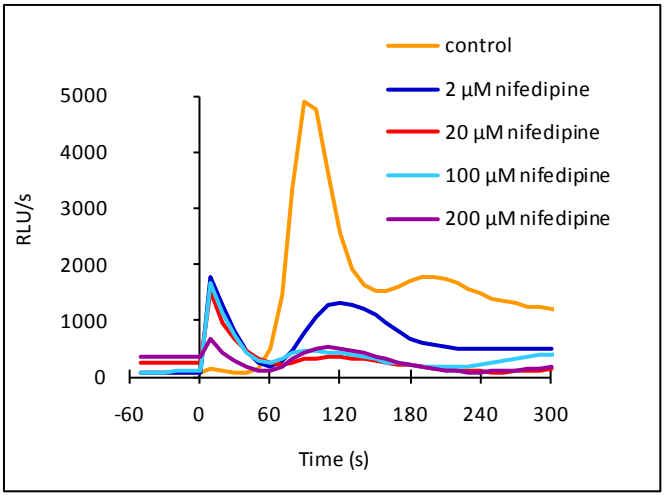
Figure 19. GIC unknown transporter is resistant to Ni^{2+} ions. *mid1Δ cch1Δ fig1Δ* aequorin expressing cells, grown in YPD, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of 1 mM $CaCl_2$ and the indicated concentrations of $NiCl_2$ (panel A). The percentage of inhibition of total calcium response was reported in panel B (squares and solid line for wild-type strain, triangles and dashed line for *mid1Δ cch1Δ fig1Δ* mutant strain).

Testing the sensitivity to many drugs, commonly used as blockers for mammalian L-type VGCCs, was revealed in the past very useful in order to characterize calcium channels, considering for example that this method demonstrated Cch1p homology to α_1 subunits of mammalian L-type VGCCs. We chose three different compounds, gadolinium which is specific for stretch-activated ion channels, nifedipine, a dihydropyridine inhibitor specific for blood vessels smooth muscle L-type Ca^{2+} channels, and verapamil, a phenylalkylamine inhibitor effective on both vascular and cardiac L-type Ca^{2+} channels (Hockerman *et al*., 1997). Furthermore, these compounds were revealed being effective in inhibiting Ca^{2+} transport also in yeast cells (Teng *et al*., 2008).
In order to verify if these compounds were effective on GIC transporter also, the following experiments were all performed in the *mid1Δ cch1Δ fig1Δ yvc1Δ* mutant strain, in order to eliminate any interference by Ca^{2+} release from vacuoles through Yvc1p activation (Fig. 20).

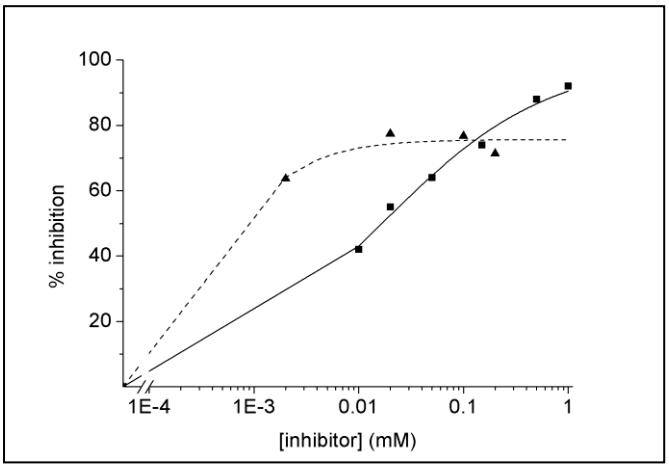
A


control
10 µM gadolinium
20 µM gadolinium
50 µM gadolinium
150 µM gadolinium
500 µM gadolinium
1 mM gadolinium
RLU/s
10000
8000
6000
4000
2000
0
-60
0
60
120
180
240
300
Time (s)

B


control
2 µM nifedipine
20 µM nifedipine
100 µM nifedipine
200 µM nifedipine
RLU/s
5000
4000
3000
2000
1000
0
-60
0
60
120
180
240
300
Time (s)

C



D

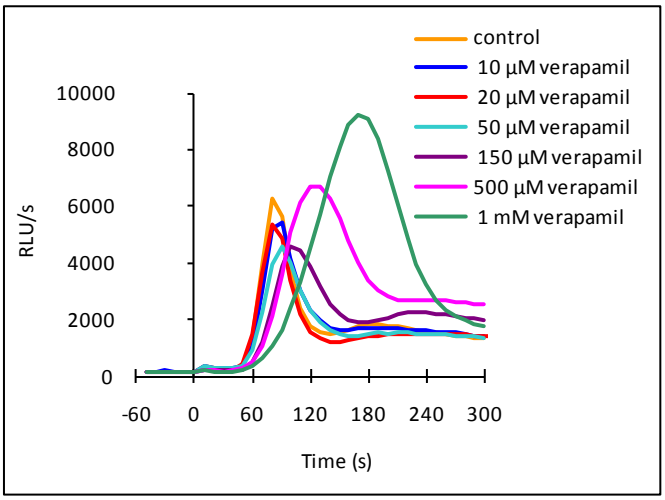


Figure 20. GIC transporter is sensitive to gadolinium and nifedipine, but not to verapamil. *mid1Δ cch1Δ fig1Δ yvc1Δ* aequorin expressing cells, grown in YPD, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of 1 mM $CaCl_2$ and the indicated concentrations of gadolinium (panel A), nifedipine (panel B) and verapamil (panel D). The percentage of inhibition of total calcium response was reported for a comparison between gadolinium (squares and solid line) and nifedipine (triangles and dashed line) in panel C.

GIC transport was found to be extremely sensitive to inhibition by gadolinium and nifedipine, revealing an apparent IC_{50} respectively of 16.7 ± 1.7 μM (Fig. 20A and C) and 0.36 ± 0.13 μM respectively (Fig. 20B and C). These values are higher than that observed for the inhibition of mammalian VGCCs, but it could be simply explained by the presence of cell wall in yeast cells requiring higher concentrations of these compounds, or maybe by a difference in the binding sites of these inhibitors in yeast Ca^{2+} channels.
The inhibitory mechanism of nifedipine was further investigated. A reliable difference in sensitivity to nifedipine was found between the wild-type and the mutant strain, suggesting that it is likely to be specific for calcium transport. The apparent IC_{50} was in fact estimated in the wild-type strain as 1.8 ± 0.005 μM, six-times higher than that calculated in the mutant strain (data not shown). Moreover, by co-varying calcium and nifedipine concentrations, inhibition mechanism for nifedipine was investigated, indicating a mixed competition affecting both K_m and V_{max} (data not shown).
Surprisingly, GIC transport was only slightly affected by 20 μM verapamil addition, which even behaves as an agonist, inducing the response up to the double than in the absence of the drug, at the highest tested concentration, higher than 500 μM (Fig. 20D). These findings together suggest that GIC transport could rely on a novel atypical class of Ca^{2+} transporters, not yet identified in yeast cells, displaying some different pharmacological features from those observed in mammalian VGCCs.

GIC-mediated response is modulated by calcineurin activity

Calcineurin, a serine-threonine phosphatase mainly involved in the response to a wide variety of cellular stress, such as high ionic conditions, pheromone response and osmotic stress, represents the major effector of intracellular calcium. It is activated following an increase in cytosolic Ca^{2+} level and, subsequently, it modulates many target genes at transcriptional level, by activating Crz1 transcription factor, its major target.
In order to assess if calcineurin was also involved in the regulation of glucose-responsive calcium transporters functionality, *CNB1* gene, encoding the calcineurin essential activating subunit, was deleted both in the wild-type and in the *mid1Δ cch1Δ fig1Δ* strains. In the *cnb1Δ* strain GIC response was similar to that observed in the wild-type strain as far as intensity and timing are concerned, suggesting that calcineurin deficiency doesn't affect GIC response in YPD growing cells (Fig. 11A and 21A). However, a reliable change in GIC response sensitivity to extracellular Ca^{2+} concentrations was

observed: *cnb1Δ* strain revealed a higher response at very low extracellular Ca^{2+} concentrations, if compared to the wild-type strain, while an inhibitory effect at higher Ca^{2+} concentrations was observed (Fig. 21A).
In contrast, in the *mid1Δ cch1Δ fig1Δ cnb1Δ* strain, the response was seriously impaired, suggesting that GIC transport system functionality requires calcineurin activity (Fig. 21B).

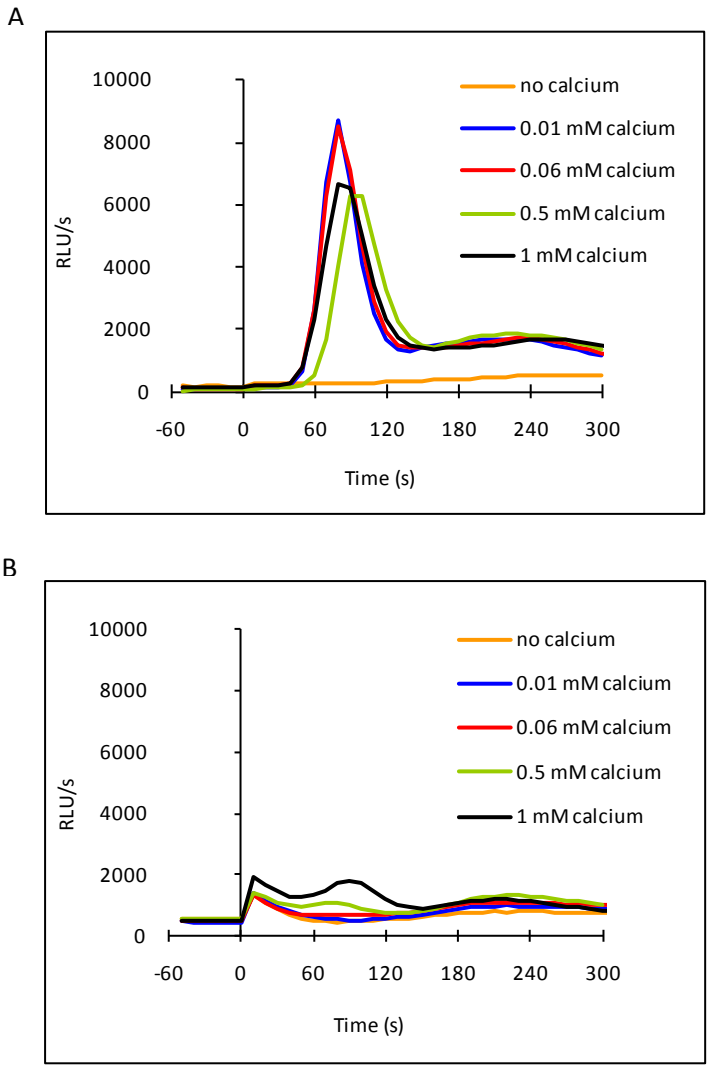


Figure 21. Calcineurin deficiency affects GIC transport functionality. *cnb1Δ* (panel A) and *mid1Δ cch1Δ fig1Δ cnb1Δ* (panel B) cells, expressing the bioluminescent aequorin protein and grown in YPD medium, were starved for nutrients and

stimulated at time zero with 100 mM glucose in presence of the indicated extracellular free calcium concentrations.

The apparent K_m was estimated for *cnb1Δ* YPD-growing cells in the order of ~ 10 µM (data not shown), revealing a value lower than that calculated for GIC transporter (Fig. 14B) and not so far from that calculated for HACS system in cells overexpressing both Mid1p and Cch1p (Teng *et al*., 2008). Thus, since calcineurin was reported to down-regulate HACS system during growth in YPD medium, it is likely that the peculiar response observed in the *cnb1Δ* strain could be due to the major contribution of the HACS transport in these conditions, characterized by higher affinity for calcium than GIC system itself.

Involvement in GIC response of novel uncharacterized proteins

Molecular identification of GIC channel is not a trivial challenge, since no yeast genes encoding mammalian calcium channels homologs were reported in literature, besides *CCH1* and *YVC1*; furthermore, setting up genetic screenings which could take advantage of GIC channel pharmacological properties identified in this work appears very hard.
Recently, a novel putative component of HACS system, encoded by *ECM7* sequence, was identified in a screening for yeast mutants lacking non-essential genes for defects in Ca^{2+} uptake during response to pheromone. Curiously, *ecm7Δ* mutant strains exhibited defects in Ca^{2+} uptake quantitatively similar to those observed in *mid1Δ* and *cch1Δ* mutants; in addition, a physical interaction between Ecm7p and Mid1p/Cch1p complex was found (Martin *et al*., 2011).
In order to assess if Ecm7p could be involved in GIC response, *ECM7* was deleted in the wild-type and *mid1Δ cch1Δ fig1Δ* strains, and GIC signalling was analysed in these mutant strains.
GIC response in the *ecm7Δ* mutant was only slightly reduced in intensity in comparison to what observed in the wild-type strain; however, the apparent K_m estimated in YPD-growing *ecm7Δ* cells was not different from that calculated for the wild-type strain in the same conditions (data not shown), suggesting that probably the lower signal observed in this mutant strain was only related to a minor activity of HACS system.
In addition, the *mid1Δ cch1Δ fig1Δ ecm7Δ* strain showed a glucose-triggered Ca^{2+} signal similar to that observed in the *mid1Δ cch1Δ fig1Δ* strain, in intensity, timing and dependence on extracellular Ca^{2+}

concentrations, excluding that Ecm7p could constitute the unknown GIC transporter itself or even a regulator subunit of this system (Fig. 22).

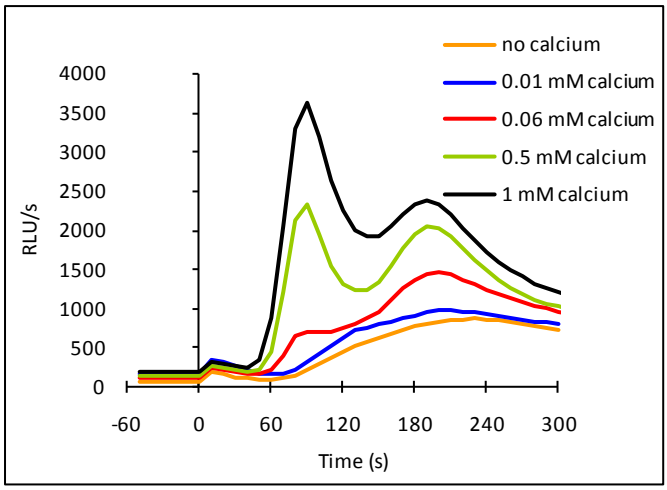


Figure 22. *ECM7* deletion doesn't affect GIC-mediated response. *mid1Δ cch1Δ fig1Δ ecm7Δ* cells, expressing the bioluminescent aequorin protein and grown in YPD medium, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of the indicated extracellular free calcium concentrations.

By performing a more detailed bioinformatic research on the whole yeast genome, focusing on still uncharacterized membrane proteins, *YOR365c* sequence was identified. It encodes a functionally uncharacterized 703 amino acids protein, presenting 7 transmembrane domains. Yor365cp displays some similarity to the mammalian TRP Ca^{2+} transporters superfamily, which includes also the yeast vacuolar Yvc1 channel: by performing an alignment of Yor365cp sequence with other TRP family members from different species, a strong conservation of TRP domains was evident, and in particular of the acidic residues in the transmembrane domains, responsible for Ca^{2+} ions binding (Fig. 23). In addition, basing on a phylogenetic classification of different yeast unknown yeast membrane proteins, Yor365cp was included as a putative ionic transporter (De Hertogh *et al*., 2002).

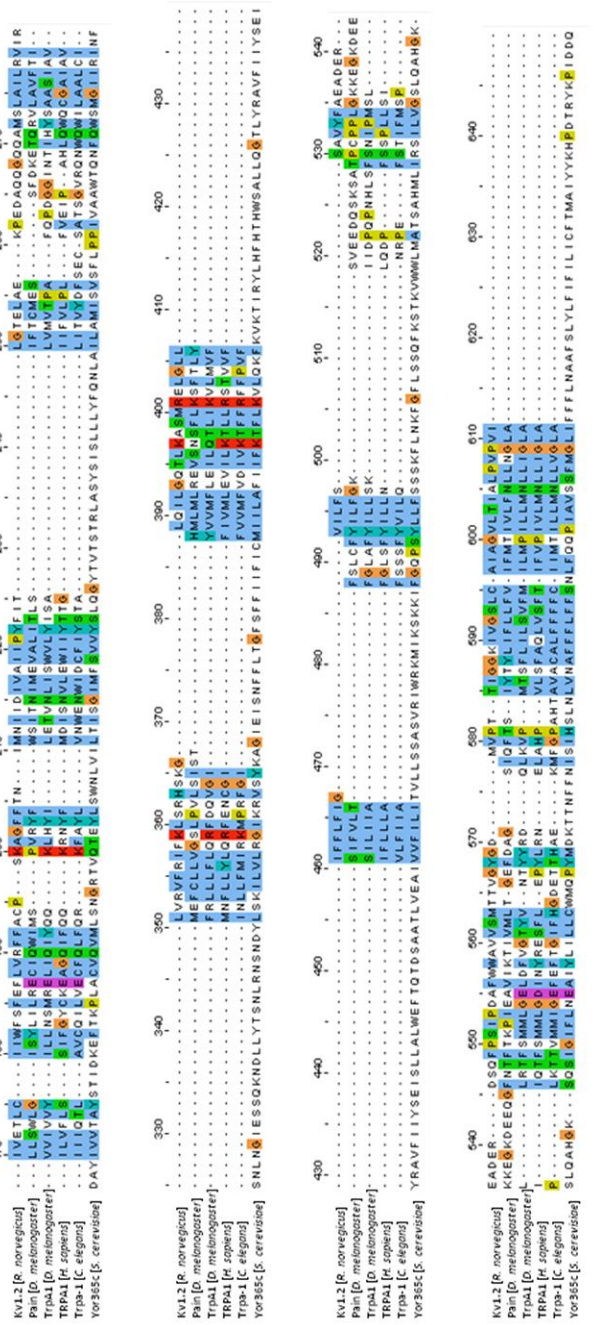
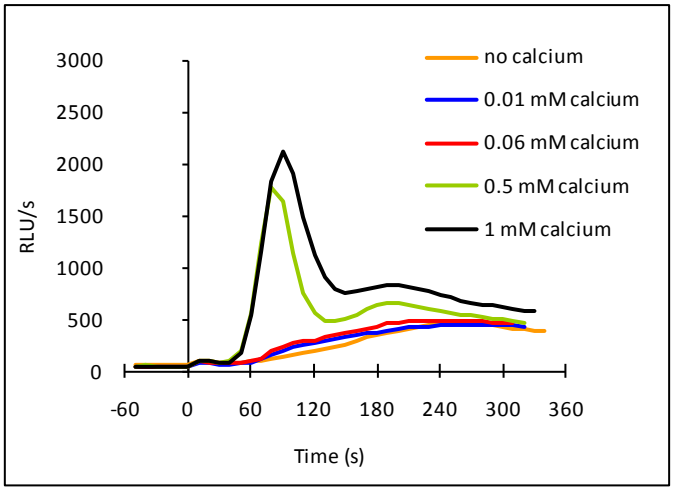


Figure 23. ClustalW alignment of *YOR365c* sequence with the transmembrane conserved domains of TRP superfamily ion channels from different species.

Basing on these findings, in order to investigate a possible role of Yor365c protein in GIC signalling, *YOR365c* gene was deleted in the wild-type and in the *mid1Δ cch1Δ fig1Δ* strains, and GIC response was analysed (Fig. 24).
yor365cΔ mutant strain showed a significantly reduced GIC signal, if compared with that observed in the wild-type strain (Fig. 24A). In order to exclude that this peculiar response was not dependent only on the intrinsic variability associated to this bioluminescent technique, the apparent K_m for calcium was estimated in *yor365cΔ* YPD-growing cells as 31 µM (data not shown), a value significantly lower than that calculated for wild-type and *mid1Δ cch1Δ fig1Δ* strains in the same growth conditions. These findings suggest that Yor365cp could play a regulatory role in GIC signalling.
However, in the *mid1Δ cch1Δ fig1Δ yor365cΔ* GIC response was still appreciable and absolutely comparable to that observed in the *mid1Δ cch1Δ fig1Δ* mutant strain (Fig. 24B). Thus, a role for Yor365cp as the unknown GIC transporter must be excluded, since in this case an almost absent glucose-induced Ca^{2+} peak would be expected.

A



B

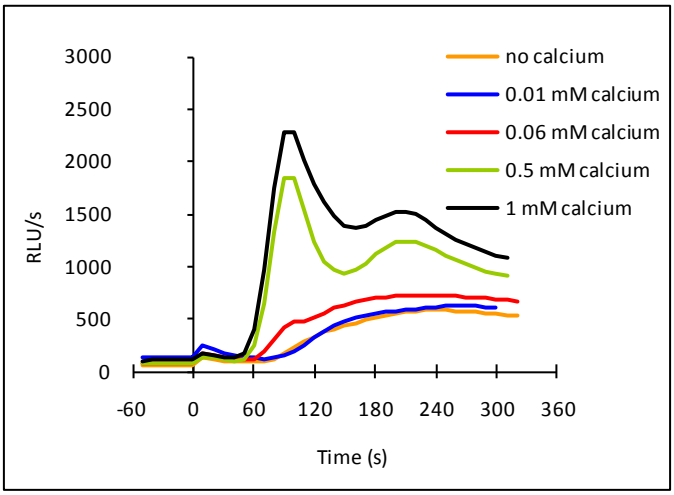
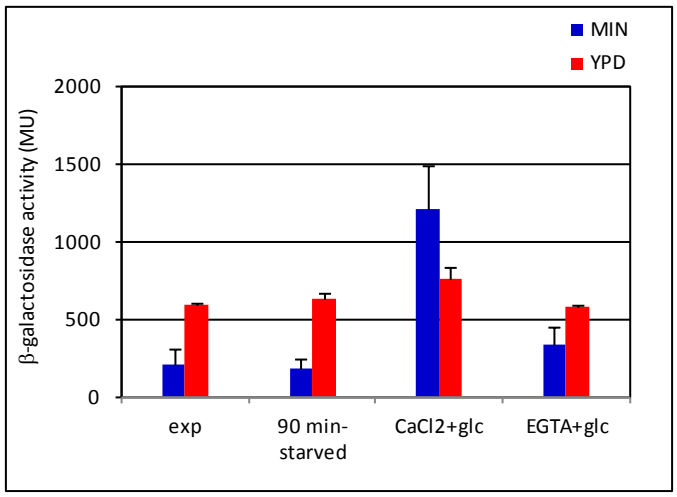


Figure 24. Yor365cp acts as a regulator of GIC-mediated response. *yor365cΔ* (panel A) and *mid1Δ cch1Δ fig1Δ yor365cΔ* cells (panel B), expressing the bioluminescent aequorin protein and grown in YPD medium, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of the indicated extracellular free calcium concentrations.

Glucose-triggered calcium signal activates calcineurin-dependent transcription

The Ca^{2+}/calmodulin/calcineurin pathway represents the main effector of calcium availability in the extracellular medium. Since calcineurin is known for being activated in response to different stress stimuli after an increase in cytosolic Ca^{2+} level, the activity of calcineurin after glucose addition to nutrient deprived cells was here investigated. The activation of calcineurin was detected by taking advantage of a reporter system, described in the Materials and Methods section, composed by the *LACZ* reporter gene put under the control of four repeated Crz1-responsive elements (CDRE): once activated, calcineurin dephosphorylates Crz1 transcription factor, causing it to accumulate into the nucleus where it activates its target genes expression, thus promoting adaptation to stress.
Primarily, calcineurin activity was monitored after glucose re-addition in wild-type cells, grown either in YPD and minimal medium and starved for nutrients (Fig. 25).

A



B

	Stimulation-fold
Minimal medium	7.1 ± 2.4
YPD medium	1.2 ± 0.2

Figure 25. Calcineurin-dependent transcription is activated by glucose re-addition after starvation in cells grown in minimal medium, but not in cells grown in rich medium. Crz1p-responsive promoter activity was assayed, as described in Materials and Methods section, in wild-type cells expressing the calcineurin responsive 4XCDRE-*LACZ* reporter, grown in minimal or YPD medium as indicated in the legend. The activity was measured in exponentially growing cells ("exp"), in nutrients-deprived cells ("90-min starved") and after 100 mM glucose addition in presence of 1 mM $CaCl_2$ ("$CaCl_2$ + glc") or of 1 mM EGTA ("EGTA + glc") (panel A). The stimulation-fold in wild-type strain grown in the indicated media, after glucose stimulus in presence of 1 mM $CaCl_2$, is reported in the table represented in panel B. The values here reported were calculated on at least three independent experiments.

Calcineurin-dependent transcriptional activity, measured in Miller Units (MU), appeared much higher in YPD medium than in minimal medium. After starvation, there was no change in the activity of β-galactosidase. However, glucose-induced transcriptional activation was observed in minimal medium, but not in YPD medium-cultured cells (Fig. 25A), reaching

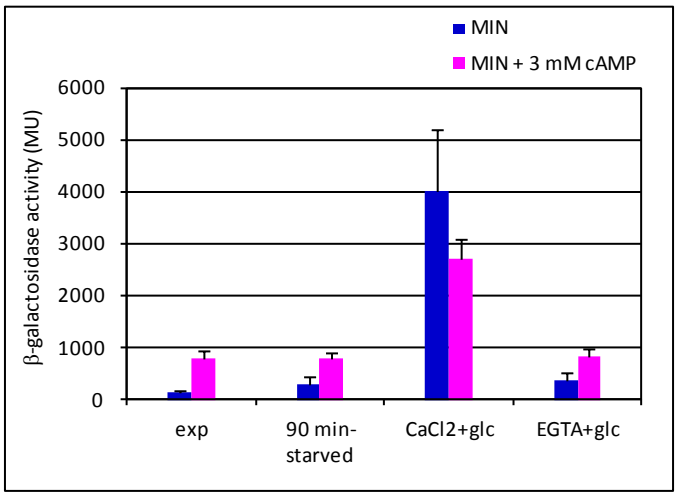
a stimulation-fold more than seven times higher than the basal level of calcineurin-responsive reporter expression, which conversely was already much higher in YPD than in minimal medium-cultured cells (Fig. 25B).
Moreover, in wild-type cells grown in synthetic complete medium, a halfway basal level of reporter expression between rich and minimal medium was observed, with a stimulation-fold of about twice over the basal level (data not shown).
Both the effects, higher basal level in YPD medium-grown cells and glucose-responsive Crz1-dependent transcription, are calcineurin-dependent since none of them was observed in a calcineurin-deficient *cnb1Δ* strain (data not shown).
In addition, the reporter gene is activated by glucose stimulus only when extracellular Ca^{2+} is added, but not by 1 mM $CaCl_2$ alone (data not shown) or by glucose in presence of the Ca^{2+} chelator agent EGTA (Fig. 25A), suggesting that glucose-induced Ca^{2+} influx is required.
However, the reporter was equally activated in all the mutant strains lacking the known Ca^{2+} transporters (*mid1Δ cch1Δ*, *mid1Δ cch1Δ fig1Δ*, *yvc1Δ*), grown in minimal medium, showing that the residual Ca^{2+} influx is sufficient to sustain the response (data not shown).
These findings together suggest that calcineurin, normally associated to cellular stress response, can also be activated by nutrients, indicating a novel correlation between calcineurin pathway and nutrient sensing.

PKA activity affects calcineurin responsiveness to nutrients

In order to investigate potential factors involved in regulating basal calcineurin-dependent Crz1p activity and its difference between minimal and rich medium, PKA signalling was considered. Actually, calcineurin and PKA signalling were reported counteracting each other in previous works, through the regulation of the same target, Crz1 transcription factor: PKA prevents Crz1p nuclear localization by direct phosphorylation on three conserved serine residues within its NLS sequence, opposing to calcineurin signalling (Kafadar and Cyert, 2004).
In order to analyze the contribute of PKA signalling on glucose-dependent activation of CDRE promoter, a *cyr1Δ pde2Δ yak1Δ* strain, impaired in adenylate cyclase activity and thus unable to synthesize cAMP and to activate PKA, was tested in minimal and rich medium with or without the addition of 3 mM cAMP in the growth medium: since this strain is cAMP

permeable, addition of cAMP activates PKA even in absence of a functional adenylate cyclase.
Actually, in this mutant strain the Crz1-dependent promoter was activated by glucose addition in minimal medium-grown cells more strongly than in the wild-type strain (Fig. 26A and C). In addition, in *cyr1Δ pde2Δ yak1Δ* YPD-growing cells, CDRE promoter activation was induced by glucose stimulus more than in wild-type cells, although weakly (Fig. 26B and C). The inhibitory effect of PKA on Crz1p activation can be observed in the presence of 3 mM cAMP added in *cyr1Δ pde2Δ yak1Δ* mutant cells, but the response is not completely abolished (Fig. 26C). Moreover, when cAMP is added to YPD-growing cells, a further increase in Crz1p basal transcriptional activity was observed, whereas Crz1-responsive promoter was less susceptible to induction by glucose stimulus, suggesting that other factors than PKA signalling are involved in the modulation of calcineurin responsiveness to nutrients.

A



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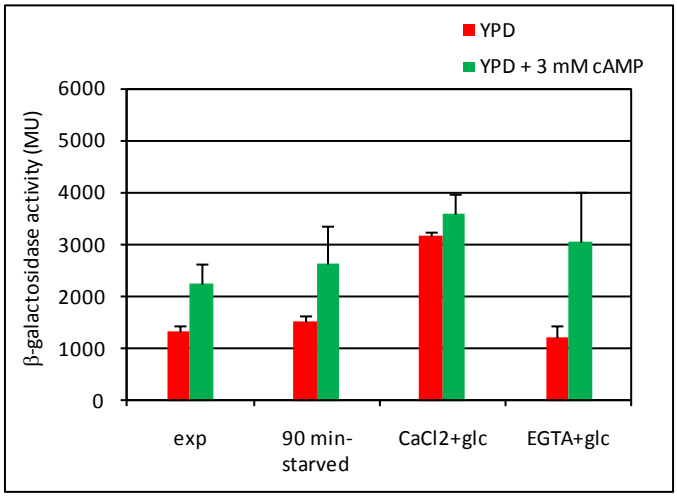


C

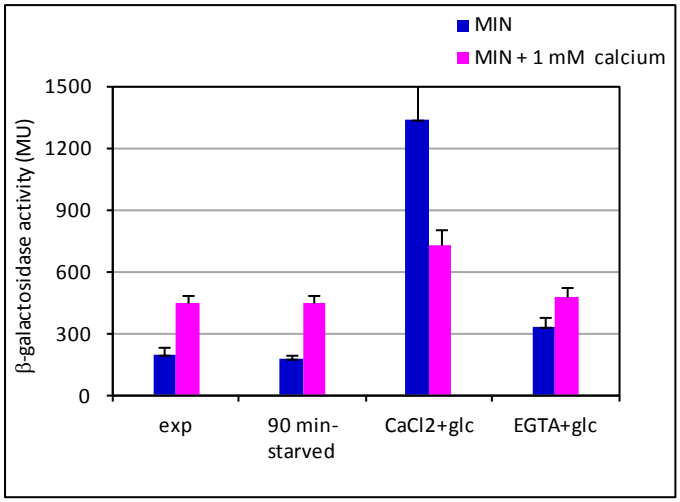
	3 mM cAMP	Stimulation-fold
Minimal medium	-	13.3 ± 1.5
	+	3.6 ± 1.0
YPD medium	-	2.1 ± 0.2
	+	1.4 ± 0.3

Figure 26. PKA activity is involved in the inhibition of glucose-induced Crz1-dependent transcription in cells grown in YPD medium. Crz1p-responsive promoter activity was assayed, as described in Materials and Methods section, in *cyr1Δ pde2Δ yak1Δ* cells expressing the calcineurin responsive 4XCDRE-*LACZ* reporter, grown in minimal (panel A) or YPD medium (panel B) with or without the addition of 3 mM cAMP in the cultural medium as indicated in the legend. The stimulation-fold in *cyr1Δ pde2Δ yak1Δ* strain grown in the indicated media supplemented or not with 3 mM cAMP when indicated, after glucose stimulus in presence of 1 mM $CaCl_2$, is reported in the table represented in panel C. The values here reported were calculated on at least three independent experiments.

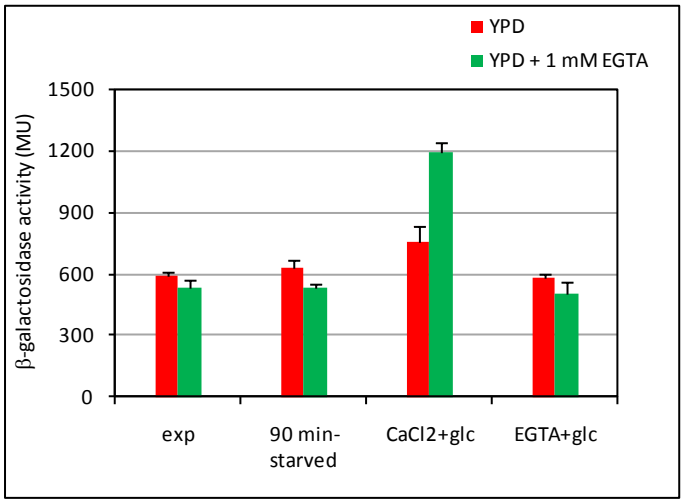
Calcium availability in the cultural medium modulates calcineurin responsiveness to nutrients

One of the relevant differences between rich and minimal medium, besides nutritional characteristics, is calcium availability, and this could affect the basal Crz1p-dependent response in differently cultivated cells, as observed in the previous sections.
In order to assess if these differences could justify what observed in glucose-induced calcineurin signals and consequently in calcineurin activity during cell growth, calcineurin activity was assayed in wild-type cells grown in YPD or minimal medium, either in exponential growth, after nutrient-starvation or after glucose addition, in presence or in absence of free extracellular calcium in order to abolish the difference in calcium content in the two cultural media (Fig. 27). In particular, calcineurin activity dependence on calcium availability in the medium was investigated adding 1 mM $CaCl_2$ to minimal medium, raising the free extracellular calcium concentration to levels comparable to YPD medium, or 1 mM EGTA to YPD medium, lowering down the free extracellular calcium concentration .

A



B



C

	$CaCl_2$	EGTA	Stimulation-fold
Minimal medium	-	-	7.1 ± 2.4
	+	-	1.7 ± 0.1
YPD medium	-	-	1.2 ± 0.2
	-	+	2.3 ± 0.2

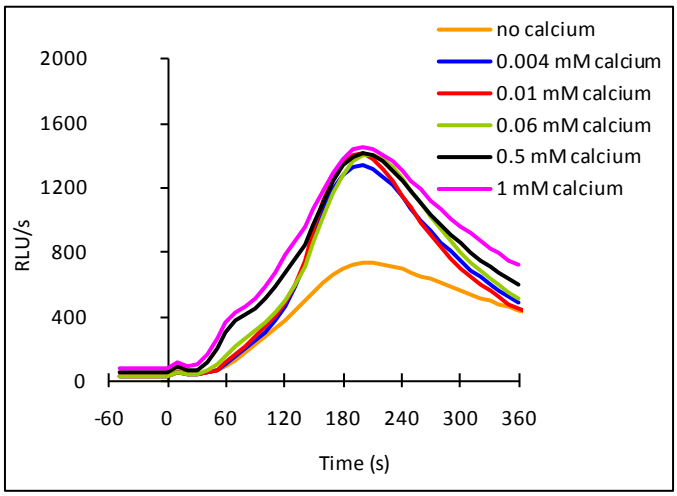
Figure 27. Calcium availability in the cultural medium regulates glucose-induced Crz1p-dependent transcription. Crz1p-responsive promoter activity was assayed, as described in Materials and Methods section, in wild-type cells expressing the calcineurin responsive 4XCDRE-*LACZ* reporter, grown in minimal (panel A) or YPD medium (panel B) with or without the addition of either 1 mM $CaCl_2$ to minimal medium or 1 mM EGTA to YPD medium during cell growth, as indicated in the legend. The stimulation-fold in the wild-type strain grown in the indicated media supplemented either with 1 mM $CaCl_2$ or 1 mM EGTA, when indicated, after glucose stimulus in presence of 1 mM $CaCl_2$, is reported in the table represented in panel C. The values here reported were calculated on at least three independent experiments.

It turned out, in fact, that increasing calcium availability during growth in minimal medium caused a rise in basal Crz1p-dependent transcriptional activity (Fig. 27A), but most of all strongly inhibited Crz1p activity stimulation upon glucose addition (Fig. 27C), in contrast to what observed in minimal medium, indicating that a high basal calcineurin activity

counteracts its glucose-responsiveness. Conversely, EGTA addition during growth in YPD medium, had almost no effect on Crz1p basal activity (Fig. 27B) and only slightly rescued calcineurin responsiveness to glucose stimulus (Fig. 27C). Basing on the data obtained in these experiments, it appears that calcium availability in the cultural medium modulates basal Crz1p-dependent transcriptional activity and calcineurin susceptibility to glucose stimulus after nutrients starvation, but that certainly other factors are involved in the regulation of these responses during growth in YPD medium.

In order to verify if what observed in glucose-induced Crz1p-dependent transcription was justified by modification in calcium fluxes caused by the modulation of calcium content in the cultural medium, the effect of calcium availability on GIC signalling was also analysed. As in the calcineurin activity assay previously described, wild-type cells grown either in minimal medium with 1 mM $CaCl_2$ added or in YPD medium supplemented with 1 mM EGTA, were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of different free extracellular calcium concentrations and GIC response was analysed. As well as Crz1p-dependent transcriptional activity, GIC response was also affected by extracellular calcium availability during cell growth: in the presence of the calcium chelator EGTA during growth in YPD medium, exposing cells to low calcium availability, a significant decrease in intensity of GIC response was observed (Fig. 28B) in comparison to that obtained in a wild-type strain grown in YPD medium, even if the timing and the sensitivity to free extracellular calcium concentrations wasn't changed. Conversely, GIC response was only slightly enhanced in wild-type cells grown in minimal medium with 1 mM $CaCl_2$ added during growth, remaining very far from the level observed in a wild-type strain grown in YPD medium (Fig. 28A). These findings suggest that calcium availability in the cultural medium is not the only factor influencing the different GIC signalling observed in YPD and minimal medium, and that none of these effects on calcium flux can justify for the effect observed on Crz1p-dependent transcription.

A



B

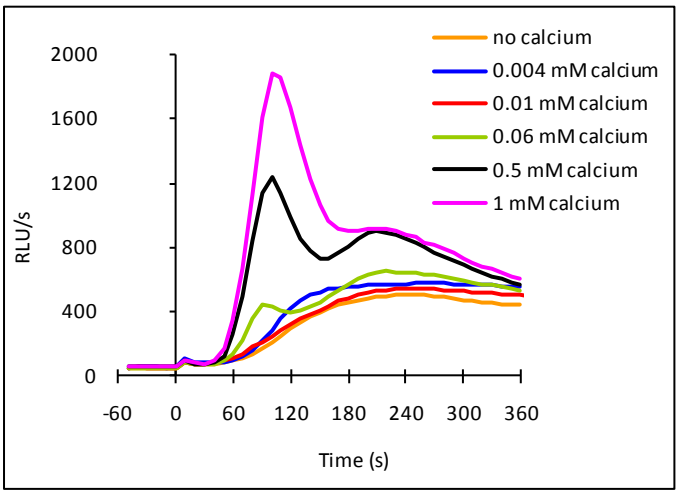


Figure 28. Calcium availability in the cultural medium affects also GIC response. Wild-type cells expressing the bioluminescent aequorin protein and grown in minimal medium with 1 mM $CaCl_2$ added (panel A) or in YPD medium with 1 mM EGTA added (panel B), were starved for nutrients and stimulated at time zero with 100 mM glucose in presence of the indicated extracellular free calcium concentrations.

From these experiments a novel role for calcineurin in response to nutrients has emerged for the first time, since no information about this relationship have ever been reported in literature.
Our data indicate that calcineurin responsiveness to nutrient signals is strongly related to growth conditions and two main factors involved in such modulation were here identified, calcium levels in the cultural medium and PKA activity.

Glucose-dependent calcineurin activation affects cell cycle progression

Calcineurin was reported to affect bud emergence in yeast cells, by negatively regulating actin polarization at the presumptive bud site: in presence of high Ca^{2+} concentrations in the growth medium (50 mM $CaCl_2$) bud emergence and actin polarization were delayed in a wild-type strain, but in a *cnb1Δ* strain this delay was abrogated indicating that it occurred in a calcineurin-dependent manner (Shitamukai *et al*., 2004).
Taking in account our previous findings demonstrating glucose-induced calcineurin activation, cell cycle progression and in particular bud emergence were monitored in wild-type and *cnb1Δ* strains, subjected to a nutritional shift-up: cells were grown in rich medium containing ethanol as the sole carbon source until the exponential phase, then glucose was added, at time zero, to the culture (see Material and Methods section). In this way cells were induced to shift from a respiratory metabolism to fermentative growth, mimicking the situation represented by glucose addition to nutrient-deprived cells responsible for calcineurin activation.
As already observed in previous experiments performed in our laboratory also, wild-type cells displayed a transient decrease in budded cells percentage after nutritional shift-up, reaching its maximum at about 80-100 min since glucose addition, indicating that cells transiently arrest the cell cycle probably in order to increase the critical cell size for bud emergence required for growth on glucose. Actually, after this observed decrease, budded cells percentage returns on level typical of growth on glucose (Belotti *et al*., 2006).
Interestingly, as shown in the following preliminary experiment represented in Fig. 29, *cnb1Δ* cells did not display such transient decrease in budding index during the nutritional shift-up from ethanol to glucose, in contrast with what observed in wild-type cells.

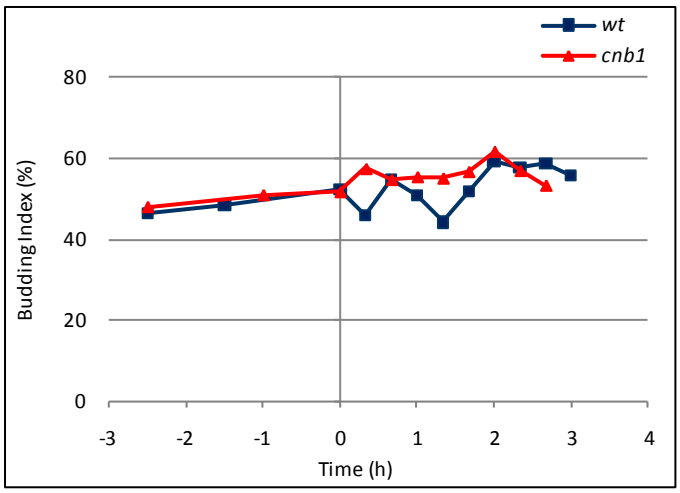


Figure 29. Glucose-induced calcineurin activation could be involved in the transient decrease in budded cells percentage during nutritional shift-up. Wild-type and *cnb1Δ* cells were grown in rich medium containing 2% ethanol as carbon source until the middle exponential phase and, at time zero, 2% glucose was added, as described in Materials and Methods section. Shift-up was monitored as budding index change for few hours after glucose addition.

These preliminary data suggest a role for calcineurin in modulating cell cycle progression during nutritional shift-up, indicating a further correlation between calcineurin pathway and nutrients sensing. It is likely that calcineurin activation after glucose stimulus delays bud emergence by negatively regulating actin polarization at the bud site, as reported in literature. However, further more detailed investigation on calcineurin involvement in regulating essential parameters for cell cycle progression in dependence on nutrient signals will must be performed in order to study calcineurin pathway and nutrient sensing crosstalks.

Characterization of hypotonic shock-induced Ca^{2+} signal

Eukaryotic cells respond to an hypotonic shock with ions or organic solute fluxes in order to restore proper volume and pressure relationships. An hypotonicity-induced temporal increase in cytosolic Ca^{2+} level has been visualized both in mammals and in budding yeast. In yeast cells hypotonic shock-triggered Ca^{2+} signal seems to be generated at the early stage by Ca^{2+} release from intracellular stores, while later a sustained increase in cytosolic Ca^{2+} concentration depends upon an influx of Ca^{2+} from the

extracellular environment (Batiza *et al.*, 1996). However, despite the understanding of the signal transduction pathways involved in transducing an hypotonic shock in yeast cells, little is known about the mechanisms involved in the activation of that pathway.
As described for glucose-induced Ca^{2+} signalling, also hypotonic shock-dependent Ca^{2+} signal was analysed by performing the bioluminescent assay *in vivo* in wild-type and mutant strains, impaired in the known Ca^{2+} transporters, in order to investigate their contribution in generating this Ca^{2+} signal (Fig. 30).

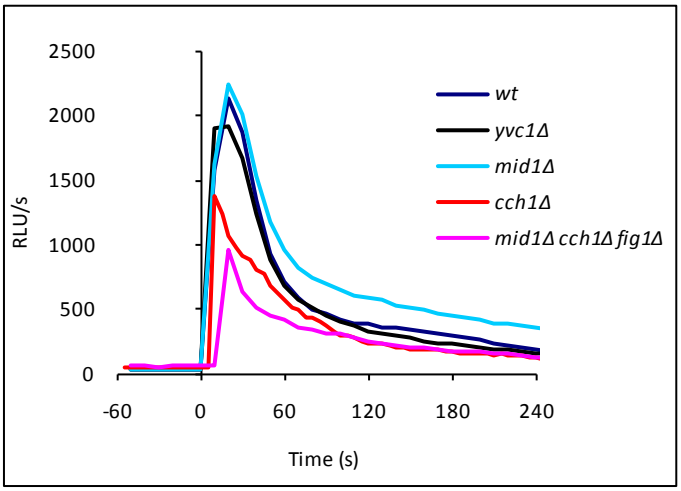
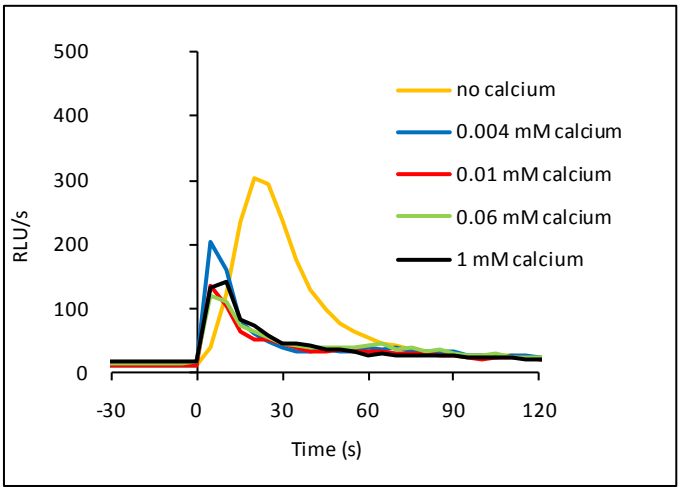


Figure 30. Cch1 HACS subunit, but not Mid1, and a still unidentified Ca^{2+} channel are involved in mediating hypotonic shock-triggered Ca^{2+} signal. Wild-type and *yvc1Δ*, *mid1Δ*, *cch1Δ*, *mid1Δ cch1Δ fig1Δ* mutant cells expressing the bioluminescent aequorin protein, grown in YPD medium, were collected and subjected at time zero to hypotonic shock by adding 4 volumes of H_2O, in presence of extracellular 1 mM $CaCl_2$.

Hypotonic shock-dependent Ca^{2+} signal differs from that triggered by glucose addition to nutrient deprived cells for timing and shape, reaching its maximum within 10-30 s immediately after the stimulus and returning to the basal level in less then 4 minutes. Hypotonic shock-induced Ca^{2+} signal appears to be mediated by Cch1 HACS subunit and by an unknown Ca^{2+} transporter, as suggested by the persistence of the response in the *mid1Δ cch1Δ fig1Δ* mutant strain. Surprisingly, in contrast with data reported in literature about pheromone-induced Ca^{2+} signalling and with the results obtained from our experiments as far as GIC signalling is concerned, in

hypotonic shock response Mid1p and Cch1p appear not to act as one complex: Mid1 HACS subunit inactivation did not affect Ca^{2+} signalling in response to hypotonic shock at all, whereas in the previous experiments *mid1Δ* and *cch1Δ* single mutants displayed the same phenotypes observed in *mid1Δ cch1Δ* double mutant. Moreover, Yvc1p is not involved in increasing cytosolic Ca^{2+} level in response to hypotonic shock, excluding a contribution of Ca^{2+} release from vacuoles through this TRP channel.
Hypotonic-shock induced Ca^{2+} signalling was also investigated in wild-type cells in presence of different extracellular calcium concentrations, revealing a peculiar feauture, never observed before: extracellular Ca^{2+} appeared to inhibit cytosolic Ca^{2+} increase in a concentration-dependent manner. Moreover, a raise was observed in Ca^{2+} signal in the absence of extracellular Ca^{2+}, indicating that hypotonic shock-induced Ca^{2+} flux depends for a large part on Ca^{2+} release from intracellular stores; this component displayed also a very different timing, intensity and shape in comparison with the response observed in presence of extracellular Ca^{2+}, reaching its maximum only 30 s after the stimulus (Fig. 31A). Both these effects, extracellular Ca^{2+}-dependent inhibition of Ca^{2+} signal and the significant response in the absence of extracellular Ca^{2+}, were also observed in all the mutant strains impaired in known Ca^{2+} transporters activity (data not shown) and, most of all, in the *mid1Δ cchΔ fig1Δ* strain, indicating that the still unidentified hypotonic shock-responsive Ca^{2+} transporter could be an intracellular channel, inhibited by extracellular Ca^{2+} in a concentration-dependent manner (Fig. 31B).

A



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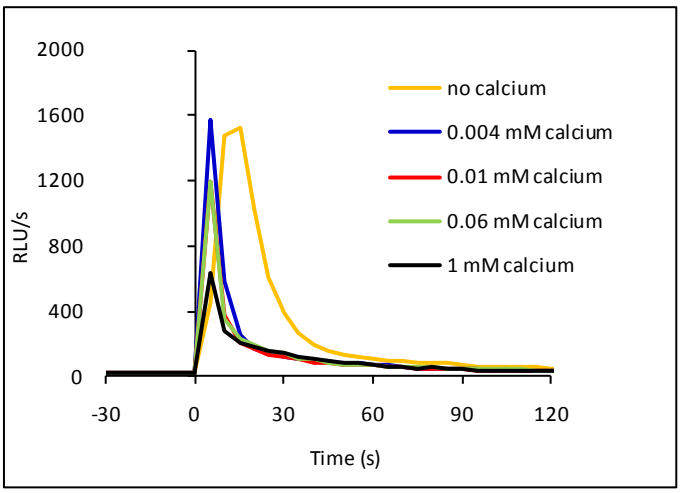

Figure 31. Hypotonic shock-triggered Ca^{2+} signal is inhibited by extracellular calcium in a concentration-dependent manner. Wild-type (panel A) and *mid1Δ cch1Δ fig1Δ* (panel B) cells expressing the bioluminescent aequorin protein, grown in YPD medium, were collected and subjected at time zero to hypotonic shock by adding 4 volumes of H_2O, in presence of the indicated free extracellular calcium concentrations.

These findings together suggest that hypotonic-shock induced Ca^{2+} response is generated both by an influx of calcium from the extracellular environment, mediated by Cch1 HACS subunit, and by Ca^{2+} release from intracellular stores, likely mediated by an unknown Ca^{2+} channel: actually, the *mid1Δ cch1Δ fig1Δ* mutant strain displayed a higher response in comparison with the wild-type strain, indicating that probably a major release from intracellular stores occurred in the absence of Cch1 membrane transporter.
The sensitivity to extracellular calcium was summarized for the different strains analysed in our experiments in figure 32: mutant strains impaired in HACS activity, *cch1Δ* and *mid1Δ cch1Δ*, displayed the same behaviour, being more sensitive to extracellular calcium-dependent inhibition than the wild-type strain, whereas when also Fig1p was inactivated the inhibition profile returned similar to that observed in the wild-type strain (Fig. 32).

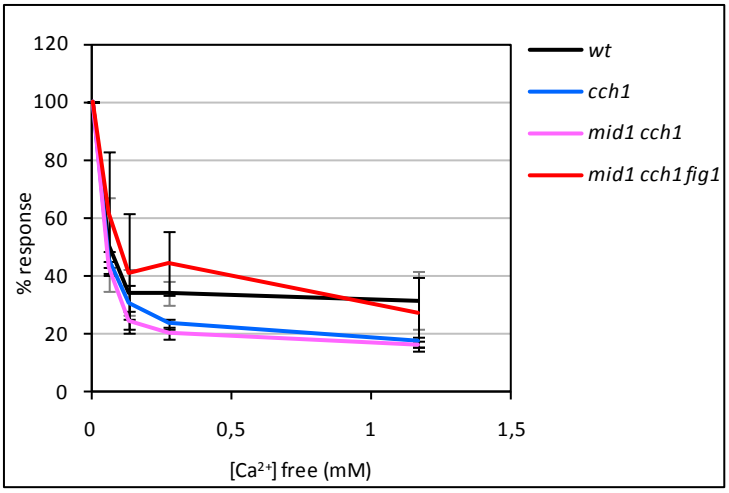

Figure 32. The relative response of hypotonic shock-induced cytosolic Ca^{2+} increase, in presence of different free extracellular calcium concentrations, is here reported for a comparison between wild-type, *cch1Δ*, *mid1Δ cch1Δ* and *mid1Δ cch1Δ fig1Δ* strains.

The unknown hypotonic shock-responsive calcium channel is different from GIC transporter

In order to better characterize the still unidentified calcium transporter, the hypotonic shock-induced calcium signalling was investigated more in detail in the *mid1Δ cch1Δ fig1Δ* mutant strain. Primarily, since this putative calcium channel seems likely to be an intracellular transporter inhibited by extracellular calcium, as described previously, it was excluded that the GIC transporter could also be the unknown transporter responsive to hypotonic shock. Thus, the sensitivity to various inhibitors of hypotonic shock response and the role of calcineurin in regulating it were analysed as previously described for GIC transporter.

In previous work from our laboratory, it was demonstrated that the hypotonic shock-responsive unknown transporter was almost resistant to magnesium: *mid1Δ cch1Δ fig1Δ* strain showed a significantly inhibited response only in presence of extracellular Mg^{2+} concentrations higher than 30 mM (unpublished data). Conversely, for GIC transporter the apparent IC_{50} for magnesium was estimated as 1.04 ± 0.05 mM (Fig. 17C).

As well as for GIC transporter pharmacological characterization, for the hypotonic shock-responsive unknown transporter the sensitivity to

gadolinium, nifedipine and verapamil VGCCs blockers was also tested. Unfortunately, this pharmacological approach was not fully applicable because no inhibition of the hypotonic shock-triggered Ca^{2+} signal was observed in presence of these compounds neither in wild-type nor in *mid1Δ cch1Δ fig1Δ* cells (data not shown). In complete lack of inhibitory effect it is hard to discriminate if the inefficacy was related to the inability of these blockers to reach their target into the cell, considering that even after an exposure of 30 min to these compounds no effect on Ca^{2+} signal was observed, or if actually these compounds were not effective on the intracellular Ca^{2+} transporters activated by hypotonic shock.

Finally, considering that a fundamental role for calcineurin in regulating GIC signalling and the activity itself of the unknown GIC transporter, as well as of many others calcium transporters, was previously demonstrated, its involvement in regulating hypotonic shock-induced response was also investigated.

Thus, Ca^{2+} signal in response to osmotic stress was analysed in a calcineurin deficient *cnb1Δ* strain: it was observed that, in absence of extracellular calcium, calcineurin inactivation did not affect Ca^{2+} signal, whereas in presence of extracellular calcium the signal was significantly decreased (Fig. 33). Thus, differently from what observed for GIC transport, the unknown hypotonic-shock-responsive Ca^{2+} transporter functionality seems not to require calcineurin activity: though the difference in sensitivity to extracellular calcium, the response was comparable in intensity and timing to that observed in the wild-type strain.

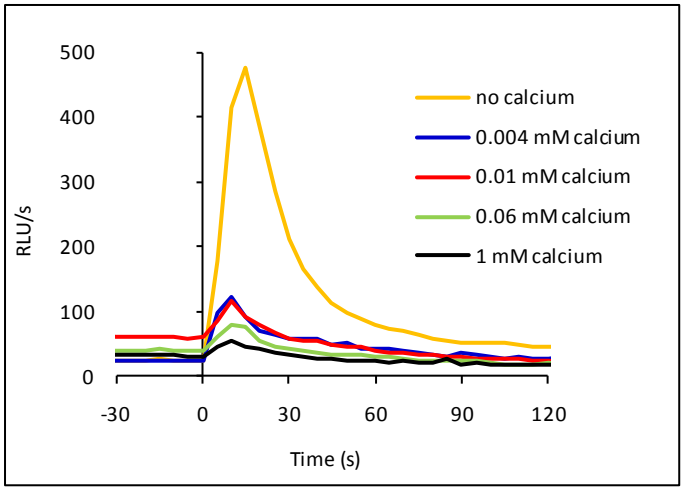

Figure 33. Calcineurin inactivation results in an enhanced sensitivity to extracellular calcium. *cnb1Δ* cells expressing the bioluminescent aequorin protein, grown in YPD medium, were collected and subjected at time zero to hypotonic shock by adding 4 volumes of H_2O, in presence of the indicated free extracellular calcium concentrations.

The inhibition profile of hypotonic shock-dependent calcium signal in dependence on extracellular calcium in mutant strains impaired in calcineurin activity was summarized in figure 34: it was observed in these experiments that calcineurin deficiency did not induce a higher sensitivity to extracellular calcium when HACS activity was impaired by deleting Mid1 and Cch1 subunits (Fig. 34). Thus, these findings seem to suggest that the enhanced inhibitory effect mediated by extracellular calcium observed in the *cnb1Δ* strain could be partially related to an effect on HACS system functionality; however, differently from the evident relationship between calcineurin activity and GIC functionality observed in glucose-triggered Ca^{2+} signalling, the data obtained from these experiments do not allow us to suppose a regulatory mechanism played by calcineurin in hypotonic shock-induced Ca^{2+} signalling.

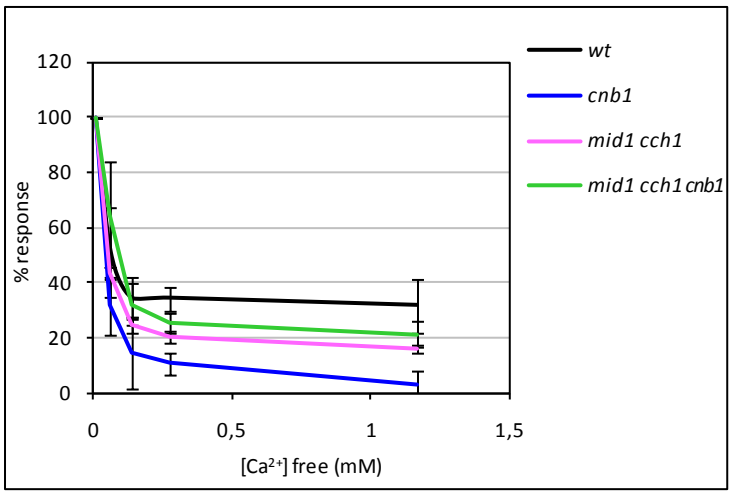

Figure 34. The relative response of hypotonic shock-induced cytosolic Ca^{2+} increase, in presence of different free extracellular calcium concentrations, is here reported for a comparison between wild-type, *cnb1Δ*, *mid1Δ cch1Δ* and *mid1Δ cch1Δ cnb1Δ* strains.

The data presented in this last section clearly indicate that in yeast cells there are at least two different unknown calcium transporters, not yet identified at the molecular level, respectively involved in glucose and hypotonic shock-induced calcium signalling. Glucose-responsive GIC transporter was more deeply characterized and it is evident that it displayed some fundamental differences from the hypotonic shock-responsive unknown transporter, which seems to be intracellular, inhibited by extracellular calcium in a concentration-dependent manner, insensitive to gadolinium, nifedipine and verapamil VGCCs blockers and independent on calcineurin activity.

Involvement in hypotonic shock response of novel uncharacterized proteins

Yor365cp, a homolog of mammalian TRP calcium channels, was previously found being involved in GIC signalling; thus, the effect of *YOR365c* deletion was also investigated in the hypotonic shock-dependent calcium signalling.
Actually, no information about Yor365cp function or localization are present in literature; thus, we verified if this putative transporter could be

responsible for calcium release from intracellular stores during the response to hypotonic shock.

Hypotonic shock response was analysed in a *yor365cΔ* mutant strain, revealing that calcium signalling was not seriously affected displaying a similar intensity and timing in comparison with a wild-type strain, and consequently that Yor365cp was not the major responsible for cytosolic Ca^{2+} level increase in response to hypotonic shock (Fig. 35).

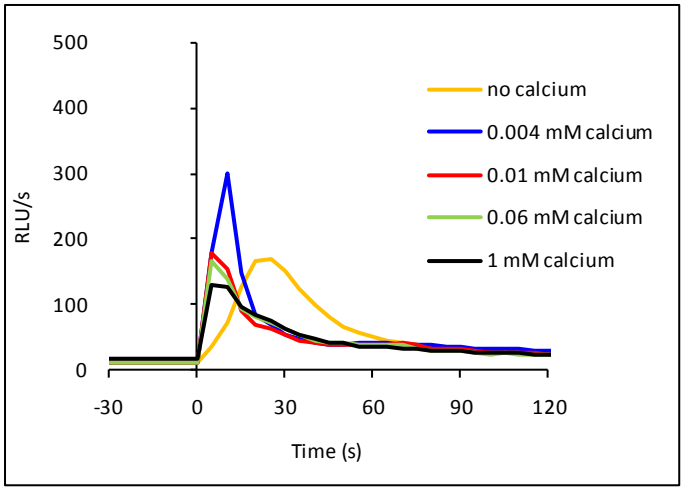

Figure 35. *YOR365c* deletion does not affect calcium release from intracellular stores in response to hypotonic shock. *yor365cΔ* cells expressing the bioluminescent aequorin protein, grown in YPD medium, were collected and subjected at time zero to hypotonic shock by adding 4 volumes of H_2O, in presence of the indicated free extracellular calcium concentrations.

However, from these data a possible regulatory role of Yor365cp in hypotonic shock-dependent calcium signalling could not be excluded at all, since the sensitivity to extracellular calcium appeared significantly reduced in comparison to a wild-type strain: while in the wild-type strain a maximum inhibition of 70% of the total response in presence of the highest extracellular [$CaCl_2$] tested was observed, in a *yor365cΔ* strain the maximum inhibition observed at the same extracellular calcium concentration reached only 30%. Moreover, by deleting *YOR365c* in a mutant strain lacking also *MID1*, *CCH1* and *FIG1*, the inhibition profile dependent on extracellular calcium concentrations returned similar to that observed in wild-type and *mid1Δ cch1Δ fig1Δ* cells (Fig. 36).

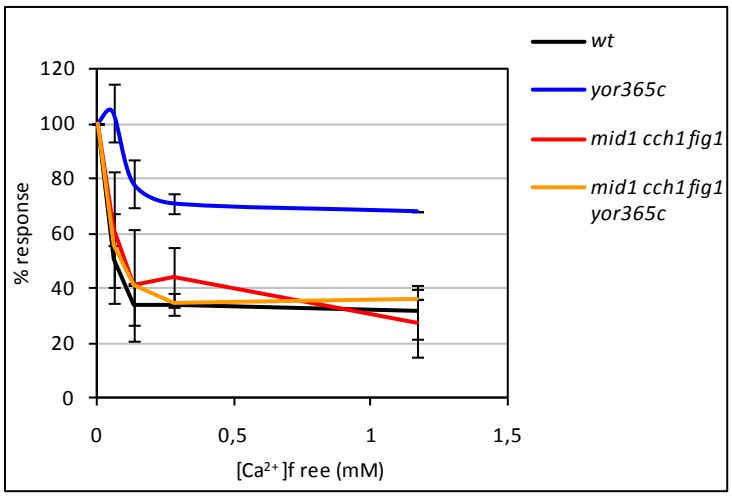

Figure 36. The relative response of hypotonic shock-induced cytosolic Ca^{2+} increase, in presence of different free extracellular calcium concentrations, is here reported for a comparison between wild-type, *yor365cΔ, mid1Δ cch1Δ fig1Δ* and *mid1Δ cch1Δ fig1Δ yor365cΔ* strains.

Curiously, a very similar, though less dramatic, inhibition profile of the hypotonic shock-induced Ca^{2+} signal, dependent on extracellular calcium, was observed in mutant strains lacking Yvc1 vacuolar calcium channel: *yvc1Δ* strains are less sensitive to inhibition by extracellular calcium than a wild-type strain, even if at the highest extracellular [$CaCl_2$] a inhibition of the total response of 60% was achieved; in addition, when *YVC1* was deleted in a *mid1Δ cch1Δ fig1Δ* strain, the inhibition profile dependent on extracellular calcium concentrations returned similar to that observed in wild-type and *mid1Δ cch1Δ fig1Δ* cells (Fig. 37).

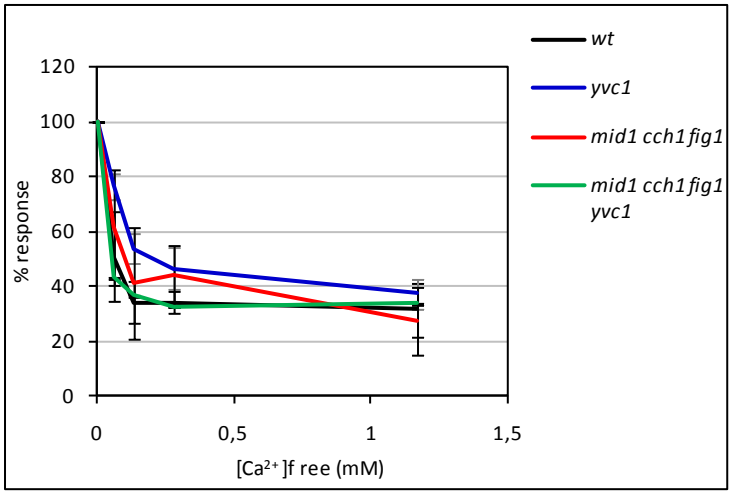

Figure 37. The relative response of hypotonic shock-induced cytosolic Ca^{2+} increase, in presence of different free extracellular calcium concentrations, is here reported for a comparison between wild-type, *yvc1Δ*, *mid1Δ cch1Δ fig1Δ* and *mid1Δ cch1Δ fig1Δ yvc1Δ* strains.

Thus, a functional homology between Yvc1p and Yor365cp could be hypothesized. Primarily, since Yvc1p is known being the major responsible for calcium signalling in response to hyperosmotic stress, the response to such stimulus was analysed also in *yor365cΔ* cells: whereas in the *yvc1Δ* strains this signal was completely abolished, in *yor365cΔ* it was not affected (data not shown), excluding that these two proteins could play the same role, at least in those conditions.

On ER membrane a putative calcium transporter, encoded by *CLS2/CSG1* sequence, was identified through a genetic screening performed in order to isolate mutants impaired in growth on medium containing high Ca^{2+} concentrations (Beeler *et al.*, 1994). In order to investigate if hypotonic shock-induced Ca^{2+} signal was mediated, at least in part, by calcium release from ER through this putative transporter, the response was analyzed in *cls2Δ* strains.

Surprisingly, *CLS2* deletion not only did not affect Ca^{2+} signal in absence of extracellular calcium, excluding a Cls2p-dependent release of calcium from ER, but a significant increase in this response was even observed in comparison to a wild-type strain. Conversely, in presence of extracellular calcium a very faint response was observed, revealing a major sensitivity to inhibition by extracellular calcium (Fig. 38).

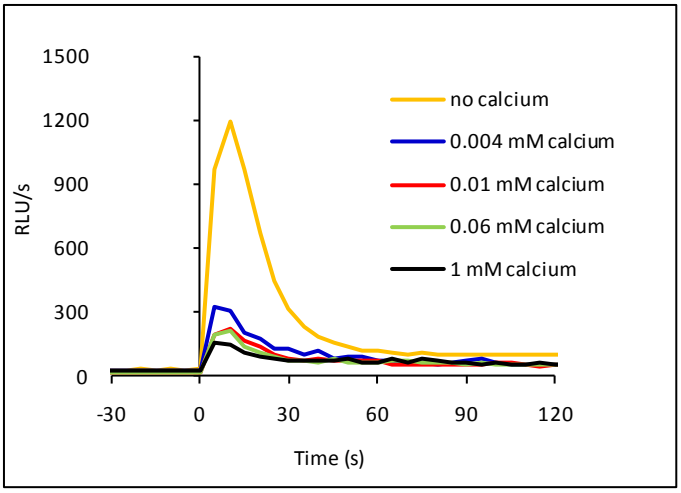

Figure 38. *CLS2* deletion does not affect calcium release from intracellular stores in response to hypotonic shock. *cls2Δ* cells expressing the bioluminescent aequorin protein, grown in YPD medium, were collected and subjected at time zero to hypotonic shock by adding 4 volumes of H_2O, in presence of the indicated free extracellular calcium concentrations.

Thus, basing on these results and keeping in account that the vacuolar Yvc1 channel is not involved in this response (Fig. 30), it is likely that another intracellular uncharacterized calcium transporter is activated in response to hypotonic shock, releasing calcium from an internal store into the cytosol. However, a role of Cls2p in regulating Ca^{2+} signal in response to hypotonic shock could not be excluded, since its inactivation confers higher sensitivity to inhibition by extracellular calcium than in a wild-type strain and in addition this increase in sensitivity is no longer observable with the concomitant deletion of *YVC1* (Fig. 39).

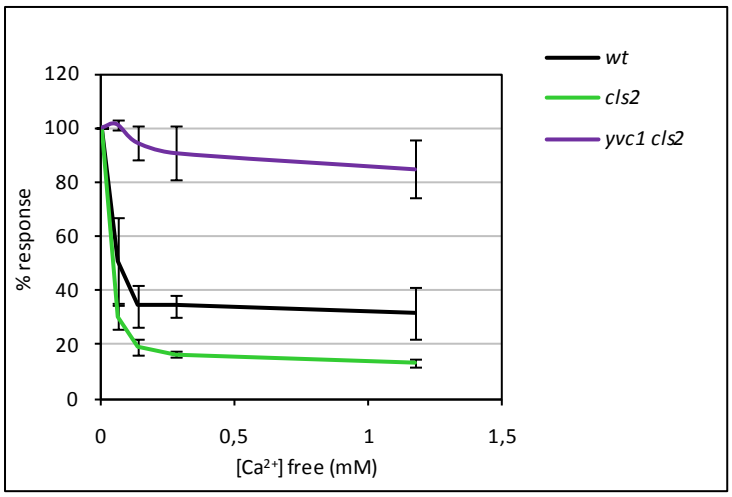

Figure 39. The relative response of hypotonic shock-induced cytosolic Ca^{2+} increase, in presence of different free extracellular calcium concentrations, is here reported for a comparison between wild-type, *cls2Δ* and *yvc1Δ cls2Δ* strains.

The release of calcium from intracellular stores seems to constitute the major component of hypotonic shock-triggered Ca^{2+} signal; thus, the response observed in the absence of extracellular calcium was analysed more in detail for a comparison between all the mutant strains used in this study.
In figure 40 the response induced by hypotonic shock, as the total amount of calcium mobilized in presence of the sole calcium chelator EGTA, is summarized for all the strains tested in our experiments.

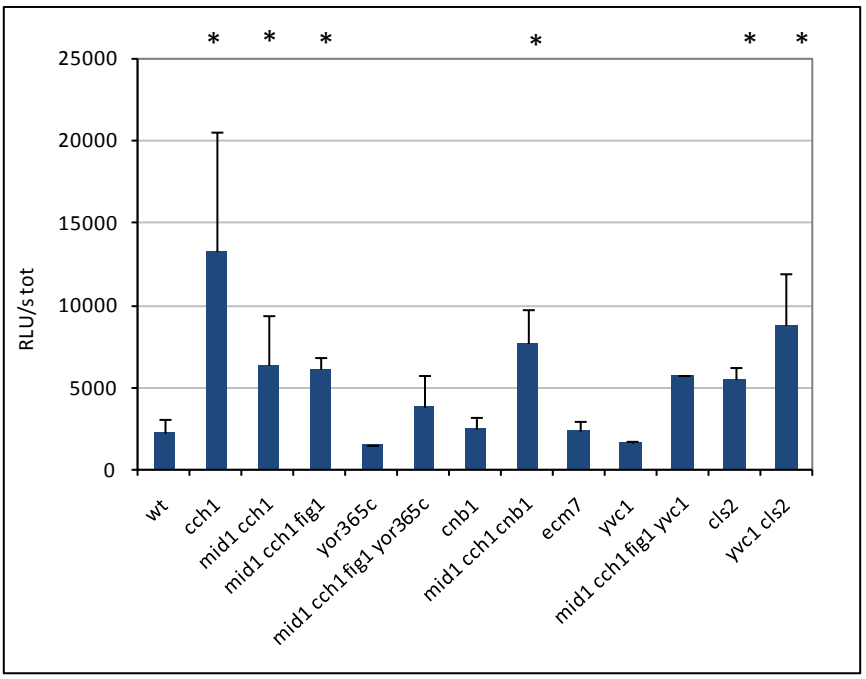

Figure 40. *CCH1* and *CLS2* deletion results in an increased release of calcium from intracellular stores in response to hypotonic shock. Wild-type and mutant cells, as indicated in the graph, grown in YPD medium, were collected and subjected at time zero to hypotonic shock by adding 4 volumes of H_2O, in presence of 1 mM EGTA and no addition of extracellular $CaCl_2$. The total amount of calcium mobilized for each strain in response to this treatment is here reported; asterisks indicate strains showing a statistically significant Ca^{2+} signal increase, in comparison to wild-type strain.

By statistically analyzing these data, we found that calcium release from intracellular stores in response to hypotonic shock, in the absence of extracellular calcium, was significantly enhanced in comparison to a wild-type strain, in mutant strains carrying either *CCH1* or *CLS2* deletion. It is not surprising that in absence of Cch1p activity, which represents the only hypotonic shock-responsive calcium channel located on the plasma membrane and consequently responsible for the Ca^{2+} influx from the extracellular environment in these conditions, an increase in Ca^{2+} release from intracellular stores was observed. However, also *CLS2* deletion, alone or together with *YVC1* deletion, resulted in the same effect observed for Cch1p inactivation, while *YVC1* deletion alone did not enhance the response being similar to what observed in a wild-type strain.

From our experiments it clearly emerged that in yeast cells there is a still unidentified intracellular calcium transporter, responsible for the major part of the hypotonic-shock-triggered Ca^{2+} signal, different from the unknown GIC transporter; however, a role of Cls2 ER membrane located and of the unknown Yor365c proteins can not be excluded.
Many aspects of this pathway remain to be elucidated, in order to identify at molecular level the unknown calcium transporters; studies on Yor365cp localization in the future could be very useful for the understanding of its role.

DISCUSSION

In all eukaryotic organisms calcium represents one of the most important second messengers, being involved in a wide variety of cellular processes. In budding yeast calcium plays essential roles in regulating many cellular processes activated by the exposure to different extracellular stimuli, as high ion concentrations, alkaline pH, pheromones or nutrients.

Several yeast proteins show homology with human calcium channels that have been implicated in channelopathies, allowing for study of the genotype/phenotype correlation in several diseases; in addition, conservation between yeast calcium system and mammalian cardiac myocytes was recently described (Wolfe and Pearce, 2006; Cui *et al*., 2009). Thus, insights in calcium homeostasis/signalling in yeast could be very useful to elucidate homologous systems in other eukaryotic cells, to help understanding of cardiac diseases or to create a model for channelopathies research.

This work focused on glucose-induced calcium signalling in yeast cells. In fact, glucose, besides constituting the preferred carbon and energy source for yeast cells, also plays a fundamental role as a signal molecule regulating a wide variety of cellular functions, such as growth rate, metabolism and stress resistance.

Glucose addition to nutrient-deprived yeast cells triggers a rapid and transient increase in cytosolic Ca^{2+} level, mediated mainly by an influx of calcium from the extracellular environment, whose kinetic and signature are strongly related to the genetic background of analyzed strains.

The main components of this pathway were formerly characterized: it is mediated by the GPCR complex, composed by the transmembrane receptor Gpr1, showing a low affinity for extracellular glucose, coupled with Gpa2 protein, homolog to α subunits of heterotrimeric G proteins (Tisi *et al*., 2002). Glucose signal transduction through GPCR complex requires the activity of *HXT* encoded hexose transporters, responsible for the transport into the cell of extracellular glucose, and glucose phosphorylation mediated by *HXK* encoded hexokinases (Tökés-Füzesi *et al*., 2002). Moreover, Gpr1-Gpa2 interaction is mediated by phospholipase C (Plc1) which, in fact, is essential for generating glucose-triggered Ca^{2+} signal: *plc1*Δ mutant strains, as well as wild-type strains treated with 3-nitrocumarin, a common inhibitor of phospholipase C, displayed a completely abolished Ca^{2+} signal in response to glucose stimulus (Tisi *et al*., 2002).

GIC (Glucose Induced Calcium) signalling, like pheromone-induced Ca^{2+} signalling, is strongly related to cultural conditions, being much higher in

YPD growing cells than in minimal medium cultured cells (Fig. 11). This is probably related to a differential expression or functionality of calcium transporters.

By taking advantage of a bioluminescent *in vivo* assay based on aequorin bioluminescent protein properties, GIC signalling was observed in wild-type and mutant aequorin expressing strains, impaired in the known calcium transporters, in order to investigate the role of these transporters in mediating GIC response. GIC signal is mainly dependent on Ca^{2+} influx from the extracellular environment rather than Ca^{2+} release from intracellular stores, and in cells growing on minimal synthetic media it is mediated by high affinity calcium transport system (HACS), composed by two components Mid1/Cch1 (Tökés-Füzesi *et al*., 2002), as suggested by the very faint signal observed in *mid1Δ cch1Δ* mutant strains in these growth conditions (Fig. 15). This is not true for cells growing in rich media, where HACS inactivation doesn't seriously affect GIC response (Fig. 12), sustaining again the hypothesis of a nutrient-dependent regulation of calcium transporters (Trópia *et al*., 2006). Actually, it was previously reported that HACS system is negatively regulated by calcineurin, a serine-threonine phosphatase representing the major effector for intracellular calcium, in rich media. This regulation mechanism is not well understood, but it is likely that calcineurin down-regulate HACS activity by directly dephosphorylating Mid1 subunit (Muller *et al*., 2001).

Differing from pheromone-induced calcium signalling, low affinity calcium transport system (LACS), whose only identified component up to now is the membrane protein Fig1, seems not to be involved in GIC response.

Calcium release from intracellular stores does not contribute significantly to GIC signaling in presence of abundant extracellular calcium: Yvc1 vacuolar calcium channel, homolog to TRP mammalian calcium transporters and located on vacuolar membrane, the major store for intracellular calcium in yeast cells, seems to be involved only in GIC signal amplification, as suggested by Ca^{2+} signal observed in *yvc1Δ* mutants (Fig. 12), especially relevant in conditions of external calcium shortage (Bouillet *et al*., 2011).

Interestingly, another calcium specific, high affinity (apparent K_m for calcium estimated 43.8 ± 10.3 μM) calcium transport has been identified in this work. This putative calcium flux, named GIC (for Glucose Induced Calcium) transport, seems to be fully functional in rich media, but not in minimal medium: known calcium transporters inactivation does not seriously affect GIC signalling in cells grown in YPD medium, suggesting that the unknown transporter is responsible for the major part of Ca^{2+} influx in these growth conditions (Fig. 12); conversely, HACS inactivation more seriously affects GIC response during growth in minimal medium, while in these conditions

mid1Δ cch1Δ fig1Δ displays a higher response than a wild-type strain, making no more any difference between the two cultural conditions tested, and suggesting that the putative GIC transporter becomes de-regulated in minimal medium only when HACS and LACS activity are impaired (Fig. 15).
Molecular identification of GIC channel is not a trivial challenge, since no genes encoding mammalian calcium channels with evident homology were identified in *S. cerevisiae* genome, besides Yvc1p and Cch1p, homolog to mammalian α subunits of L-type voltage-gated calcium channels. Anyway, low similarity observed among calcium channels sequences could have impaired identification of homologs.
Thus, a pharmacological approach was used in order to better characterize GIC transporter, studying Ca^{2+} signal in *mid1Δ cch1Δ fig1Δ* mutant strain. It proved to be sensitive to magnesium, with an apparent IC_{50} of 1.04 ± 0.05 mM, which was demonstrated acting as a competitive inhibitor by co-varying calcium and magnesium concentrations (Fig. 17 and 18). Moreover, GIC transporter resulted sensitive to gadolinium (apparent IC_{50} 16.7 ± 1.7 μM) and to nifedipine (apparent IC_{50} 0.36 ± 0.13 μM), two of the most common blockers of mammalian L type VGCCs (Fig. 20A, B and C). Curiously, GIC transporter resulted insensitive to verapamil, which even acts as an agonist at high concentration (Fig. 20D), and, differently from known calcium transporters in yeast, also to nickel (Fig. 19). This is peculiar since both verapamil and nifedipine are inhibitors of L-type VGCCs, even if with a slightly different specificity. Anyway, these two classes of inhibitors act on different target sites in these L-type calcium channels, consequently there could be no relation between their effect on non-conventional calcium channel (Hockerman *et al*., 1997). In the future the sensitivity to other common blockers with specificity for mammalian P/Q, R, N and T-type VGCCs could be tested. A reason for the failure of verapamil as inhibitor of yeast calcium channels could be the presence of cell wall, which constitutes a barrier for exogenous compounds; however, verapamil was demonstrated able to inhibit HACS system in *MID1/CCH1* overexpressing cells (Teng *et* al., 2008).
These findings together suggest that GIC transport could rely on a novel class of calcium channels, displaying atypical pharmacological traits, in according to the fact that *S. cerevisiae* genome does not encode for any protein homolog to known calcium channels, besides the already characterized channels.
Recently, a mathematical model was proposed which reproduces calcium transients in yeast, which suggested the existence of two Mg^{2+}-sensitive influx pathways (indicated as X and M transporters), both targets of rapid Ca^{2+}-dependent feedback inhibition (Cui *et al*., 2009). Computational

analysis revealed the existence of a transporter, called X, which should be inhibited by magnesium at very low concentrations and characterized by high affinity for calcium, consistently with the characteristics revealed for the unknown transporter involved in glucose response here identified.
By performing a computational analysis on the whole yeast genome, the sequence *YOR365c*, encoding for a still uncharacterized protein carrying the TRP conserved domains typical of mammalian TRP superfamily of ion channels, was identified. Thus, in order to understand if this sequence could encode for the unknown GIC transporter, GIC signalling was investigated in *yor365cΔ* mutant strains. Since in the *mid1Δ cch1Δ fig1Δ yor365cΔ* strain glucose-induced Ca^{2+} peak was still significant and similar in intensity and timing to that observed in the *mid1Δ cch1Δ fig1Δ* strain (Fig. 24B), it was excluded that the uncharacterized Yor365c protein could represent the unknown GIC transporter, considering that in that case a completely abolished signal had been expected. However, Yor365cp was previously classified as a putative transporter by combining mechanistic, functional and phylogenetic analyses (De Hertogh *et al*., 2002); in addition, by performing a research in yeast fitness databases, it was found that *yor365c* mutant strains displayed some phenotypic features related to alterations in Ca^{2+} signalling, such as high sensitivity to high ion conditions (i. e. to high LiCl concentrations) and high sensitivity to FK506, the most common inhibitor of calcineurin. Consistently with these findings, from our experiments a regulatory role of Yor365cp in GIC signalling emerged: *yor365cΔ* single mutants showed a significant decrease in the apparent K_m for calcium (estimated as 31μM in YPD-growing cells) (Fig. 24A), a somewhat lower value if compared with that obtained in wild-type and *mid1Δ cch1Δ fig1Δ* strains in the same conditions.
GIC transporter was found being positively regulated by calcineurin activity: in a *mid1Δ cch1Δ fig1Δ cnb1Δ*, inactivated in the *CNB1* encoded calcineurin activating subunit, GIC signal was completely abolished (Fig. 21B), suggesting that GIC transporter requires calcineurin activity to be fully functional. Calcineurin was already reported to be responsible for calcium transporters regulation: in fact, it was demonstrated that HACS system is down-regulated by calcineurin during growth in minimal medium, at least in pheromone-induced Ca^{2+} signalling (Muller *et al*., 2001). Actually, in a *cnb1Δ* single mutant strain GIC signal was not affected, but a significant decrease in the apparent K_m for calcium was observed in comparison to a wild-type strain (Fig. 21A), consistently with a major contribution of HACS system in these conditions, which in fact is characterized by higher affinity for calcium than GIC transporter.

The mechanism by which calcineurin regulates GIC transporter is still unknown: calcineurin could down-regulate GIC transporter directly by dephosphorylating it or indirectly through an activator, as well as it could regulate GIC channel at transcriptional level.
In literature calcineurin activation has been recognized as being essential for survival under diverse stress conditions, such as pheromones or ions-induced stress; however, in this work, it is reported for the first time that calcineurin can be also responsive to nutrients. Actually, by taking advantage of a reporter system composed by *LACZ* gene put under the control of 4 CDRE sequences, recognized by Crz1 transcription factor, which in turn is activated by calcineurin, calcineurin activity was measured upon glucose addition in yeast nutrient-deprived cells: it was found that Crz1p-dependent transcriptional activity was strongly induced by GIC signal, in presence of extracellular calcium, during growth in minimal medium, but this induction was not observed in rich medium cultured cells, where a significantly higher basal calcineurin activity was evident (Fig. 25A and B).
Calcium availability is one of the most important differences, besides the nutritional properties, between rich and minimal media; thus, we investigated if this factor could justify the difference observed in basal Crz1p-dependent transcriptional activity observed in our experiments. Actually, calcium addition to minimal medium, in order to raise the free calcium concentration to levels comparable to YPD medium, activated calcineurin-dependent transcription, even if weakly (Fig. 27A and C), and also raised the faint calcium influx which is typical in minimal medium (Fig. 28A). However, EGTA addition to YPD medium in order to low down the free calcium concentration in this medium, did not significantly rescue calcineurin susceptibility to glucose stimulus after nutrient starvation nor affected basal Crz1p-dependent activity (Fig. 27B and C), suggesting that other factors than calcium availability in the culture medium regulate calcineurin activity in exponential phase.
Keeping in account that GIC transporter appeared fully functional in rich medium and almost completely inactivated in minimal medium, and also considering that glucose-induced Ca^{2+} signal seems to be enhanced by calcineurin activity since calcium addition to minimal medium activates calcineurin-dependent transcription and can raise the faint calcium influx typical in this medium, it is evident that calcineurin activity is essential for GIC transporter functionality.
A relation between calcineurin and carbon source was already suggested, since many genes encoding carbohydrate-metabolizing enzymes were reported to be regulated by calcium/calcineurin pathway (Ruiz *et al.*, 2008). Consistently, PKA and calcineurin signalling converge on regulation of Crz1

transcription factor. PKA was in fact found constituting another important factor in regulating calcineurin basal activity in different growth conditions: the inhibitory effect of PKA signalling on calcineurin responsiveness to nutrients was demonstrated by monitoring calcineurin activity in a *cyr1Δ pde2Δ yak1Δ* strain, impaired in PKA activity, grown either in minimal or rich medium with or without the addition of 3 mM cAMP, in order to activate PKA even in absence of adenylate cyclase activity. Actually, in this mutant strain impaired in PKA activity, Crz1p-dependent transcriptional activity was more strongly induced by GIC signalling during growth in minimal medium than in a wild-type strain, whereas in YPD-growing cells calcineurin responsiveness to glucose was only slightly rescued. However, when cAMP was added Crz1p-dependent basal transcriptional activity was increased and calcineurin was poorly induced by glucose stimulus, even if the response was not completely abolished (Fig. 26A, B and C). Thus, also PKA signalling, besides calcium availability in the culture medium, can play an important role in regulating calcineurin responsiveness to nutrients.

The phosphoinositide specific phospholipase C, Plc1, acts together with GPCR complex in a pathway separated from Ras1/Ras2 pathway, converging on activation of adenylate cyclase and PKA: actually, *plc1Δ* mutants display some phenotypic features which are typical of cells with decreased PKA activity, such as decreased PKA-dependent inhibitory phosphorylation of Msn2 stress-responsive transcription factor (Demczuck *et al*., 2008), while calcium stress seems to act both on Msn2/Msn4 transcription factors and Snf1 protein kinase regulatory phosphorylation (Ohdate *et al*., 2010). In this work an interaction of glucose-induced calcineurin activation and classical glucose activated PKA pathway has emerged, but this complex relationship is far from being elucidated.

Calcineurin was already reported to be involved in cell cycle regulation also, through a positive regulation of Swe1 transcription factor, which acts as negative regulator of G_2/M transition: by activating Swe1p both at transcriptional and post-translational level, calcineurin inhibits G_2/M transition in response to cell membrane alterations that cause Ca^{2+} signalling activation (Miyakawa and Mizunuma, 2007).

Calcineurin activation by glucose stimulus after nutrients depletion, demonstrated in this work, could suggest a role of calcineurin also in G_1/S transition: in yeast cells, in fact, nutrient availability affects growth rate and the critical cell size (P_s), which represents the protein content per cell at the onset of DNA replication (Barberis *et al*., 2007). Actually, different components of Ca^{2+} signalling were reported to be involved in the progression through START at G_1/S transition. In particular, *PKC1* gene, essential for yeast cells viability and encoding a homolog of the Ca^{2+}-

dependent isozymes of the mammalian protein kinase C (PKC) family of enzymes, was identified in a screening for mutants that decreased the effectiveness of signalling by Cdc28, a member of the cyclin-dependent kinase superfamily which, by associating to its positive regulatory subunits known as cyclins, regulates the progression through different phases of the cell cycle (Marini *et al*., 1996). Although in budding yeast there is no evidence that Pkc1 is activated by Ca^{2+} signalling, Marini and co-workers demonstrated a cell cycle-dependent activation of type C phospholipases, mediated by Cdc28 kinase: at START, Cdc28p, by activating these phospholipases, leads to DAG production, the major activator of mammalian PKC, via phosphatidylcholine hydrolysis and the subsequent activation of Pkc1p, responsible for cell wall remodelling or maintenance as cells enter the budded phase of the cell cycle. A direct relationship between Pkc1p activity and DAG in *S. cerevisiae* has not been documented; however, all PKC isozymes structurally related to Pkc1p are DAG-stimulable and Pkc1p contains consensus DAG-binding sites (Levin *et al*., 1990). In addition, it was demonstrated that glucose addition to nutrient-deprived cells is able to stimulate phosphatidylinositol turnover in a Plc1p-dependent manner, by monitoring DAG level increase, and also to activate plasma membrane H^+/ATPase, which in turn can also be activated by Pkc1p (Coccetti *et al*., 1998).

A role for Pkc1p pathway specifically at the G_1/S transition has been also suggested by the finding that *PKC1* overexpression suppressed the growth defect in bud emergence of *swi4* mutants, inactivated in the major transcription factor involved in activating transcription of many genes at the G_1/S transition (Gray *et al*., 1997).

Calcineurin itself was previously reported to be involved in the regulation of actin polarization at the bud site during bud emergence at G_1/S transition: in presence of high Ca^{2+} concentrations in the medium (50 mM $CaCl_2$), bud emergence and actin polarization at the presumptive bud site were delayed in a wild-type strain, but in a *cnb1Δ* strain this delay was abrogated indicating that it occurred in a calcineurin-dependent manner. Thus, a negative calcineurin-dependent regulation of actin polarization was proposed (Shitamukai *et al*., 2004).

Keeping in account these literature data and our previous findings demonstrating glucose-induced calcineurin activation, cell cycle progression, and in particular bud emergence, were monitored in wild-type and *cnb1Δ* strains, subjected to nutritional shift-up from ethanol to glucose, in order to mimick the situation represented by glucose addition to nutrient-deprived cells responsible for calcineurin activation. Consistently with the previously reported role of calcineurin as negative regulator of

actin polarization at the bud site, *cnb1Δ* strains did not display the physiological decrease in budding index after nutritional shift-up, normally observed in a wild-type strain (Fig. 29), necessary to reset the critical cell size for bud emergence required for growth on glucose. It is possible that calcineurin activation by glucose addition to cells grown in ethanol containing cultural medium could be responsible for a delay in bud emergence, due to inhibition of actin polarization at the bud site.

However, further and more detailed investigation on calcineurin involvement in regulating essential parameters for cell cycle progression in dependence on nutrient signals, will have to be performed in order to understand if calcineurin activation by nutrients could regulate growth in dependence on nutrient availability by another still unknown mechanism.

In the second part of this work we focused on calcium signalling induced by hypotonic shock: yeast cells respond to an hypotonic shock with a temporal increase in cytosolic Ca^{2+} level , which seems to be generated at the early stage by Ca^{2+} release from intracellular stores, while later a sustained increase in cytosolic Ca^{2+} concentration depends upon an influx of Ca^{2+} from the extracellular environment (Batiza *et al*., 1996).

Hypotonic shock-triggered Ca^{2+} signal appeared to be mediated for 50% of the total response by Cch1 HACS subunit, whereas Mid1 subunit seems not to be involved in such response: *mid1Δ* strain was not affected in Ca^{2+} signalling (Fig. 30), suggesting for the first time that Mid1p and Cch1p can act independently and not as a sole complex. In pheromone-induced Ca^{2+} signalling, as well as in GIC signalling, *mid1Δ* and *cch1Δ* single mutant strains displayed the same defects, indicating that these two proteins act in the same complex, playing the same role. Conversely, Cch1p appeared to be the only known membrane calcium transporter responsible for Ca^{2+} influx from the extracellular environment in response to hypotonic shock. *mid1Δ cch1Δ fig1Δ* strains, impaired in HACS and LACS activity, displayed a significant increase in cytosolic Ca^{2+} level after hyptonic shock, suggesting that another not yet identified calcium transporter can mediate this Ca^{2+} flux, contributing for 50% to the total response in the wild-type strain (Fig. 30). Our data suggest that Ca^{2+} release from extracellular stores in response to hypotonic shock is not mediated by Yvc1 vacuolar channel, since *YVC1* deletion did not affect Ca^{2+} signal in these conditions (Fig. 30).

In order to better investigate the role of Ca^{2+} release from intracellular stores in response to hypotonic shock, Ca^{2+} signal was analysed in the absence of extracellular calcium: in the *mid1Δ cch1Δ fig1Δ* mutant strain, Ca^{2+} signal seemed to be almost exclusively dependent on release of calcium from intracellular stores (Fig. 31B), suggesting that the unknown

hypotonic shock-responsive calcium transporter is likely an intracellular channel.

Differently from all the others calcium fluxes described hitherto, hypotonic shock-induced calcium release from intracellular stores is inhibited by extracellular calcium in a concentration-dependent manner, both in wild-type and in all the mutant strains analysed (Fig. 31): this peculiar feature seems to be related partially to HACS system, since *cch1Δ* and *mid1Δ cch1Δ* mutants were slighlty more sensitive to inhibition by extracellular calcium than a wild-type strain (Fig. 32), even if in absence of Cch1p activity a major release of calcium from intracellular stores was observed (Fig. 40). It is likely that in absence of the calcium channel responsible for calcium influx from the extracellular environment, an enhanced release of calcium into the cytosol mediated by intracellular calcium transporters occurred, and that this response is inhibited by an unknown mechanism when extracellular calcium is present in the culture medium. This mechanism could be mediated by Fig1 LACS component, since *FIG1* deletion in a *mid1Δ cch1Δ* strain restored an inhibition profile dependent on extracellular calcium similar to that observed in wild-type cells (Fig. 32).

Calcineurin involvement in regulating hypotonic shock-induced Ca^{2+} signalling was hypothesized, since calcineurin is strongly related to osmotic stress response. However, differently from what observed in GIC signalling, calcineurin seems not to be involved in regulating the unidentified hypotonic shock-responsive transporter: *CNB1* encoding calcineurin activating subunit deletion, did not affect Ca^{2+} signal in absence of extracellular calcium, but resulted in a significantly higher sensitivity to inhibition by extracellular calcium (Fig. 33). In the absence of extracellular calcium calcineurin activity could be very low even in wild-type cells, making no difference with a *cnb1Δ* strain; however, a role for calcineurin as positive regulator of a putative intracellular calcium channel could be hypothesized, since in the absence of calcineurin activity a very faint Ca^{2+} signal in presence of extracellular calcium was observed. Alternatively, calcineurin inactivation could affect calcium homeostasis maintaining high HACS activity, causing an increase in cytosolic calcium levels: actually, when HACS system was impaired in a *cnb1Δ* strain, the sensitivity to extracellular calcium was partially relieved (Fig. 34), with a parallel increase in calcium release from intracellular stores (Fig. 40).

Even if a role of calcineurin in regulating calcium flux in response to hypotonic shock can not be excluded, the unknown hypotonic-shock responsive calcium transporter, differently from GIC transporter, seems not to require calcineurin activity for its functionality. These findings together lead to hypothesize that in budding yeast there are at least two different,

still unidentified at the molecular level, calcium transporters responsive to nutrients and to hypotonic shock, differently regulated.
Unfortunately a pharmacological approach in order to characterize the unknown hypotonic shock-responsive calcium channel was abandoned: neither gadolinium nor nifedipine, previously resulted effective in inhibiting HACS system and GIC transporter, displayed any effect in our conditions on osmotic stress-dependent calcium signalling. However, it is possible that the lack of inhibitory effect could be related to these drugs inability to access to their intracellular target.
For hypotonic shock-dependent Ca^{2+} signalling also, a possible involvement of the uncharacterized Yor365c protein was investigated. Although in *yor365cΔ* strains Ca^{2+} signal was not affected (Fig. 35), excluding that Yor365cp could be the unknown intracellular calcium channel, a regulatory role in this response for Yor365cp emerged: *YOR365c* deletion resulted in a significant decrease in sensitivity to inhibition by extracellular calcium, but the inhibition profile returned similar to that observed in wild-type cells when *YOR365c* was deleted together with *MID1*, *CCH1* and *FIG1* (Fig. 36).
CLS2 encoded putative calcium transporter, located on ER membrane, was also investigated in order to understand if Ca^{2+} signal observed in response to hypotonic shock in absence of extracellular calcium was related to Ca^{2+} release from ER. *cls2*Δ mutants not only were not affected in Ca^{2+} signalling (Fig. 38), but even displayed an increasing Ca^{2+} peak in absence of extracellular calcium (Fig. 40). It is likely that in the absence of Cls2p and Yvc1p intracellular calcium transporters, another intracellular channel could be hyperactivated in order to sustain the response.
However, *CLS2* and *YVC1* deletion resulted in opposite effects on Ca^{2+} signal inhibition by extracellular calcium, causing, at the highest extracellular concentration of calcium chloride tested, a maximum inhibition of total response respectively by nearly 80% (Fig. 39) and by 60% (Fig. 37), whereas in the wild-type strain an inhibition by 70% was reached. The concomitant deletion of *CLS2* and *YVC1* sequences, resulted in a strong relieve in sensitivity to extracellular calcium: double mutants displayed a maximum inhibition of the total response of less than 20% (Fig. 39), indicating an inhibition profile very similar to that observed in *yor365cΔ* strains (Fig. 36).
Thus, a functional homology between Yvc1p and Yor365cp was hypothesized, keeping in account that their inactivation resulted in a similar inhibition profile of hypotonic shock-induced Ca^{2+} signalling; however these two proteins seem to play differential roles, at least in our conditions, i. e. in hyperosmotic stress response.
Molecular identification of the unknown glucose and hypotonic shock-responsive calcium transporters is not a trivial challenge, considering also

that for this last intracellular transporter a pharmacological characterization was not certain. Although mammalian and yeast calcium channels differ both for structure and for regulation mechanism, budding yeast could be a very useful model to understand the molecular mechanisms of various channelopathies, by inactivating known yeast calcium transporters or by expressing mammalian sequences encoding different calcium transporters in yeast cells. One of the most important goals, besides the discovery of potential drugs for the treatment of such diseases, could be also represented by the identification of novel compounds specific for yeast calcium channels that could be developed for treatment of several disorders caused by different fungal species.

RIASSUNTO

Il calcio è uno dei principali secondi messaggeri nelle cellule eucariotiche, dove regola svariate funzioni cellulari. Nel lievito *Saccharomyces cerevisiae* tale ione è coinvolto nella regolazione di diversi processi attivati nella cellula in seguito all'esposizione a vari stimoli extracellulari, quali elevate concentrazioni saline, pH alcalino, stress osmotici, feromoni e nutrienti.
La concentrazione citosolica di calcio è mantenuta a livelli estremamente bassi (50-200 nm) grazie ad un fine meccanismo deputato al mantenimento dell'omeostasi del calcio, mediato dall'azione di una serie di pompe e trasportatori localizzati sulla membrana plasmatica e sulle membrane degli organelli intracellulari. In particolare, nelle cellule di lievito la principale riserva di calcio intracellulare è costituita dal vacuolo, sulla cui membrana è stato identificato il canale Yvc1p, omologo dei canali TRP (Transient Receptor Potential) di mammifero, che viene attivato in risposta ad uno stress iperosmotico rilasciando Ca^{2+} dal vacuolo nel citosol. Al contrario di quanto accade in cellule di mammifero, il reticolo endoplasmico sembra svolgere invece solo un ruolo marginale come magazzino di Ca^{2+} in lievito.
In lievito sono noti solo due sistemi di trasporto per il calcio, localizzati sulla membrana plasmatica, che siano responsabili dell'influsso di Ca^{2+} nella cellula dall'ambiente extracellulare. Il sistema ad alta affinità e bassa capacità HACS (High Affinity Calcium System) è composto dalle subunità Mid1 e Cch1, omologa alle subunità α_1 dei canali del calcio voltaggio-dipendenti di mammifero, ed è pienamente funzionale durante la crescita in terreno minimo mentre è regolato negativamente dalla calcineurina, uno dei principali effettori intracellulari del calcio, durante la crescita in terreno ricco. Il sistema a bassa affinità ed alta capacità LACS (Low Affinity Calcium System) è invece meno caratterizzato sia dal punto di vista funzionale che molecolare: ad oggi ne è stata identificata un'unica componente, la proteina di membrana Fig1, identificata in uno *screening* per mutanti difettivi nella risposta ai feromoni a causa di un'alterazione del *signalling* del calcio, richiesto per la sopravvivenza cellulare in seguito ad esposizione ai feromoni in assenza di coniugazione.
La prima parte di questo lavoro di tesi si è focalizzata sul segnale del calcio dipendente dai nutrienti. È noto, infatti, che l'aggiunta di glucosio a cellule di lievito precedentemente deprivate dei nutrienti induce un rapido e transiente incremento della concentrazione citosolica di calcio, che raggiunge il picco massimo entro 100-120 secondi dallo stimolo, con una cinetica strettamente correlata al *background* genetico del ceppo, e che è essenzialmente mediato dall'influsso di calcio dall'ambiente extracellulare.

Ricorrendo ad un saggio di bioluminescenza *in vivo* basato sulle proprietà della proteina bioluminescente equorina che, in presenza del suo cofattore sintetico e di ioni Ca^{2+}, emette luce in modo dipendente dalla concentrazione di Ca^{2+} citosolica, è stato possibile indagare il ruolo di questi trasportatori nella generazione del segnale del Ca^{2+} dipendente da glucosio. E' emerso che tale segnale è mediato in parte dal sistema HACS, mentre il sistema LACS non sembra essere coinvolto; tuttavia, monitorando il segnale del calcio in risposta al glucosio in ceppi *mid1Δ cch1Δ fig1Δ* si evince l'esistenza di un nuovo trasportatore del calcio, responsivo ai nutrienti, in grado di sostituire i canali noti quando questi sono inattivati. Il trasportatore ignoto, chiamato GIC (Glucose-Induced Calcium Channel), sembra mediare la maggior parte dell'influsso di Ca^{2+} in risposta ai nutrienti durante la crescita in terreno ricco, mentre in terreno minimo esso sembra divenire pienamente funzionale solo quando tutti gli altri trasportatori noti sono inattivati.

Il trasportatore GIC si è rivelato un canale ad alta affinità per il calcio, infatti la sua K_m apparente stimata in cellule *mid1Δ cch1Δ fig1Δ* in crescita in terreno ricco è di 43.8 ± 10.3 µM.

Non è ancora stato possibile mettere a punto uno *screening* genetico che permettesse di identificare il trasportatore GIC a livello molecolare, ma ne abbiamo intrapreso la caratterizzazione da un punto di vista farmacologico. Per prima cosa, ne è stata testata la sensibiltà al magnesio, il più abbondante catione divalente presente nella cellula che è noto inibire in modo competitivo molti sistemi di trasporto del calcio in cellule di mammifero. Il segnale del calcio indotto da glucosio è risultato sensibile anche a basse concentrazioni di magnesio presenti nell'ambiente extracellulare; in particolare, la costante IC_{50} apparente in cellule *mid1Δ cch1Δ fig1Δ* cresciute in terreno ricco è stata stimata 1.04 ± 0.05 mM.

Inoltre è stata testata la sensibilità del trasportatore GIC ad alcuni tra i più comuni bloccanti dei canali voltaggio-dipendenti di tipo L di mammifero: nifedipina, gadolinio e verapamil. La nifedipina e il gadolinio si sono rivelati due potenti inibitori dei canali del calcio anche in cellule di lievito, rivelando una IC_{50} apparente per il trasportatore GIC, calcolata in cellule *mid1Δ cch1Δ fig1Δ* cresciute in terreno ricco e trattate con diverse concentrazioni di questi composti, rispettivamente di 0.36 ± 0.13 µM e 16.7 ± 1.7 µM. Sorprendentemente il trasportatore GIC è risultato insensibile al verapamil, che al contrario alle più alte concentrazioni testate si comporta da agonista: questa osservazione, sommata alla dimostrata resistenza del trasportatore GIC al nichel, un altro comune inibitore dei canali del calcio voltaggio-dipendenti di tipo T/L di mammifero, sembra suggerire che il trasportatore

GIC possa essere costituito da un canale atipico, con caratterisitiche farmacologiche e funzionali diverse da quelle dei canali del calcio di mammifero. Al fine di meglio caratterizzare i meccanismi di regolazione di tale trasportatore, è stato studiato il segnale del calcio dipendente da nutrienti in ceppi mutanti *mid1Δ cch1Δ fig1Δ cnb1Δ*, inattivati anche nella subunità regolatoria della calcineurina: l'influsso di calcio in tali ceppi è completamente abolito, suggerendo che il trasportatore GIC è regolato positivamente dalla calcineurina.

Nella seconda parte del lavoro ci siamo focalizzati sull'effetto del segnale del Ca^{2+} indotto da glucosio sulla calcineurina stessa, una serina-treonina fosfatasi in grado di trasdurre vari segnali all'interno della cellula in risposta ad un aumento del livello citosolico di Ca^{2+}, grazie alla capacità di controllare la trascrizione di una grande quantità di geni attraverso la regolazione della localizzazione del fattore trascrizionale Crz1p. Sfruttando un saggio per la misurazione dell'attività β-galattosidasica basato sul gene reporter *LACZ* posto sotto il controllo di quattro sequenze CDRE, riconosciute dal fattore trascrizionale Crz1 e quindi responsive alla calcineurina, è stato possibile monitorare l'attività della calcineurina in seguito allo stimolo con glucosio: per la prima volta è stato dimostrato che l'aggiunta di glucosio a cellule precedentemente deprivate dei nutrienti, in presenza di calcio extracellulare, è in grado di attivare la calcineurina durante la crescita in terreno minimo, suggerendo una nuova relazione tra l'attività della calcineurina, normalmente associata a stress, ed il *sensing* dei nutrienti. In terreno ricco tale induzione nell'attività della calcineurina non è osservabile, tuttavia in queste condizioni di crescita si osserva un livello di attività trascrizionale basale calcineurina-dipendente molto più elevato di quello osservato durante la crescita in terreno minimo. E' stato successivamente dimostrato che vi sono due importanti fattori responsabili di questa differenza osservata nelle due condizioni di crescita testate: la disponibilità di calcio nel mezzo di crescita, che è di gran lunga superiore in terreno ricco, e l'attività della PKA, che regolando negativamente il fattore trascrizionale Crz1 si oppone al *signalling* della calcineurina.

Infine, ci siamo focalizzati anche sul segnale del calcio indotto da stress osmotico: è noto, infatti, che cellule di lievito rispondono ad uno shock ipotonico con un rapido e transiente incremento del livello citosolico di Ca^{2+}, mediato in parte dall'ingresso di calcio dall'ambiente extracellulare e in parte dal rilascio di calcio dai depositi intracellulari. E' stato dimostrato che anche nella risposta allo shock ipotonico è coinvolto un trasportatore intracellulare ignoto. Tale trasportatore ignoto è stato caratterizzato da un punto di vista farmacologico e funzionale : esso si è rivelato insensibile agli inibitori dei canali del calcio voltaggio-dipendenti mostratisi attivi anche sul

trasportatore GIC responsivo al glucosio, inibito dal calcio extracellulare presente nel mezzo di crescita in modo concentrazione-dipendente e infine non dipendente dall'attività della calcineurina. Tutte queste osservazioni indicano chiaramente che il trasportatore GIC e il trasportatore intracellulare responsivo allo shock ipotonico, non ancora identificati a livello molecolare, sono due trasportatori del calcio differenti.
Sulla base di una ricerca bioinfromatica in banca dati ed in letteratura, abbiamo identificato delle putative sequenze riconducibili a trasportatori ionici e ne abbiamo verificato il coinvolgimento nel *signalling* del calcio indotto da nutrienti e da shock ipotonico. Abbiamo identificato così la sequenza *YOR365c*, codificante per una proteina ignota che presenta un motivo conservato TRP peculiare della superfamiglia di canali TRP di mammifero, che sembra svolgere un ruolo regolatorio nella risposta al glucosio e che influenza anche le caratteristiche del rilascio di calcio intracellulare indotto da shock ipotonico, e potrebbe quindi essere coinvolto nella regolazione generale dei flussi di calcio.
Purtroppo non è stato possibile identificare a livello molecolare I trasportatori del calcio ignoti, ma in futuro le proprietà farmacologiche di tali trasportatori identificate in questo lavoro potranno costituire un ottimo punto di partenza per mettere a punto *screening* genetici volti a identificare tali sequenze.

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