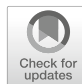

Calcium Cation Cycling and Signaling Pathways in Fungi

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I. Introduction

Ca^{2+} is chosen by all organisms as the second intracellular messenger based on its own physical and chemical properties (Carafoli et al. 2001; Haiech and Moreau 2011). Different from other second intracellular messengers, such as the cyclic adenosine monophosphate (cAMP), the inositol 1,4,5-trisphosphate (IP3), and the diacylglycerol (DAG), Ca^{2+} cannot be degraded to terminate its carried signals (Chakraborty and Hasan 2012; Hartwig et al. 2014; Hohendanner et al. 2014; Ueda et al. 2014). In contrast, Ca^{2+} ions are pumped out of the cytoplasm or conserved in the intracellular Ca^{2+} stores via a series of Ca^{2+} transporters or Ca^{2+}/anion exchangers localized in the plasma membrane or the intracellular organelle membrane, respectively. In eukaryotic cells, the vacuole, the lysosome, the endoplasmic reticulum (ER), the Golgi apparatus (GA), the mitochondria, and even the nucleus envelope are employed to function as the intracellular Ca^{2+} reservoirs (Dunn et al. 1994; Ermak and Davies 2002; Van Baelen et al. 2004; Michelangeli et al. 2005; Lustoza et al. 2011; Takeuchi et al. 2015). Upon stimulation, Ca^{2+} ions are rapidly released from the intracellular Ca^{2+} stores, resulting in the Ca^{2+} concentration increase in the cytoplasm (Cui et al. 2009). Depletion of Ca^{2+} from the intracellular Ca^{2+} stores triggers the influx of Ca^{2+} from the extracellular environments, which further increase cytosolic Ca^{2+} concentrations (Locke et al. 2000; Liu et al. 2006). These Ca^{2+} ions are caught by Ca^{2+} sensors such as calmodulin (CaM) to activate itself and subsequently the downstream enzymes such as calcineurin. The transcription factor Crz1 is consequently dephosphorylated by calcineurin and translocated from the cytoplasm into the nucleus to initiate special transcriptions in order to deal with the abovementioned stimulations (Matheos et al. 1997; Onyewu et al. 2004). Finally, the Ca^{2+} is pumped out of the cytoplasm or into the intracellular Ca^{2+} reservoirs to set the stage for the next cycle. This Ca^{2+} signaling pathway is highly conserved from prokaryotes to eukaryotes, but there are different editions in various kinds of cell types (Dominguez 2004; Tuteja and Mahajan 2007; Luan 2009; Hashimoto and Kudla 2011; Batistic and Kudla 2012). In fungal kingdom, knowledge on Ca^{2+} regulation is primarily obtained from the model fungus *Saccharomyces cerevisiae*, in spite of accumulation of parallel advances in other fungi. Here, we outline the participators or potential players in the

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Ca^{2+} regulation network, focusing on Ca^{2+} circling and signaling mechanisms.

II. The Vacuole and Ca^{2+} Cycling Pathways Against the Cytoplasm

Similar to plants but different from animals, the vacuole is the principal intracellular **Ca^{2+} reservoir in fungi** (Pittman 2011). *S. cerevisiae* has one to several vacuoles in which 90% of the intracellular Ca^{2+} ions are stored (Cunningham and Fink 1994; Tanida et al. 1995). In *Neurospora crassa*, there are different types of vacuoles with distinct morphogenesis and composition, and it is not clear now whether they are just the intermediates prior to maturation or already mature, final vacuoles (Bowman et al. 2009; Cunningham 2011). Besides playing as the intracellular Ca^{2+} stores, fungal vacuoles also participate in toxic substance sequestration, osmotic pressure regulation, and pH value accommodation (Wada and Anraku 1994; Zhou et al. 2003; Moreno and Docampo 2009). Oxalate, phosphate, and organic acids inside the vacuole can chelate a bulk of Ca^{2+} ions, and only a small proportion of Ca^{2+} is ready for release in response to different stimulations (Moreno and Docampo 2009; Docampo and Moreno 2011). For example, in *S. cerevisiae* cultured in standard conditions, the free Ca^{2+} concentration in the vacuole is about 30 μM, while the total Ca^{2+} concentration is about at the level of 3 mM, indicating 99% of the Ca^{2+} has been immobilized in the vacuolar lumen (Cunningham 2011).

Membrane fusion of the vacuole with the secretory vesicles derived from the GA can contribute to Ca^{2+} uptake of the vacuole (Cunningham 2011), but two other Ca^{2+} transporting mechanisms play main roles in providing the vacuole with Ca^{2+}: a primary energized **Ca^{2+}-ATPase pathway** mediated by the plasma membrane Ca^{2+} ATPase (PMC) and a secondary energized Ca^{2+} exchanger pathway carried out by the Ca^{2+}/H^{+} exchanger (Cunningham and Fink 1996; Miseta et al. 1999b; Samarao et al. 2009; Li et al. 2011; Pittman 2011). Ca^{2+}-ATPases belong to the P-type ATPase superfamily characterized by the formation of a phosphorylated intermediate during the transportation cycle (Okamura et al. 2003; Bublitz et al. 2010). This superfamily is divided into five families based on sequence similarities, phylogenetic analyses, and especially substrate specificities, with Ca^{2+}-ATPase being grouped as type II P-type ATPase (Okamura et al. 2003). In contrast to the high-affinity property of the Ca^{2+}-ATPase, Ca^{2+}/H^{+} exchanger has the characteristics of low affinity but accompanied by high capacity, which can transport Ca^{2+} to the vacuole with high efficiency soon after a burst of cytosolic Ca^{2+} elevation (Pittman 2011).

S. cerevisiae **Pmc1** was the first Ca^{2+} ATPase identified and cloned from all organisms, which displays 40% identical to the mammalian plasma membrane Ca^{2+} ATPases (PMCAs). In *S. cerevisiae*, *pmc1* mutants sequestrate Ca^{2+} in the vacuole at less than 20% of the wild type in the standard medium (Cunningham and Fink 1994), and the mutants demonstrate sensitivity to high concentrations of environmental Ca^{2+} (Cunningham and Fink 1996), suggesting that Pmc1 probably plays predominant roles in Ca^{2+} sequestration in the vacuole. In the opportunistic pathogen *Cryptococcus neoformans*, deletion of *pmc1* impairs blood-brain barrier transmigration and alters the global gene expression profile, including *ure1* which encodes the virulence factor urease (Squizani et al. 2018). In *S. cerevisiae*, Pmc1 almost settles exclusively in the vacuolar membrane, whereas in *N. crassa*, the Pmc1 counterparts NCA-2 and NCA-3 localize in the vacuolar membrane as well as in the plasma membrane (Bowman et al. 2011). Mammalian PMCAs are solely positioned in the plasma membrane and possess an autoinhibition domain at the C-terminus, and binding of the Ca^{2+} sensor calmodulin with this regulatory domain can release the activities of PMCAs (Brini and Carafoli 2009). In *S. cerevisiae*, Pmc1 has no C-terminal inhibitory region, and the regulation takes place at the transcription level (Cunningham and Fink 1996; Matheos et al. 1997). Hence, the expression level of *pmc1* mRNA is upregulated as a response to the elevated cytosolic Ca^{2+} level.

Vcx1 (vacuolar calcium exchanger), as the first Ca^{2+}/H^{+} exchanger identified in eukaryotes,

was originally discovered to be important for *S. cerevisiae pmc1* mutants to survive in high levels of Ca^{2+} (Cunningham and Fink 1996; Miseta et al. 1999b). Vcx1 had been demonstrated to play a primary role in transporting Ca^{2+} to the vacuole in *S. cerevisiae*. A similar phenomenon is also observed in *C. neoformans*, which showed that *C. neoformans vcx1* null mutants had an increased intracellular calcium concentration (Kmetzsch et al. 2010). However, deletion of *S. cerevisiae vcx1* does not lead to obvious decrease of vacuolar Ca^{2+} concentrations, and the mutant does not exhibit sensitivity to high environmental Ca^{2+} (Cunningham and Fink 1996; Miseta et al. 1999b), which is in sharp contrast to the phenotypes of *pmc1* mutant. In fact, Ca^{2+} transport into the vacuole is mainly mediated by Pmc1 and to a lower extent by Vcx1 resulting from the regulation of calcineurin, the calmodulin-dependent serine/threonine phosphatase. Calcineurin increases the expression levels of Pmc1 and inhibits the activity of Vcx1 simultaneously (Cunningham and Fink 1996; Kmetzsch et al. 2010). The exact reason for this phenomenon remains unexplored, but one can hypothesize that cells avoid Ca^{2+} overload by not activating both Ca^{2+} transporters simultaneously.

Ca^{2+} can rapidly enter the vacuole via Vcx1, with the stoichiometry of 1 Ca^{2+} ion per 2 or 3 H^+ ions (Dunn et al. 1994), immediately after the cytosolic Ca^{2+} concentrations are increased and prior to the complete activation of calcineurin. The pH gradient across the membrane of the vacuole is the prerequisite for Vcx1 to function normally, which is administered by the H^+ ATPase (V-type ATPase) in the vacuolar membrane (Kakinuma et al. 1981). A sudden loss of vacuole acidity might not only prevent Ca^{2+} influx into the vacuole but also oppositely induce the Ca^{2+} efflux from the vacuole via Vcx1 (Forster and Kane 2000). When the gene encoding the H^+ ATPase was mutated, yeast cannot tolerate high concentration of Ca^{2+}, further confirming that acidification of vacuole is indispensable for Ca^{2+} efflux into lumen (Forster and Kane 2000).

Ca^{2+} could possibly be released from the vacuole via Vcx1 in response to special conditions (Cunningham 2011); however, the vacuole is equipped with at least one Ca^{2+} channel specially responsible for Ca^{2+} release. **Yvc1 (yeast vacuolar conductance)** is a homologue of mammalian Ca^{2+} channels from TRPC (transient receptor potential canonical) family (Palmer et al. 2001; Cunningham 2011; Yu et al. 2014), which can convey Ca^{2+} and some other ions such as K^+ and Na^+ across the membrane (Chang et al. 2010). To date, three kinds of Ca^{2+} channels have been identified: voltage-gated channels, stretch-activated channels, and ligand-gated channels (Carafoli et al. 2001). Studies on Yvc1 from isolated *S. cerevisiae* vacuoles indicate that it can be activated by membrane stretch, suggesting that Yvc1 is a mechanosensitive (stretch-activated) ion channel (Zhou et al. 2003; Loukin et al. 2008; Chang et al. 2010). Another evidence suggests that Yvc1 might also be a ligand-activated channel, because phosphatidylinositol-3,5 bisphosphate (PIP_2) and Ca^{2+} itself can trigger Ca^{2+} release from the vacuole via binding to Yvc1 (Dong et al. 2010). Ca^{2+}-induced Ca^{2+} release is common with the Ca^{2+} efflux from sarcoplasmic/endoplasmic reticulum of mammalian cells (Hernandez-Cruz et al. 1997; Ji et al. 2006). Considering the fact that the vacuole is the primary Ca^{2+} detoxifying organelle in the cell, this mechanism might render Pmc1 and Vcx1 a huge mission to sequester Ca^{2+} from the cytoplasm. At least one other Ca^{2+} channel, rather than Yvc1, is situated in fungal vacuolar membrane, because the ligand IP_3 is able to trigger Ca^{2+} release from the vacuole via the IP_3 receptor Ca^{2+} channel (IP_3R) (Calvert and Sanders 1995; Silverman-Gavrila and Lew 2002), but the isolated Yvc1 does not respond to IP_3 stimulation under numbers of patch-clamp conditions in *S. cerevisiae* (Palmer et al. 2001). This IP_3-mediated Ca^{2+} channel induced by IP_3 has not been identified to date and needs further exploitation.

Ca^{2+} Circulation Between the Golgi and the Cytoplasm

The **Golgi apparatus (or Golgi complex, GA)** was first discovered in 1898 by the Italian physician Camillo Golgi (Bentivoglio et al. 2011; Li et al. 2013). During the next several decades, debates still exit focusing on the existence of this organelle in the cell. Now, it is clear that the GA is an independent intracellular organelle,

and it is constituted of stacks of flattened cisternae with polarity, which are primarily organized into three compartments: the cis-Golgi network (CGN), the medial-Golgi, and the trans-Golgi network (TGN) (Pizzo et al. 2011; Day et al. 2013). Although the overall morphogenesis of the GA might vary slightly, for example, the cisternae seem relatively disperse in *S. cerevisiae* whereas compact in *Schizosaccharomyces pombe*, their functions are believed to be similar (Suda and Nakano 2012).

The GA is the processing factory and sorting center in the cell, guaranteeing cargo proteins to be modified, packaged, and sent to their final destinations correctly (Nakano 2004; Pizzo et al. 2011). Some processing enzymes in the medial-Golgi or TGN utilized for glycosylation need Ca^{2+} as the cofactor, while correct aggregation of secretory proteins in the TGN relies on the Ca^{2+} concentration in lumen, indicating the GA might be an intracellular Ca^{2+} reservoir (Micaroni and Mironov 2010; Pizzo et al. 2011; von Blume et al. 2011). The GA contains Ca^{2+} ions at the high concentration, ranging from 10^{-4} to 10^{-3} M in mammalian cells (Li et al. 2013), which is distributed unevenly in different subcompartments from CGN to TGN. In the membrane of the GA, there exist the Ca^{2+} ATPase(s), responsible for transporting Ca^{2+} from cytoplasm to the GA lumen and the Ca^{2+} channel(s), releasing Ca^{2+} from the GA lumen to the cytoplasm (Van Baelen et al. 2004; Pizzo et al. 2011; Li et al. 2013).

Pmr1 (plasma membrane ATPase related) was identified in *S. cerevisiae* (Rudolph et al. 1989) as the first Ca^{2+} ATPase found to be situated in the GA, especially the medial-Golgi, TGN, and secretory vesicles derived from TGN (Antebi and Fink 1992; Durr et al. 1998). Further homologues of Pmr1 have been identified from a variety of organisms, such as other fungi, worms, insects, mammals, and even bacteria (Yagodin et al. 1999; Van Baelen et al. 2001; Raeymaekers et al. 2002; Bates et al. 2005; Cho et al. 2005; Buttner et al. 2013). Pmr1 belongs to the type II P-type ATPase, but it is distinguished from another member of the type II P-type ATPase, i.e., SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase) in at least three aspects: (1) Pmr1 translocates one Ca^{2+} ion at the expense of one molecule of ATP, whereas SERCA can simultaneously transport two Ca^{2+} per ATP molecule (Wuytack et al. 2003); (2) Pmr1 is able to transfer not only Ca^{2+} but also Mn^{2+} (Ton et al. 2002); and (3) ion transportation of Pmr1 is thapsigargin-insensitive, whereas Ca^{2+} transport action of SERCA is sensitive to thapsigargin (Wuytack et al. 2003). The possible reason for the difference in transport efficiency between Pmr1 and SERCA is that Pmr1 has only one Ca^{2+}-binding site, while SERCA has two (Wuytack et al. 2003). The fact that the unique ion-binding site in Pmr1 can be occupied by either Ca^{2+} or Mn^{2+} is probably due to the relatively low substrate specificity of this Ca^{2+}/Mn^{2+} ATPase (He and Hu 2012).

S. cerevisiae lacks Ca^{2+} ATPase(s) in the plasma membrane and ER membrane (i.e., PMCA and SERCA), and Vcx1 function is inhibited by calcineurin activation. These may suggest that Pmr1 and Pmc1 play the predominant role in maintaining the cytosolic homeostasis. Deletion of *pmr1* in *S. cerevisiae* leads to 16-fold increase of cytosolic Ca^{2+} levels relative to wild type (Ton et al. 2002). In the *S. cerevisiae pmr1* mutants, the expression level of *pmc1* rises about fivefold, and Pmc1-splicing variants might be localized in the GA, as the compensatory mechanisms (Marchi et al. 1999; Miseta et al. 1999a). Double deletion of *pmr1* and *pmc1* in *S. cerevisiae* is lethal in standard medium. However, repression of the calcineurin activity, which abolishes the inhibition of Vcx1, will recover the viability (Kellermayer et al. 2003). Different from the case in *S. cerevisiae*, double deletion of *pmr1* and *pmc1* homologues in *Aspergillus nidulans* is not lethal, which probably results from Ca^{2+} detoxification carried out by the Ca^{2+} ATPase(s) in the plasma membrane or in ER membrane (Jiang et al. 2014a).

The GA, possessing about 5% of total intracellular Ca^{2+} in LLC-PK1 cells (Pizzo et al. 2011), is suggested to be an exchangeable Ca^{2+} store; therefore, at least one channel is needed to mediate Ca^{2+} release from the lumen to the cytoplasm. IP_3 receptor (IP_3R) is a Ca^{2+} channel situated primarily in the sarcoplasmic/endoplasmic reticulum membrane, mediating Ca^{2+} influx from matrix to cytoplasm (Krizanova and Ondrias 2003; Bezprozvanny 2005). Recently, IP_3R is found to reside in the GA as well, because the intracellular second messen-

ger IP_3 is able to trigger Ca^{2+} mobilization from the GA, especially the CGN subcompartment (Li et al. 2013). Ryanodine receptors (RyR) is sarcoplasmic/endoplasmic reticulum-localized Ca^{2+} channel that is responsible for the release of Ca^{2+} from intracellular stores (Lanner et al. 2010). It has been demonstrated that RyR may participate in Ca^{2+} release from the GA in rat sympathetic neurons and exist in neonatal cardiac myocytes as well (Cifuentes et al. 2001; Lissandron et al. 2010). Therefore, it is possible that RyR might also exist in GA membrane. However, no IP_3R and RyR Ca^{2+} channels have been reported in the fungal kingdom.

III. Ca^{2+} Circuit from and to the Endoplasmic Reticulum

Compared to the vacuole and the GA, **ER (endoplasmic reticulum)** is less important in sequestering Ca^{2+} from the cytoplasm in fungi. Nevertheless, Ca^{2+} is maintained in the fungal ER lumen, e.g., *S. cerevisiae* has about 10 μM free Ca^{2+} in ER matrix (Ton et al. 2002; Li et al. 2013), which is crucial to the organelle's function, especially in the perspective of protein modification and folding, because a variety of ER-resident enzymes are Ca^{2+}-dependent (Uemura et al. 2007; Losev et al. 2008). For example, Calreticulin is the molecule chaperon in ER contributing to correct protein folding, which is also a Ca^{2+}-binding protein with high capacity (Parlati et al. 1995; Groenendyk et al. 2004; Brunner et al. 2012). There are many other chaperons in the ER lumen, such as Calnexin, endoplasmin, PDI (protein disulfide isomerase), ERp (ER protein), and BiP (immunoglobulin-binding protein) (Shnyder et al. 2008). They corporately support correct protein folding in a Ca^{2+}-dependent matter [for details, please refer to (Michalak et al. 2002; Groenendyk et al. 2004; Coe and Michalak 2009)].

In mammalian cells, the Ca^{2+} ATPase replenishing ER with Ca^{2+} is dependent on SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase), which was first discovered in 1962 when sarcoplasmic reticulum fractions were shown to accumulate Ca^{2+} at the expenses of ATP hydrolysis (Carafoli and Brini 2000). SERCA belongs to type II P-type ATPase, translocating Ca^{2+} and/or Mn^{2+} across the membrane at the expense of ATP hydrolysis. In *S. cerevisiae*, no SERCA homologues have been identified from the ER membrane, and partial ER luminal Ca^{2+} is contributed by Pmr1 located mainly in the GA. However, type V P-type ATPase Cod1/Spf1 localized in the ER membrane can supplement ER with Ca^{2+}, although the substrate transfer and the in vivo function of this P-type ATPase are not clear yet (Cronin et al. 2002). *S. pombe* type V P-type ATPase Cta4p is proven to have Ca^{2+}-transporting activity coupled with ATPase activity, and a *S. pombe* mutant lacking *cta4* has a sixfold excess of cytosolic Ca^{2+} concentration when compared to wild type (Lustoza et al. 2011). On the contrary, there is SERCA homologue in the ER membrane in *N. crassa*, namely, NCA-1, which has been proved to be essential in maintaining the calcium level (Bowman et al. 2011).

IV. Ca^{2+} Exchange Between Mitochondria and the Cytoplasm

The **mitochondrion** is an essential intracellular organelle for energy production, because the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation coupled by the electron transfer reactions take place in the lumen or across the membrane (Pan et al. 2007; Katoh et al. 2008). Some dehydrogenases such as the pyruvate dehydrogenase, the oxoglutarate dehydrogenase, and the isocitrate dehydrogenase employed in the ATP creation procedures need Ca^{2+} at high concentrations to increase enzyme activities (Denton 2009; Santo-Domingo and Demaurex 2010). Therefore, it is reasonable to deduce that the inner Ca^{2+} concentration of the mitochondria can be regulated to satisfy with different physiological demands. In fact, it has been established for a long period of time that the mitochondria can sequester and release Ca^{2+} as a dynamic Ca^{2+} store (Ganitkevich 2003). As other intracellular Ca^{2+} sinks, only a small percentage of Ca^{2+} is freely available, and most of it is bound by the buffers in the matrix, probably membrane phospholipids and/or the inner phosphate ions (Horikawa et al. 1998; Pivovarova et al. 1999; Ganitkevich 2003).

To date, two routes mediating Ca^{2+} entering the mitochondria have been identified: a saturable **low-affinity uniporter pathway** and

a saturable **rapid uptake mode (RaM)** (Gunter and Gunter 2001; Adiele et al. 2012). The **mitochondrial Ca^{2+} uniporter (MCU)** located in the inner membrane is believed to be mainly responsible for Ca^{2+} accumulation in this organelle, which is driven by the mitochondrial membrane potential established by the electron-transport chain (Santo-Domingo and Demaurex 2010; Samanta et al. 2014). MCU has a high preference for Ca^{2+} and requires a high concentration of external Ca^{2+} to open the gate. This seems to be the reason why mitochondria are always localized closely to the plasma membrane and the ER membrane (in mammalian cells), because clusters of mitochondrial Ca^{2+} channels can provide bursts of high concentrations of Ca^{2+} (Michelangeli et al. 2005; Prole and Taylor 2012; Takeuchi et al. 2015). MCU have been identified in many eukaryotic cells but are absent in *S. cerevisiae* (Kovacs-Bogdan et al. 2014), while a recent report in the fungal pathogen *Aspergillus fumigatus* demonstrated that deletion of *mcuA*, which is a putative mammalian MCU gene homolog, impaired the mitochondrial Ca^{2+} homeostasis and environmental stress adaptation (Song et al. 2016).

RaM acts as another Ca^{2+} entry pathway in the membrane of the mitochondria and probably owns more important physiological functions than MCU (Buntinas et al. 2001; Bazil and Dash 2011). Different from MCU, RaM can mediate Ca^{2+} influx in the resting state of cytosolic Ca^{2+} concentrations, supplying enough Ca^{2+} to activate the dehydrogenases using Ca^{2+} as a cofactor. The RaM-mediated Ca^{2+} influx is terminated immediately after its initiation, preventing overload of Ca^{2+} to the mitochondrial matrix (Ganitkevich 2003). A considerable increase of mitochondrial Ca^{2+} leads to the formation of the permeability transition pore (PTP) in the mitochondrial membrane, a voltage-gating non-specific channel with numerous subunits, which can release a wide range of matrix materials out of the mitochondria into the cytoplasm, including Ca^{2+}, via PTP itself or the impaired mitochondrial membrane induced by environmental stresses (Bernardi and Di Lisa 2014; De Marchi et al. 2014).

Ca^{2+} can be released from the mitochondrion via PTP; however, Ca^{2+} efflux from the mitochondrion is primarily mediated by two other saturable pathways, a **Na^+-dependent pathway (Ca^{2+}/Na^+ exchanger)** and a **Na^+-independent pathway (Ca^{2+}/H^+ exchanger)** (Pfeiffer et al. 2001; Molinaro et al. 2013; Tsai et al. 2014). The Ca^{2+}/Na^+ exchanger transports one Ca^{2+} ion out of the mitochondria matrix and in return three Na^+ ions into the lumen (Pfeiffer et al. 2001). Therefore, Ca^{2+} release from the mitochondria via the Ca^{2+}/Na^+ exchanger is accompanied by the accumulation of the mitochondrial Na^+ ions, which are subsequently extruded out of the matrix via Na^+/H^+ exchanger for recovering the previous ion gradients (Blomeyer et al. 2013; Rueda et al. 2014). Distinct from the Ca^{2+}/Na^+ exchanger, the H^+/Ca^{2+} exchanger directly utilizes the energy derived from the H^+ potential across the membrane to drive Ca^{2+} efflux out of the mitochondria with the stoichiometry of n H^+: Ca^{2+}, $n \geq 2$ (Pfeiffer et al. 2001; Ganitkevich 2003).

V. Ca^{2+} Signaling Pathways and Its Primary Components in the Cytoplasm

In addition to sequestering cytosolic Ca^{2+} in the intracellular stores, eukaryotic cells also clear Ca^{2+} out of the cytoplasm via the **plasma membrane Ca^{2+} ATPase (PMCA)** and a Ca^{2+}/ion exchanger localized in the plasma membrane (Herchuelz et al. 2013; Roome and Empson 2013). The first PMCA was identified from erythrocytes by Schatzmann in 1966. Afterwards, these kind of Ca^{2+} pumps are found to exist in almost all other eukaryotic cells (Carafoli and Brini 2000). However, no PMCA homologues have been characterized in fungi yet. In contrast, many researches have been performed on the Ca^{2+} channels in the fungal plasma membrane. There are two Ca^{2+} entry systems responsible for extracellular Ca^{2+} influx: (1) the **high-affinity calcium channel (HACS)**, which has high affinity for Ca^{2+} and works in the environment containing Ca^{2+} at low concentration, and (2) the **low-affinity calcium channel (LACS)**, which has relatively reduced

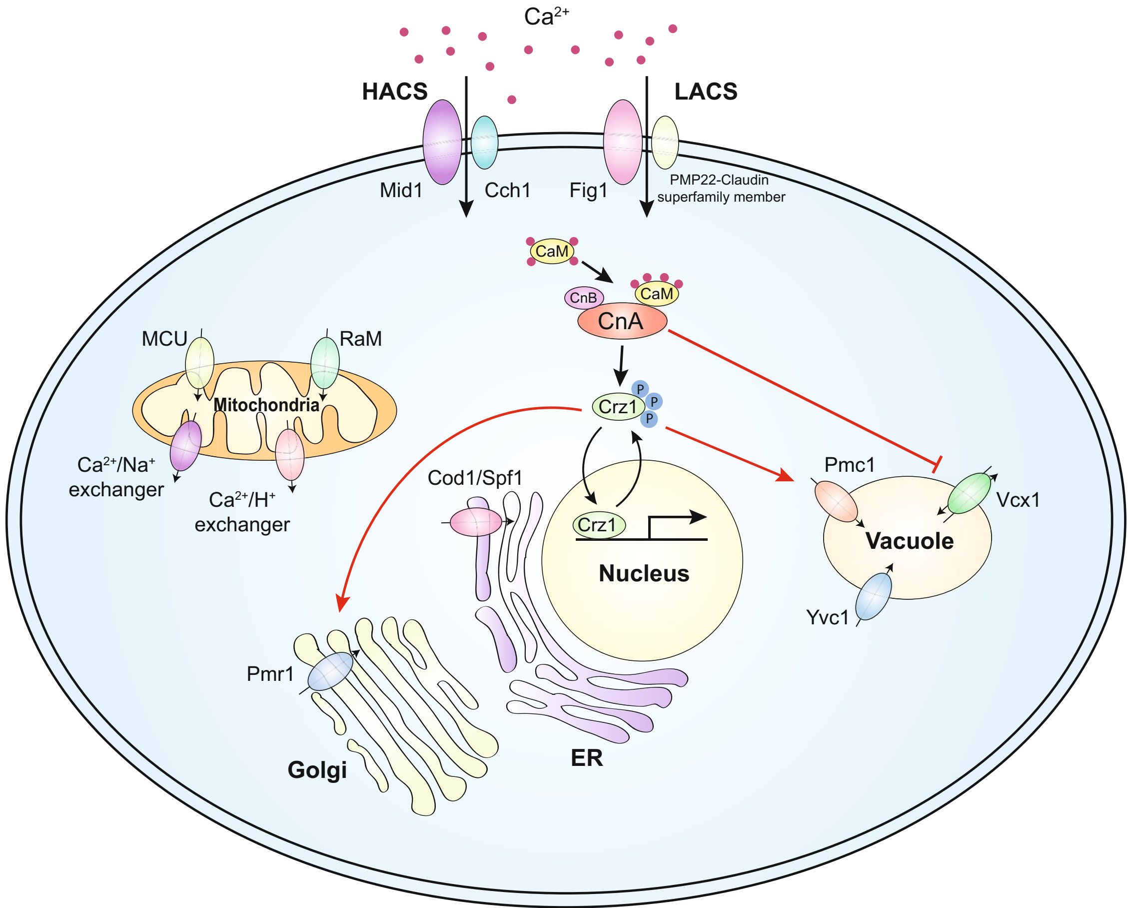

Fig. 1 Scheme model of calcium signaling in eukaryotic cells. Calcium enters through the HACS and/or LACS or is released from calcium stores, mitochondria, and vacuole. Then, Calcineurin binds to the calmodulin-Ca^{2+} complex and dephosphorylates nuclear the transcription factor Crz1 in the cytoplasm, leading to its nuclear translocation. Crz1 subsequently activates downstream genes

affinity for Ca^{2+} and works in circumstances with a higher Ca^{2+} concentration (Muller et al. 2001; Cavinder et al. 2011; Martin et al. 2011) (Fig. 1). The HACS is constituted of at least three subunits: the stretch-activated Ca^{2+} channel **Mid1 (mating-induced death)**; the **voltage-gated calcium channel Cch1 (calcium-channel homologue)**; and the **regulatory protein Ecm7 (extracellular matrix)** (Ding et al. 2013). The LACS is minimally comprised of the Fig1 protein (mating factor-induced gene) and a PMP22-Claudin superfamily member (Brand et al. 2007; Jiang et al. 2014b).

Mid1 was first identified in *S. cerevisiae* and is found to be essential for this organism to mate in presence of environmental Ca^{2+} at low concentrations (Iida et al. 1994; Kanzaki et al. 1999). During the mating cycle, the two haploid cells of opposite mating types (MATa and MATα) fuse to form a diploid zygote (Fischer et al. 1997), which is initiated by binding of pheromone (a factor or α factor secreted by MATa and MATα) to the receptor in the membrane of the other mating type. Then, a series of programmed actions are induced, such as agglutination of cells, regulation of the cell cycle, and differentiation of the cell into shmoos (differentiated cells with projects for mating), accompanied by Ca^{2+} influx from extracellular environments. *S. cerevisiae* lacking Mid1, Cch1, or both cannot complete the mating procedure and finally die upon the pheromone treatment (Iida et al. 1994; Muller et al. 2001). In *Aspergillus* species, deletion of *cchA* or *midA* causes the defects in the conidiation, hyphal polarity, and cell wall integrity in *A. nidulans* (Wang et al. 2012). In comparison, the *A. fumigatus midA* deletion mutant

is hypervirulent in the immunosuppressed mice model (Jiang et al. 2014b). In addition, a coimmunoprecipitation test demonstrated that Mid1 and Cch1 in *S. cerevisiae* might assemble to form a Ca^{2+} channel (Liu et al. 2006). It is suggested that Mid1 acts as a regulatory subunit, whereas Cch1 is the catalytic subunit in the HACS; however, Mid1 expressed in COS7 or CHO cells demonstrates the ability of Ca^{2+} transfer, indicating that Mid1 can function independently (Liu et al. 2006). In rich media containing Ca^{2+} at high concentrations, the HACS activity to mediate Ca^{2+} influx is inhibited by calcineurin, while the LACS is stimulated to play the role as Ca^{2+} channel in response to pheromone treatment (Muller et al. 2003; Aguilar et al. 2007; Yang et al. 2011), although its affinity for Ca^{2+} is 16-fold lower than the HACS (Muller et al. 2001). In *A. nidulans*, lacking of the putative LACS components FigA leads to defects in hyphal growth and development (Zhang et al. 2014).

One important question regarding Ca^{2+} signaling is how signals and cellular events couple specifically, i.e., how Ca^{2+} signaling is encoded and decoded. Recent studies demonstrate that Ca^{2+} signaling is not only transmitted by Ca^{2+} concentrations but also by temporal and spatial messages (Flegg et al. 2013). The specific Ca^{2+} profile corresponding to a given stimulation is often termed Ca^{2+} signature or Ca^{2+} code (White and Broadley 2003). Those Ca^{2+} pumps, exchangers, antiporters, and channels all participate in shaping Ca^{2+} signals. In the cytoplasm, a set of EF-hand proteins function as Ca^{2+} buffers in shaping the Ca^{2+} signals. However, the majority of the EF-hand proteins acts as Ca^{2+} sensors, which deliver Ca^{2+} signals downstream via interaction with target enzymes after conformational changes (Schwaller 2010). **Calmodulin (CaM)** is the well-known representative of Ca^{2+} sensors, which has a wide range of downstream target proteins, including calcineurin, a conserved serine/threonine phosphatase composed of the catalytic subunit calcineurin-A (CnA) and the regulatory subunit calcineurin-B (CnB) (Rusnak and Mertz 2000). CnA contains four primary regions: the N-terminal catalytic domain similar with other phosphatases; CnB-binding region; CaM-binding segment; and C-terminal autoinhibitory region (Baksh and Burakoff 2000; Rusnak and Mertz 2000). CnA alone has relatively a low phosphatase activity, but association with CnB enhances its activity greatly, probably resulting from repressing the autoinhibition (Perrino et al. 1992). CnB is homologous to CaM, and they may play similar roles in CnA activation (Stemmer and Klee 1994). Calcineurin also has a broad range of downstream substrates, i.e., the ion channels, the RCN (regulators of calcineurin) family, and the transcription factor, such as Crz1 (crazy). Dephosphorylation of Crz1 by calcineurin results in its translocation from the cytoplasm to the nucleus to initiate a number of target genes, including *pmr1* and *pmc1*, whose encoding proteins are Ca^{2+} ATPases pumping Ca^{2+} back into their respective Ca^{2+} stores as a feedback response (Liu et al. 2015; Chatfield-Reed et al. 2016). In *S. cerevisiae*, Ca^{2+} signals are always transmitted by the calmodulin-calcineurin-Crz1 pathway and finally translated into particular cellular events (Cyert 2003). A recent study in *C. neoformans* revealed that Crz1 is also capable of activating some downstream targets independently of calcineurin activation during thermal stress (Chow et al. 2017). In addition to the transcription factor Crz1, our previous work showed that AkrA, a homologue of *S. cerevisiae* palmitoyl transferase Akr1, plays a vital role in maintaining calcium homeostasis in *Aspergilli*. The *akrA* deletion mutant showed significantly decreased cytosolic-free Ca^{2+} levels induced by external stresses including high extracellular Ca^{2+} stress, ER stress, and membrane stress, suggesting that posttranslational modification may also be involved in cytosolic Ca^{2+} homeostasis in *Aspergilli* (Zhang et al. 2016).

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