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# Calcium and Cell Response to Heavy Metals: Can Yeast Provide an Answer?

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## Abstract

Despite constant efforts to maintain a clean environment, heavy metal pollution continues to raise challenges to the industrialized world. Exposure to heavy metals is detrimental to living organisms, and it is of utmost importance that cells find rapid and efficient ways to respond to and eventually adapt to surplus metals for survival under severe stress. This chapter focuses on the attempts done so far to elucidate the calcium-mediated response to heavy metal stress using the model organism *Saccharomyces cerevisiae*. The possibilities to record the transient elevations of calcium within yeast cells concomitantly with the heavy metal exposure are presented, and the limitations imposed by interference between calcium and heavy metals are discussed.

**Keywords:** heavy metal, calcium, stress adaptation, *Saccharomyces cerevisiae*, aequorin

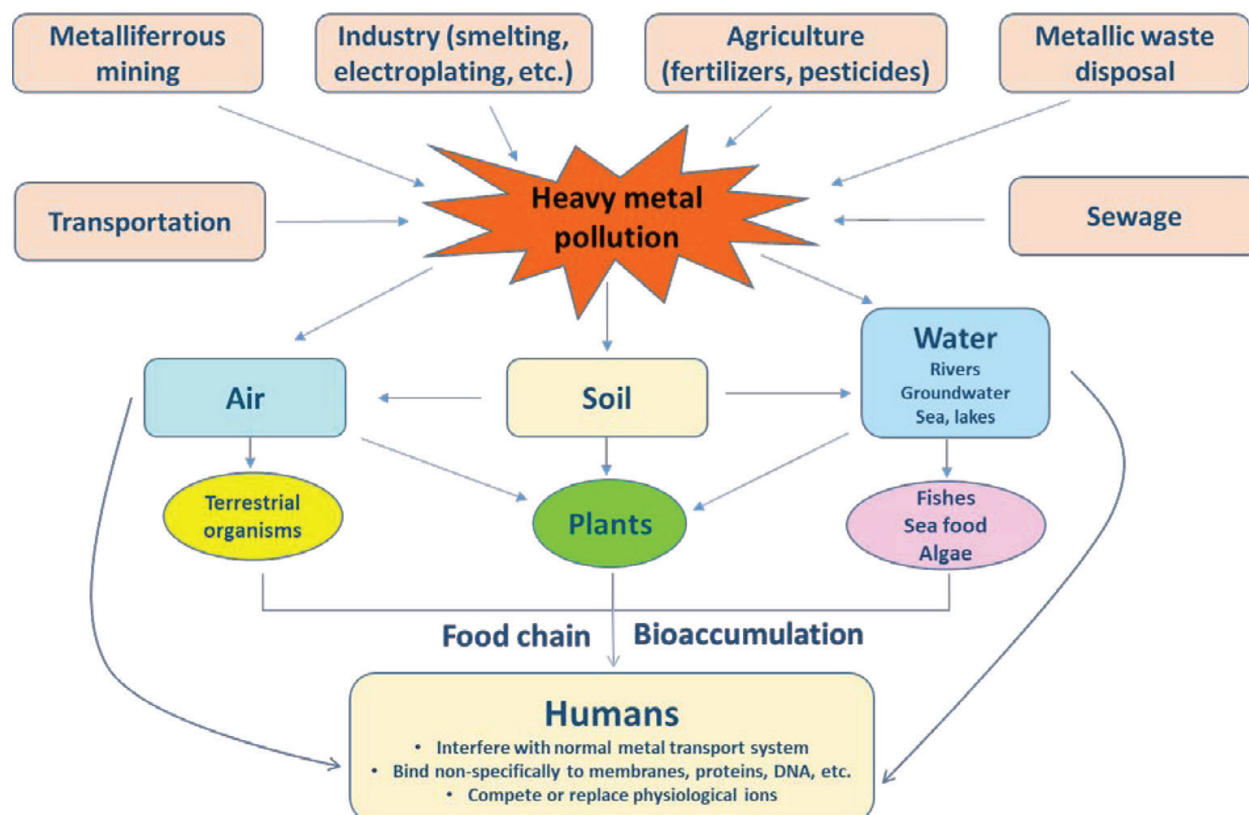
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## 1. Introduction

Responding to environmental stimuli is a prerequisite for cell adaptation to the ever-changing conditions in the cell surroundings. Stress conditions such as sudden changes of temperature, pH, irradiation, or elevations in various chemicals concentration need to be sensed by the cell in order to respond and adapt to these changes. Calcium ions are one of the most widespread second messengers in the eukaryotic cell, being responsible for triggering many responses to external stress conditions [1]. Various biotic and abiotic stresses induce an increase in cytosolic calcium ions ( $[Ca^{2+}]_{cyt}$ ), which in turn activate many proteins involved in signaling pathways, from yeast to humans [2]. Thanks to easy manipulation, rapid growth, genetic

amenability and with many genes bearing resemblance with higher eukaryotic genes, the yeast *Saccharomyces cerevisiae* is one of the widely used model organisms which helped in elucidating a wide variety of molecular mechanisms conserved along evolution, related to cell cycle and cell proliferation, homeostasis, adaptation and survival [3]. Among many others studies, *S. cerevisiae* was used as a model to investigate the  $\text{Ca}^{2+}$ -mediated responses to a variety of stimuli: hypotonic stress [4–6], hypertonic and salt stress [7], cold stress [8], high ethanol [9],  $\beta$ -phenylethylamine [10], glucose [11, 12], high pH [13–15], amidarone and anti-fungal drugs [16, 17], oxidative stress [18], eugenol [19, 20], essential oils [21, 22], or heavy metals [23, 24]. This chapter focuses on the studies made on *S. cerevisiae* cells in the effort to understand the role of calcium in cell response to heavy metal exposure.

Heavy metals represent a constant threat to clean environments as they are constantly released in the course of various anthropogenic activities (**Figure 1**), both industrial (mining, electroplating, smelting, metallurgical processes, nanoparticles, unsafe agricultural practices) and domestic (sewage and waste, metal corrosion), all in the context of rapid industrialization and urbanization [25]. Heavy metals as contaminants are included in the category of persistent pollutants, because they cannot be destroyed or degraded. Being natural components of the earth crust, the environmental contamination becomes serious when heavy metals have the possibility to leach into surface or underground water, or undergo atmospheric deposition and metal evaporation from the water resources [26–28]. The ultimate threat imposed by the spread of heavy metals into the environment is their accumulation in the living organisms (**Figure 1**) via the food chain [29], inducing serious illnesses in animals and humans [30–34].



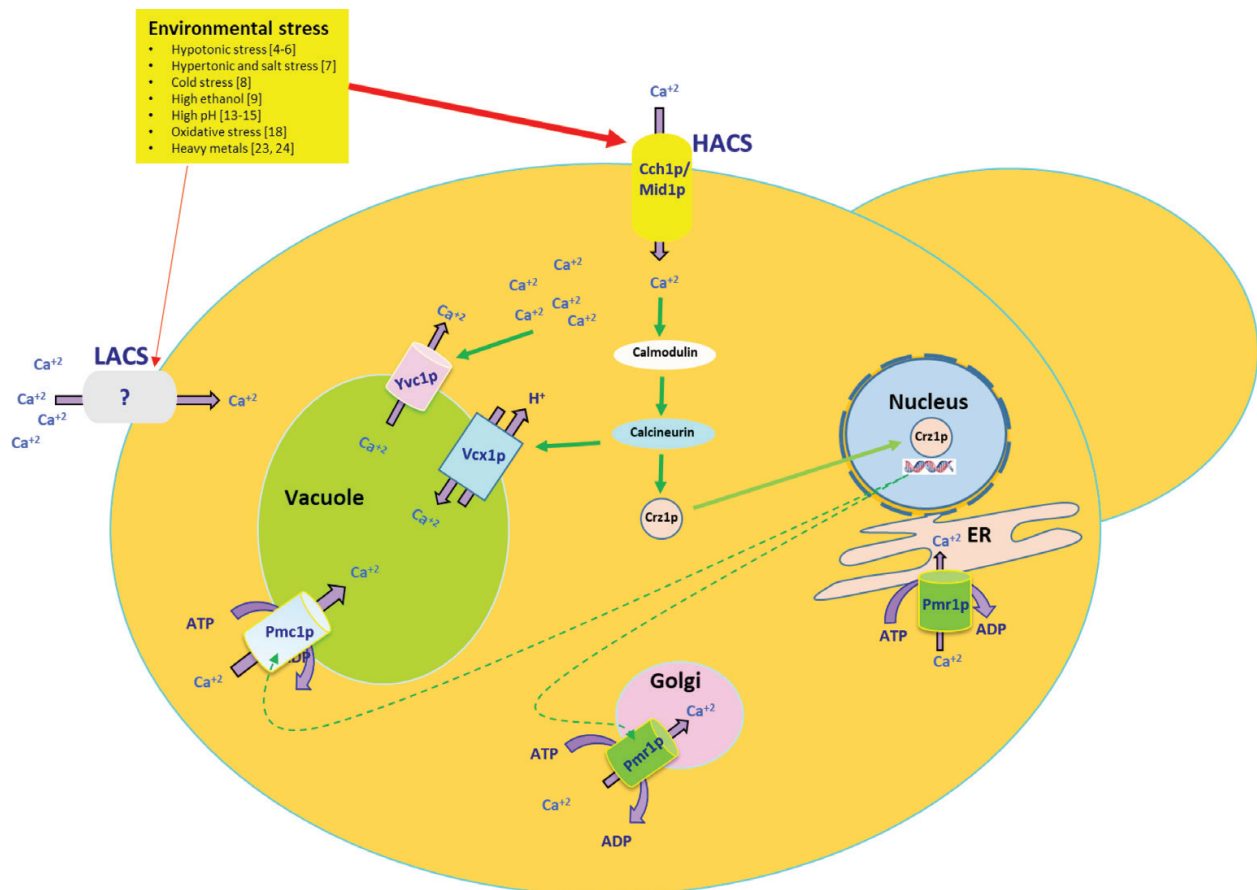
**Figure 1.** Schematic representation depicting the sources of heavy metal pollution and the impact on the environment and organisms.

Some heavy metals ( $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Mo}^{2+}$ , and  $\text{Zn}^{2+}$ ) are essential for life, contributing to various biochemical and physiological functions in the living organisms. The nutritional requirements of these elements are generally low and they must be present in food in trace concentrations [35]. However, excessive exposure to higher concentrations is deleterious, representing a threat to living organisms [36]. Other heavy metals ( $\text{Ag}^+$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ) are not essential for life and have no established biological roles, but they are highly toxic because they compete with the essential metals for their biological targets or they simply bind nonspecifically to biomolecules; these metals are able to induce toxicity at low doses [37]. Essential or not, the hazardous heavy metals such as  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$  are known to be major threats to the environment [38]. The molecular mechanisms involved in heavy metal transport and homeostasis have been intensively studied in *S. cerevisiae* [3], along with many aspects regarding their toxicity, tolerance, accumulation, or extrusion [38–47]. Some of the relevant studies performed in *S. cerevisiae* correlating heavy metal exposure to calcium-related mechanisms are presented in the following section.

## 2. Calcium transport and homeostasis in *S. cerevisiae*

Intracellular calcium ions are important second messengers in all organisms, including yeast. The mechanisms involved in calcium transport and homeostasis in *S. cerevisiae* cells have been extensively studied [48–50]. Under normal conditions, the  $[\text{Ca}^{2+}]_{\text{cyt}}$  is maintained very low (50–200 nM) at external  $\text{Ca}^{2+}$  concentrations ranging from  $<1 \mu\text{M}$  to  $>100 \text{mM}$  [51, 52]. Abrupt changes in the environment can be transduced inside the yeast cells by sudden elevations in  $[\text{Ca}^{2+}]_{\text{cyt}}$  which can be the result of  $\text{Ca}^{2+}$  influx from outside the cell,  $\text{Ca}^{2+}$  release from internal stores (usually vacuole), or both (**Figure 2**). The yeast plasma membranes contain at least two different  $\text{Ca}^{2+}$  influx systems, the high-affinity  $\text{Ca}^{2+}$  influx system (HACS) and the low-affinity  $\text{Ca}^{2+}$  influx system (LACS), the former being responsible for  $\text{Ca}^{2+}$  influx under stress conditions [50]. The HACS consists of two proteins, Cch1p and Mid1p, which are expressed and colocalize to the plasma membrane. These two subunits form a stable complex that is activated in response to sudden stimulation, boosting the influx of  $\text{Ca}^{2+}$  from the extracellular space. In *S. cerevisiae*, Cch1p is similar to the pore-forming  $\alpha 1$  subunit of mammalian L-type voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs) [53], while Mid1p is as a stretch-activated  $\text{Ca}^{2+}$ -permeable cation channel homologous to  $\alpha 2\delta$  subunit of animal VGCCs [54]. HACS is regulated by Ecm7p, a member of the PMP-22/EMP/MP20/Claudin superfamily of transmembrane proteins that includes the  $\lambda$  subunits of VGCCs. Ecm7p is stabilized by Mid1p, and Mid1p is stabilized by Cch1p under non-signaling conditions [55].

Changes in the cell environment are signaled by a sudden increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  which can be a consequence of either external  $\text{Ca}^{2+}$  influx via the Cch1p/Mid1p channel on the plasma membrane [4–14, 56], release of vacuolar  $\text{Ca}^{2+}$  into the cytosol through the vacuole-located  $\text{Ca}^{2+}$  channel Yvc1p [18, 57], or both (**Figure 2**). After delivering the message, the level of  $[\text{Ca}^{2+}]_{\text{cyt}}$  is restored to the normal very low levels through the action of  $\text{Ca}^{2+}$  pumps and exchangers. Thus, the  $\text{Ca}^{2+}$ -ATPase Pmc1p [58, 59] and a vacuolar  $\text{Ca}^{2+}/\text{H}^+$  exchanger Vcx1p [60, 61] independently transport  $[\text{Ca}^{2+}]_{\text{cyt}}$  into the vacuole, while Pmr1p, the secretory  $\text{Ca}^{2+}$ -ATPase, pumps  $[\text{Ca}^{2+}]_{\text{cyt}}$  into endoplasmic reticulum (ER) and Golgi along with  $\text{Ca}^{2+}$  extrusion from the cell [62, 63]. These responses are mediated by the universal  $\text{Ca}^{2+}$  sensor protein calmodulin that



**Figure 2.** The mechanisms by which yeast cell regulate cell calcium. Under external stresses, the plasma membrane Ca<sup>2+</sup> influx systems HACS (high-affinity Ca<sup>2+</sup> influx system) and to a lesser extent LACS (low-affinity Ca<sup>2+</sup> influx system) are activated, resulting in a rapid influx of Ca<sup>2+</sup> into the cytosol. Transient increases in intracellular Ca<sup>2+</sup> concentrations may also be due to release from internal compartments, mainly the vacuole, via Yvc1p. Unlike mammalian cells, where the main Ca<sup>2+</sup> stores reside in the endoplasmic reticulum (ER), in yeast the intracellular stores are situated in the vacuole compartment. The increased cytosolic Ca<sup>2+</sup> concentrations ( $[Ca^{2+}]_{\text{cyt}}$ ) are sensed by calmodulin, activating calcineurin. Activated calcineurin acts on its downstream target Crz1p, inducing its translocation from cytoplasm to nucleus to further induces the expression of a set of Ca<sup>2+</sup>/calcineurin-dependent target genes, including *PMC1* and *PMR1*. Calcineurin also regulates *Vcx1p* at post-transcriptional level. Subsequently, the  $[Ca^{2+}]_{\text{cyt}}$  concentration is reduced to basal levels via uptake by organelles, especially vacuole (by means of *Pmc1p* and *Vcx1p*) and Golgi (by means of *Pmr1p*).

can bind and activate calcineurin, which inhibits at the post-transcriptional level the function of *Vcx1p* [60, 64, 65] and induces the expression of *PMC1* and *PMR1* genes via activation of the *Crz1p* transcription factor [64, 65]. The release of Ca<sup>2+</sup> from intracellular stores stimulates the extracellular Ca<sup>2+</sup> influx, a process known as capacitative calcium entry [66]. Inversely, the release of vacuolar Ca<sup>2+</sup> via *Yvc1p* can be further stimulated by the Ca<sup>2+</sup> from outside the cell as well as that released from the vacuole by *Yvc1p* itself in a positive feedback called Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR) [67–70].

### 3. Aequorin, a transgenic molecular tool for detecting $[Ca^{2+}]_{\text{cyt}}$ changes in *S. cerevisiae*

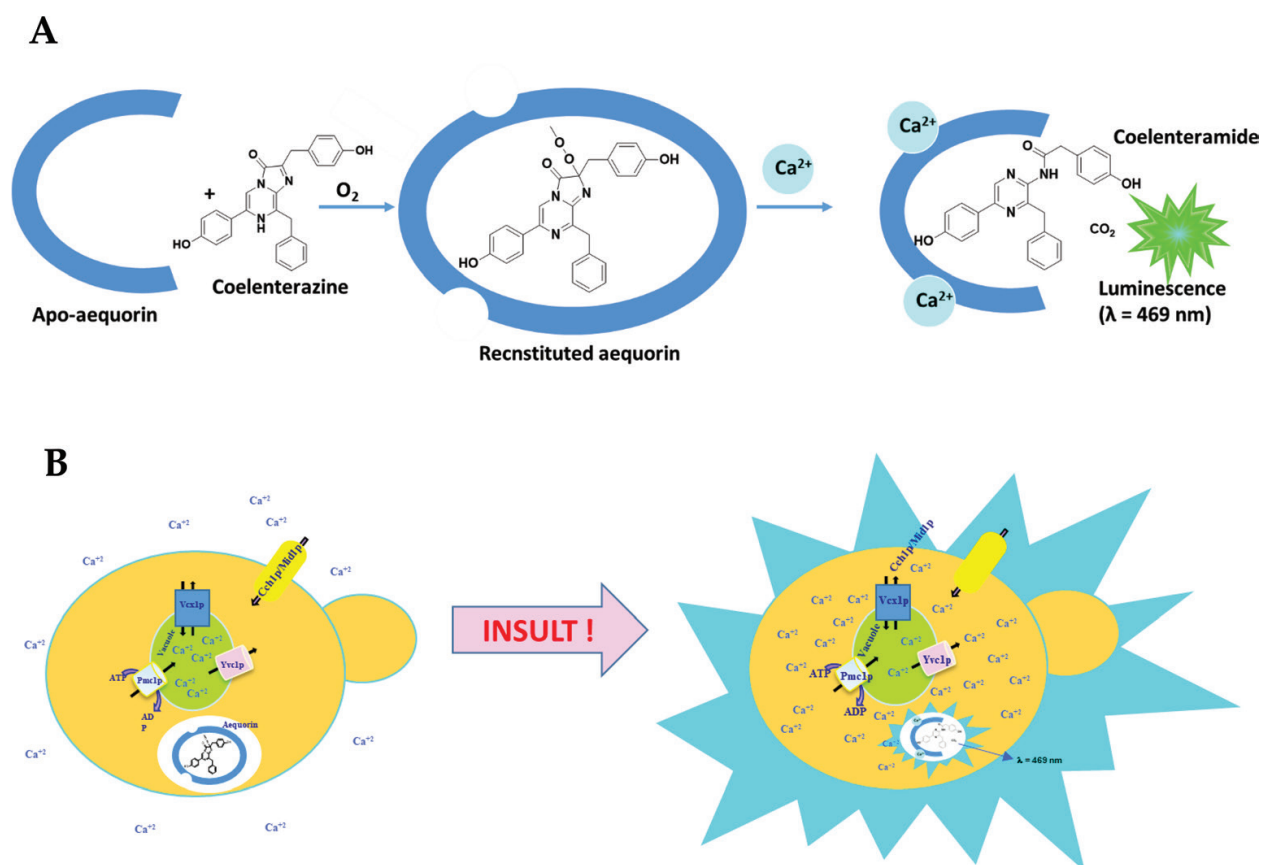
As a second messenger, Ca<sup>2+</sup> triggers a variety of cascade responses by temporarily activating Ca<sup>2+</sup>-binding components of signaling pathways which can lead either to adaptation to



the environmental changes or to cell death [71]. To determine the  $[Ca^{2+}]_{\text{cyt}}$  fluctuations during cell exposure to environmental changes, it is necessary to have a system capable to detect the sudden and transient elevations in  $[Ca^{2+}]_{\text{cyt}}$ . This was made possible by the isolation of aequorin, a  $Ca^{2+}$ -binding photoprotein, isolated from the luminescent jellyfish, *Aequorea victoria*. Aequorin consists of two distinct units, the apoprotein apoaequorin (22 kDa) and the prosthetic group, coelenterazine, which reconstitute spontaneously in the presence of molecular oxygen, forming the functional protein [72–74]. Aequorin has become a useful instrument for the measurement of intracellular  $Ca^{2+}$  levels, since it has binding sites for  $Ca^{2+}$  ions responsible for protein conformational changes that convert through oxidation its prosthetic group, coelenterazine, into excited coelenteramide and  $CO_2$  (**Figure 3A**). As the excited coelenteramide relaxes to the ground state, blue light ( $\lambda_{\text{max}}$  469 nm) is emitted and can be easily detected with a luminometer [75].

The expression of cDNA for apoaequorin in yeast cells and subsequent regeneration of apoaequorin into aequorin provide a noninvasive, nontoxic and effective method to detect the transient variations in yeast  $[Ca^{2+}]_{\text{cyt}}$  [76]. The yeast strains to be analysed must express the *A. victoria* apoaequorin, and they need to be reconstituted into fully active aequorin by association with coelenterazine (**Figure 3B**). The latter cannot be synthesized by yeast itself; therefore, the way to achieve reconstitution is to incubate the apoaequorin-expressing cells with coelenterazine, prior to  $Ca^{2+}$  determination. Coelenterazine is a hydrophobic molecule, and therefore, it is easily taken up across yeast cell wall and membrane, making aequorin suitable as a  $Ca^{2+}$  reporter [52, 77]. Aequorin has a number of advantages over other  $Ca^{2+}$  indicators as follows: because the protein is large, it has a low leakage rate from cells compared to lipophilic dyes and it does not undergo intracellular compartmentalization or sequestration. Also, it does not disrupt cell functions, and the light emitted by the oxidation of coelenterazine does not depend on any optical excitation, so problems with auto-fluorescence are eliminated [78]. The primary limitation of aequorin is that the prosthetic group coelenterazine is irreversibly consumed to produce light. Such issues led to developments of other genetically encoded calcium sensors including the calmodulin-based sensor cameleon, which were less successful in yeast, due to their size [79].

In *S. cerevisiae*, the reconstituted aequorin is used primarily to detect the  $Ca^{2+}$  fluctuations in the cytosol [76]; there have been few attempts to obtain apoaequorins targeted to various cell compartment in yeast. One notable example was the construction of a recombinant apoaequorin cDNA whose product localizes in the ER lumen; using this product, a steady state of 10  $\mu\text{M}$   $Ca^{2+}$  was detected in the ER lumen of wild type cells, and it was possible to demonstrate that the Golgi pump Pmr1p also controls, at least in part, the ER luminal concentration of  $Ca^{2+}$  [63]. Nevertheless, no reports on  $Ca^{2+}$  fluctuation in the ER in response to environmental stress are available in yeast. Surprisingly, no vacuole-targeted aequorin has been reported in yeast, in spite of the fact that the vacuole is the main storage compartment for  $Ca^{2+}$  in yeast; instead, the vacuolar  $Ca^{2+}$  traffic was determined indirectly, using genetic approaches (knockout mutants of various  $Ca^{2+}$  pumps and transporters) [61, 80] or blockers of the  $Ca^{2+}$  influx across the plasma membrane. This latter approach makes use of cell-impermeant  $Ca^{2+}$  chelators such as 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) [18] or of lanthanide ( $Ln^{3+}$ ) ions, which are efficient blockers of ion channels due to size similarity between  $Ca^{2+}$  and  $Ln^{3+}$  [80]. Of all  $Ln^{3+}$ ,  $Gd^{3+}$  is the most widely used as  $Ca^{2+}$ -channel blocker. It was shown that at 1 mM concentration in the medium all the cations from the  $Ln^{3+}$  series block  $Ca^{2+}$  entry into



**Figure 3.** Transgenic aequorin as a tool for measuring intracellular  $\text{Ca}^{2+}$ . **A.** Schematic representation of aequorin bioluminescence [72–74]. Cells expressing apo-aequorin are first incubated with the cell-permeant coelenterazine to produce functional aequorin. When  $\text{Ca}^{2+}$  binds to aequorin, the protein undergoes a conformational change leading to the destabilization of the peroxide group ( $-\text{O}-\text{O}-$ ), linking apoaequorin to coelenterazine, decomposing it to coelenteramide and  $\text{CO}_2$ ; the coelenteramide, which is in an excited state, generates blue light ( $\lambda_{\text{max}} = 469 \text{ nm}$ ). **B.** Schematic representation of  $\text{Ca}^{2+}$ -induced bioluminescence of yeast cells expressing reconstituted aequorin in the cytosol. When cells are exposed to an insult (e. g., environmental stress) the secondary messenger  $\text{Ca}^{2+}$  ions enter the cytosol and bind to aequorin, rendering the cell luminescent. Luminescence traces indicate the intensity and the duration of the  $[\text{Ca}^{2+}]_{\text{cyt}}$  wave [75, 76].

cytosol with the exception of  $\text{La}^{3+}$  (lanthanum) and to a lesser extent,  $\text{Pr}^{3+}$  and  $\text{Nd}^{3+}$  [81]. Care must be taken when using  $\text{Ln}^{3+}$  as channel blockers, as it was shown that at low concentrations  $\text{Ln}^{3+}$  may leak into the cytosol via the Cch1p/Mid1p system [82].

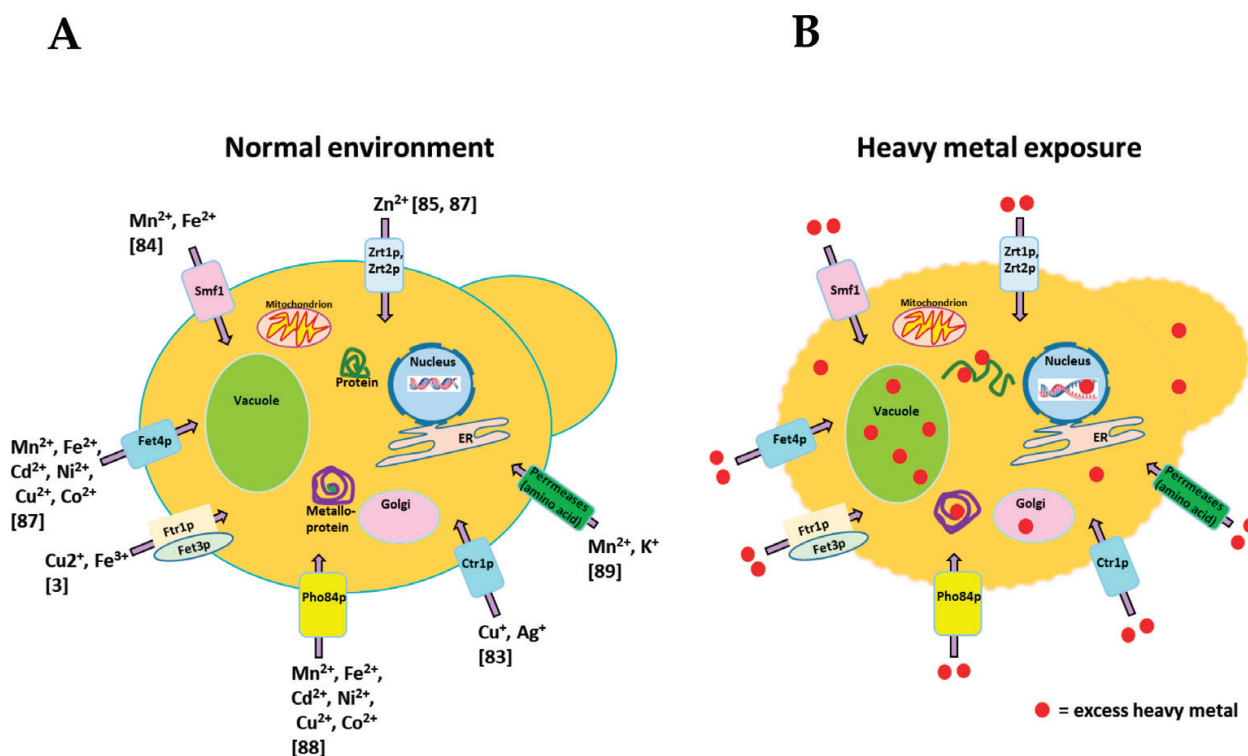
#### 4. Correlations between calcium and heavy metal exposure as seen in *S. cerevisiae* cells

When grown in media contaminated with heavy metals, the yeast cell wall is the first to get in contact with the surplus cations present in the cell surroundings. If the contamination is not excessive, the cations would probably get stuck at this level, due to the mannoproteins that compose the outer layer of the cell wall (alongside of  $\beta$ -glucans and chitin) which are heavily phosphorylated and carboxylated, decorating the cell façade with a negatively charged shield prone to bind to positively charged species, such as the metal cations [83]. Excess metal ions which escape the negatively charged groups on the cell wall surface penetrate the porous cell

wall and reach the membrane to exert their toxic effect by disrupting the lipid bilayer or by assaulting the membrane transporters.

Several heavy metals ( $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Zn}^{2+}$ ) are essential for life in their ionic forms, acting mainly as cofactors for a variety of enzymes. They are necessary only in minute amounts inside the cell (hence their denomination as “trace” elements); if their concentration goes beyond the physiological threshold they become toxic by nonspecifically binding to any biomolecule bearing a negative charge or a metal-chelator fragment. The bipolar nature of trace metals determined the development of intricate cellular systems dedicated to their uptake, buffering, sequestration, intracellular trafficking, compartmentalization and excretion. As in many other directions of study, *S. cerevisiae* brought a considerable contribution to the understanding of the molecular mechanism involved in trace metal transport and homeostasis [3, 38–47]. Several heavy metal transporters were identified at the plasma membrane level (**Figure 4A**), with both high and low affinity. For example, Ctr1p, Smf1p and Zrt1p are involved in the high-affinity uptake of  $\text{Cu}^+$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ , respectively [84–86]. Low-affinity plasma membrane transporters are more numerous and less specific: Fet4p for  $\text{Fe}^{2+}$ , but also for  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$ ; Zrt2p for  $\text{Zn}^{2+}$ , but also for  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$  [87, 88]. Transporters for phosphate or amino acids were also shown to participate in the low-affinity transport of  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ni}^{2+}$  [89, 90]. All these transporters are likely to be assaulted by surplus metals (**Figure 4B**) when cells are exposed to contaminated environments [91].

To have any chance of survival under heavy metal stress, the cell needs to be one step ahead of the “villain” ions and to get prepared for defense by using various strategies. The attempts



**Figure 4.** Toxicity of heavy metal exposure. A. Schematic representation of transporters involved in the uptake of essential metals under normal conditions. B. Under high surplus of heavy metals, the transporters will carry the excess cations into the cell, where they bind non-specifically to biomolecules, altering their structure and functionality [91].

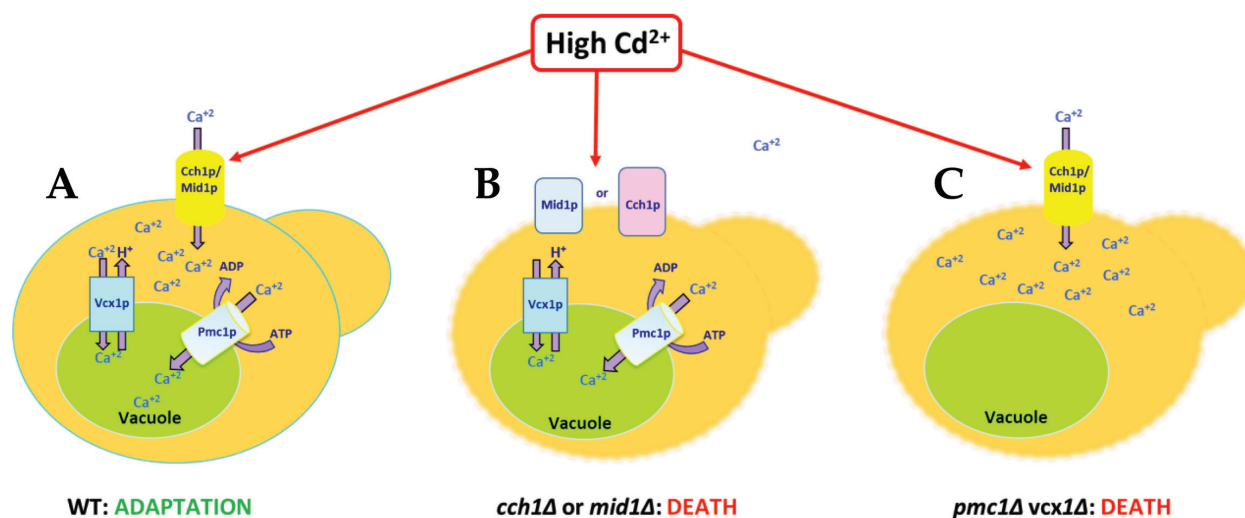


to understand the role of calcium in preparing the yeast cell to resist the heavy metal attack are summarized in the following sections.

#### 4.1. Cd<sup>2+</sup>

Cd<sup>2+</sup> is one of the most studied non-essential heavy metals as it is a global environmental pollutant present in soil, air, water, and food, representing a major hazard to human health [92]. External Cd<sup>2+</sup> was shown to unequivocally induce the [Ca<sup>2+</sup>]<sub>cyt</sub> elevations in *S. cerevisiae*, as recorded in aequorin-expressing cells, which responded through a sharp increase in the [Ca<sup>2+</sup>]<sub>cyt</sub> just a few seconds after being exposed to high Cd<sup>2+</sup> [23]. Interestingly, the chemically similar Zn<sup>2+</sup> and Hg<sup>2+</sup> failed to elicit [Ca<sup>2+</sup>]<sub>cyt</sub> elevations under the same conditions [23]. The response to high Cd<sup>2+</sup> depended mainly on external Ca<sup>2+</sup> (transported through the Cch1p/Mid1p channel) and to a lesser extent on the vacuolar Ca<sup>2+</sup> (released into the cytosol through the Yvc1p channel). The adaptation to high Cd<sup>2+</sup> was influenced by perturbations in Ca<sup>2+</sup> homeostasis in that the tolerance to Cd<sup>2+</sup> often correlated with sharp Cd<sup>2+</sup>-induced [Ca<sup>2+</sup>]<sub>cyt</sub> pulses (**Figure 5A, B**), while the Cd<sup>2+</sup> sensitivity was accompanied by the incapacity to rapidly restore the low levels of [Ca<sup>2+</sup>]<sub>cyt</sub> [23] (**Figure 5C**).

It had been suggested that Cd<sup>2+</sup> toxicity was a direct consequence of Cd<sup>2+</sup> accumulation in the ER and that Cd<sup>2+</sup> does not inhibit disulphide bond formation (which could account for the lack of response in the case of Zn<sup>2+</sup> and Hg<sup>2+</sup>) but perturbs calcium metabolism. Cd<sup>2+</sup> activates the calcium channel Cch1/Mid1 under low external Ca<sup>2+</sup>, which also contributes to Cd<sup>2+</sup> entry into the cell [93]; the protective effect of Ca<sup>2+</sup> may be the result of competitive uptake between the two cations at the plasma membrane. In this line of evidence, it was shown that excess concentration of extracellular Ca<sup>2+</sup> attenuates the Cd<sup>2+</sup>-induced ER stress [94]. It was



**Figure 5.** Cd<sup>2+</sup>-induced [Ca<sup>2+</sup>]<sub>cyt</sub> elevations mediate cell adaptation or cell death under Cd<sup>2+</sup> stress. A. In normal (WT, wild type) cells, surplus Cd<sup>2+</sup> induces Ca<sup>2+</sup> entry via Cch1p/Mid1p channel, then [Ca<sup>2+</sup>]<sub>cyt</sub> is rapidly restored to low levels by the action of vacuolar Pmc1p and Vcx1p, allowing adaptation to high Cd<sup>2+</sup>. B. Cells lacking Cch1p or Mid1p (knock-out mutants *cch1Δ* or *mid1Δ*) die under Cd<sup>2+</sup> stress, as Ca<sup>2+</sup> does not enter the cell in sufficient quantity to signal the Cd<sup>2+</sup> excess. C. Cells lacking both Pmc1p and Vcx1p (double knock-out mutant *pmc1Δ vcx1Δ*) die under Cd<sup>2+</sup> stress, as [Ca<sup>2+</sup>]<sub>cyt</sub> cannot be rapidly restored to the low physiological levels [23].

determined that divalent  $\text{Cd}^{2+}$  and  $\text{Ca}^{2+}$  have very similar physical properties, with ionic radii of  $\text{Ca}^{2+}$  (0.97 Å) and  $\text{Cd}^{2+}$  (0.99 Å) giving similar charge/radius ratios, meaning that these ions are able to exert strong electrostatic forces on biological macromolecules [95]. Under such circumstances, the  $\text{Cd}^{2+}$ -induced aequorin luminescence observed could also be the result of aequorin binding to  $\text{Cd}^{2+}$  instead of  $\text{Ca}^{2+}$ . This was not the case though: when measuring the  $\text{Cd}^{2+}$  accumulation in yeast cells, it was revealed that the  $\text{Cd}^{2+}$ -induced aequorin luminescence occurred significantly faster than the  $\text{Cd}^{2+}$  uptake, indicating that the luminescence produced was the result of increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  [23].

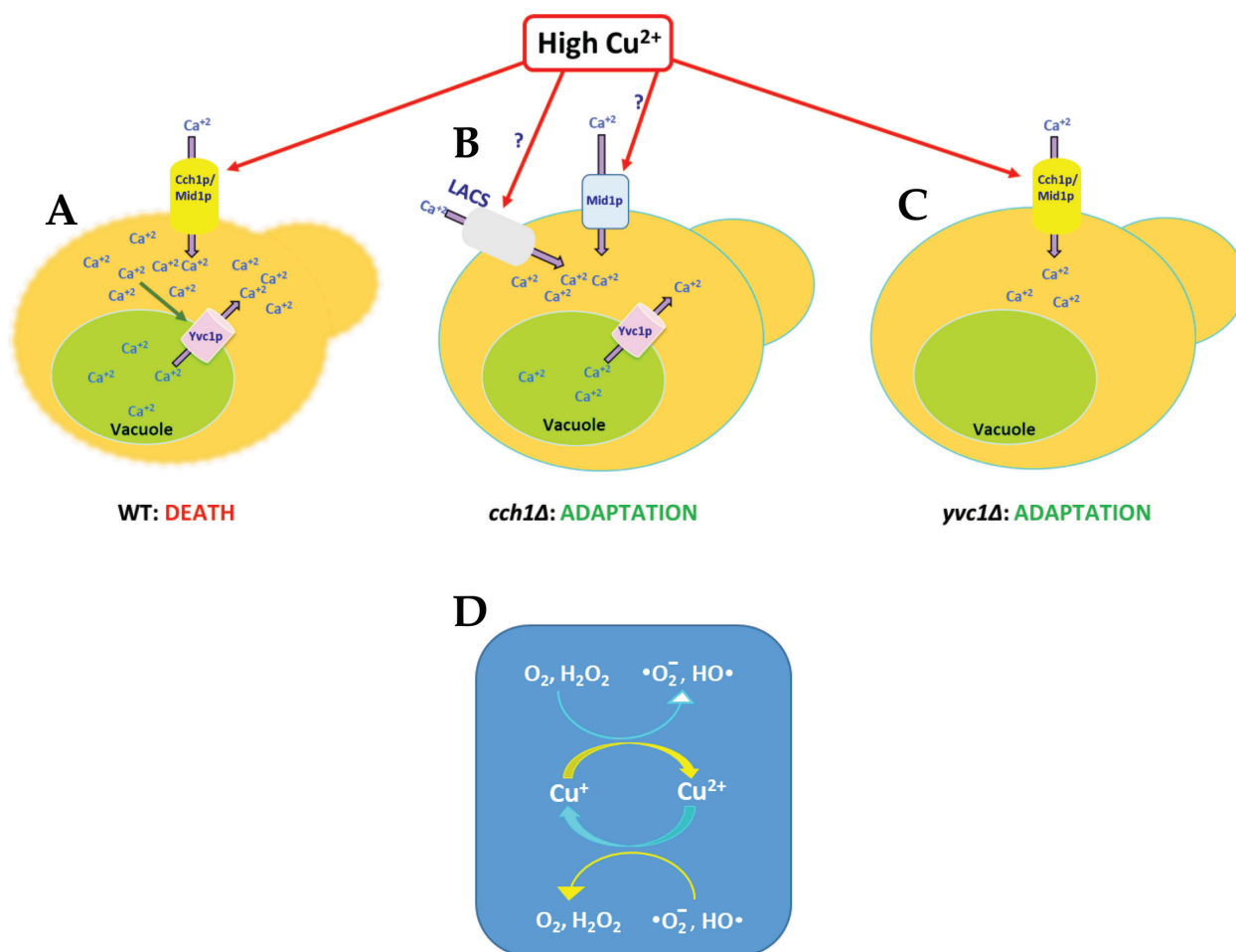
#### 4.2. $\text{Cu}^{2+}$

$\text{Cu}^{2+}$  is one of the most important essential metals: a variety of enzymes require copper as a cofactor for electron transfer reactions [96]. Nevertheless, when in excess,  $\text{Cu}^{2+}$  is very toxic in the free form because of its ability to produce free radicals when cycling between oxidized  $\text{Cu}^{2+}$  and reduced  $\text{Cu}^+$ . Studies correlating  $\text{Ca}^{2+}$  with  $\text{Cu}^{2+}$  toxicity in yeast are scarce, but it had been known that the inhibitory effect of  $\text{Cu}^{2+}$  on glucose-dependent  $\text{H}^+$  efflux from *S. cerevisiae* could be alleviated by  $\text{Ca}^{2+}$  [97]. The role of  $\text{Ca}^{2+}$  in mediating the cell response to high concentrations of  $\text{Cu}^{2+}$  was investigated in parallel with  $\text{Cd}^{2+}$ , and it was noted that exposure to high  $\text{Cu}^{2+}$  determined broad and prolonged  $[\text{Ca}^{2+}]_{\text{cyt}}$  waves which showed a different pattern from the  $[\text{Ca}^{2+}]_{\text{cyt}}$  pulses induced by high  $\text{Cd}^{2+}$  [23]. In contrast to  $\text{Cd}^{2+}$ ,  $\text{Ca}^{2+}$  – mediated responses to high  $\text{Cu}^{2+}$  depend predominantly on internal  $\text{Ca}^{2+}$  stores [24] (**Figure 6A**).

It was found that the cell exposure to high  $\text{Cu}^{2+}$ -induced broad  $\text{Ca}^{2+}$  waves into the cytosol which were accompanied by elevations in cytosolic  $\text{Ca}^{2+}$  with patterns that were influenced by the  $\text{Cu}^{2+}$  concentration but also by the oxidative state of the cell [18, 24]. When  $\text{Ca}^{2+}$  channel deletion mutants were used, it was revealed that the main contributor to the cytosolic  $\text{Ca}^{2+}$  pool under  $\text{Cu}^{2+}$  stress was the vacuolar  $\text{Ca}^{2+}$  channel, Yvc1p, also activated by the Cch1p-mediated  $\text{Ca}^{2+}$  influx (**Figure 6**). Using yeast mutants defective in the  $\text{Cu}^{2+}$  transport across the plasma membrane, it was found that the  $\text{Cu}^{2+}$ -dependent  $\text{Ca}^{2+}$  elevation could correlate with the accumulated metal, but also with the  $\text{Cu}^{2+}$  – induced oxidative stress and the overall oxidative status. Moreover, it was revealed that  $\text{Cu}^{2+}$  and  $\text{H}_2\text{O}_2$  acted in synergy to induce  $\text{Ca}^{2+}$ -mediated responses to external stress [24]. Interestingly, other redox active metals such as  $\text{Mn}^{2+}$  or  $\text{Fe}^{2+}$  were inactive in inducing  $[\text{Ca}^{2+}]_{\text{cyt}}$  waves ([23], unpublished observations), probably because these metals are less redox-reactive than the  $\text{Cu}^{2+}/\text{Cu}^+$  couple (**Figure 6D**) under aerobic conditions [98].

#### 4.3. $\text{Mn}^{2+}$

High manganese failed to elicit  $\text{Ca}^{2+}$  elevations irrespective of the magnitude of the insult applied ([23]; unpublished observations). The response was monitored over a wide range of concentrations (from the quasi-physiological 0.5 mM to the super lethal 50 mM) and times (up to 60 min of exposure). Of all the cations,  $\text{Mn}^{2+}$  is the closest to  $\text{Ca}^{2+}$  in terms of ionic radius and charge. This similarity is so relevant that  $\text{Mn}^{2+}$  effectively supports yeast cell-cycle progression in place of  $\text{Ca}^{2+}$  [99]. This similarity probably renders the cell irresponsive to high concentrations of an otherwise toxic metal. A more subtle  $\text{Mn}^{2+}$ - $\text{Ca}^{2+}$  interplay exists though, being



**Figure 6.**  $\text{Cu}^{2+}$ -induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevations mediate cell adaptation or cell death under  $\text{Cu}^{2+}$  stress. A. In normal (WT, wild type) cells, surplus  $\text{Cu}^{2+}$  induces  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevations as  $\text{Ca}^{2+}$  enters via Cch1p/Mid1p channel or is released from the vacuole via Yvc1p, in a positive feed-back. The normal low levels of  $[\text{Ca}^{2+}]_{\text{cyt}}$  are not rapidly restored as in the case of  $\text{Cd}^{2+}$ -exposure, and the cells die. B. Cells lacking Cch1p (but not Mid1p) exhibit lower elevations in  $\text{Cu}^{2+}$ -induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  and are more tolerant to  $\text{Cu}^{2+}$  stress. C. Cells lacking Yvc1p (knock-out mutant *yvc1Δ*) exhibit very low elevations in  $\text{Cu}^{2+}$ -induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  and adapt easily to  $\text{Cu}^{2+}$  stress [24]. The cell behavior described in A-C is similar to the  $\text{Ca}^{2+}$ -mediated response to oxidative stress [18], suggesting that the  $\text{Cu}^{2+}$ -induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  changes may be indirectly mediated by the formation of reactive oxygen species during copper shuffling between oxidative states  $\text{Cu}^{2+}$ - $\text{Cu}^+$  (D).

manifested at several levels [41]. For example, high  $\text{Mn}^{2+}$  is controlled by calcineurin/Crz1p-regulated Pmr1p and Pun1p [100]. Importantly, the tolerance of yeast cells to  $\text{Mn}^{2+}$  is related to both Pmr1p and Vcx1p [41, 64, 65, 101] two determinants of maintaining low  $[\text{Ca}^{2+}]_{\text{cyt}}$  by transporting the ions to the vacuole and Golgi/ER, respectively [60–63]. The  $\text{Ca}^{2+}$ -dependent response to  $\text{Mn}^{2+}$  surplus seems to be induced not by external  $\text{Mn}^{2+}$ , but by the cations accumulated inside the cell. For example, it was found that internal  $\text{Mn}^{2+}$  can be redistributed by calcium-stimulated vesicle trafficking [102].

#### 4.4. $\text{Fe}^{2+}$

$\text{Fe}^{2+}$  toxicity can be the result of direct ionic effect, but the indirect effect of catalyzing Fenton reactions, in which highly reactive oxygen species arise, represents the main concern raised

by  $\text{Fe}^{2+}$  surplus. As in the case of  $\text{Mn}^{2+}$ , excess  $\text{Fe}^{2+}$  did not elicit sudden elevations in  $[\text{Ca}^{2+}]_{\text{cyt}}$  upon exposure [23]. It had been reported that yeast strains lacking the components of the Cch1p/Mid1p plasma membrane channel were hypersensitive to  $\text{Fe}^{2+}$ . When measuring the relative  $\text{Ca}^{2+}$  accumulation, it was noted that iron stress also increased the residual  $\text{Ca}^{2+}$  uptake in the *cch1 $\Delta$  mid1 $\Delta$*  double knockout mutant [8]. As the  $\text{Ca}^{2+}$  measurements in this study were done radiometrically, there must have been a considerable lag between application of the stimulus and  $\text{Ca}^{2+}$  measurement (unlike aequorin determinations, which allow  $\text{Ca}^{2+}$  detection simultaneously with stimulus application), and the mutant's sensitivity towards  $\text{Fe}^{2+}$  might have been caused by  $\text{Ca}^{2+}$  lingering in the cytosol, as in the case of  $\text{Cd}^{2+}$ -sensitive mutants [23].

#### 4.5. Other metals

The surplus of heavy metals such as  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Ag}^+$  did not have the ability to rapidly induce elevations in  $[\text{Ca}^{2+}]_{\text{cyt}}$ . In some cases, ( $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$ ) exogenous  $\text{Ca}^{2+}$  alleviated the toxicity of the metal ions, but this effect was rather related to the inhibition of  $\text{Co}^{2+}$  or  $\text{Ni}^{2+}$  uptake by  $\text{Ca}^{2+}$  [103].

### 5. Concluding remarks

In this chapter, we attempted to highlight the studies made in *S. cerevisiae* which correlate the exposure to high concentrations of heavy metals with the  $\text{Ca}^{2+}$ -mediated cellular responses. *S. cerevisiae* is a very good model to study the cell response to sudden changes of metal concentration in the environment; such studies were greatly facilitated by the ease of obtaining yeast cells expressing aequorin in the cytosol, thus allowing the real-time detection of  $[\text{Ca}^{2+}]_{\text{cyt}}$  fluctuations. By combining  $\text{Ca}^{2+}$  monitoring under metal stress with the genetic approaches that make use of mutants with perturbed heavy metal or  $\text{Ca}^{2+}$  homeostasis, important aspects related to cell adaptation or cell death under heavy metal stress have been elucidated. Using yeast cells expressing aequorin in the cytosol provides answers regarding the immediate  $\text{Ca}^{2+}$ -mediated responses, which are crucial for deciding the cell fate. Nevertheless, to understand the  $\text{Ca}^{2+}$ -mediated cell responses which occur at later phases, developing sensitive  $\text{Ca}^{2+}$  sensors targeted to specific compartments is still a desiderate for future studies.

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