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# Exchangeable zinc ions transiently accumulate in a vesicular compartment in the yeast *Saccharomyces cerevisiae*

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**Abstract**

The baker's yeast *Saccharomyces cerevisiae* was used as a model to visualize intracellular labile zinc under conditions of nutritional zinc imbalance. Zinc-specific staining was performed in yeast cells using both Zinquin fluorescence and zinc–selenium autometallography. Both techniques resulted in specific labeling of an intracellular vesicular compartment that was present in wild type cells as well as in the vacuolar Zn transporter mutants Δ*zrc1* and Δ*cot1*. This compartment, that closely resembles mammalian zincosomes, appeared rapidly under conditions of zinc availability and was independent of endocytosis. However, persistence of the zinc loaded vesicles in nutritional zinc deficiency was dependent on the presence of functional Zrc1 and Cot1 vacuolar transporters. Overall our findings indicate that labile zinc in yeast cells enters a dynamic vesicular compartment which could represent an extremely important defence to buffer both zinc excess and deficiency.


*Keywords:* Zinc transport; Zinquin; Autometallography; Zincosomes; Zrc1

Zinc is a micronutrient required by several cellular processes related to growth and differentiation, transcription, and apoptosis [1,2]. Therefore, zinc is essential to life but, as is the case for other mineral micronutrients, when its intracellular concentration reaches a threshold, toxic effects are manifested. Maintenance of intracellular zinc homeostasis is a crucial process in all living cells. Homeostatic mechanisms have evolved to handle intracellular zinc levels, to supply different cellular compartments with this vital metal, and to protect cells from dangerous levels of zinc ions. Such mechanisms include safe metabolic pathways where zinc ions are vehicled to target sites, passing membranes by way of specific transporter proteins and accumulating in distinct intracellular compartments. The yeast *Saccharomyces cerevisiae* is a useful model organism to study micronutrient metabolism, having provided important insights into the molecular genetics of metal ion transport. Several transporters of zinc, copper, and iron are strongly conserved between yeast and mammals and often share the same function. The proteins involved in maintaining intracellular zinc homeostasis in higher eukaryotes are divided into two families based on sequence and structural homologies: the ZIP family (recently renamed Solute Carrier Family 39, SLC39 [3]), comprising the yeast ZRTs and the mammalian hZIPs, responsible for zinc uptake in different cell types; the CDF family [4], recently renamed SLC30 [5], whose members are predominantly involved in zinc efflux and intracellular compartmentalization. Proteins within each family share several common features such as structural configuration (eight transmembrane domains in ZIP proteins and six transmembrane domains in CDFs) [6]. The first eukaryotic zinc transporters to be identified were the yeast Zrc1 and Cot1 proteins [7,8]. Later, a

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whole family of mammalian transporters related to Cot1 and Zrc1 have been described and named ZnTs [9,10]. They represent an important mammalian sub-family of CDF transporters. Their number has suddenly increased from 4 to 9 after completion of the mouse and human genome sequencing projects, and there may be more to be uncovered yet. The nine ZnT proteins identified to date share significant sequence and structural similarities with the two yeast transporters [5,11]. With the possible exception of ZnT1 and ZnT5, the remaining ZnTs display vesicular localization.

The regulation of zinc transport has been widely studied in *S. cerevisiae*. Two ZIP proteins, named Zrt1 and Zrt2, are involved in zinc uptake from the extracellular environment, with high and low affinities, respectively [12,13]. Other CDF proteins are responsible for intracellular compartmentalization of zinc ions: Zrc1 and Cot1, required for sequestration of excess zinc into the vacuole; Msc2, localized in the endoplasmic reticulum/nucleus [14]. The corresponding deletion mutants display sensitivity to zinc (Δ*zrc1*) or zinc and cobalt (Δ*cot1*), or increased accumulation of zinc ions (Δ*msc2*). Zrc1 was extensively characterized at the molecular level; it was shown to reside in the vacuolar membrane and to affect cytoplasmic–vacuolar zinc transport [15]. A recent work describes the biochemical properties of zinc accumulation within the vacuole—the major yeast intracellular organelle responsible for storage of excess zinc as well as other heavy metal ions [16]—and the Zrc1-dependence of this transport system [17]. The Zrt3 protein, on the other hand, can mobilize zinc ions back to the cytoplasm when needed [18]. With the exception of *COT1* and *MSC2*, all these genes have been shown to respond to intracellular zinc levels by transcriptional regulation mediated by the zinc responsive transcription factor Zap1 [19,20].

Zinc ions are present in all cells as tightly bound, poorly exchangeable pools involved in metalloenzyme catalysis, as well as structural elements in a number of proteins, including transcription factors [21]. However, in addition to these stable pools some cells, even in the absence of externally added zinc, contain pools of dynamic, free or loosely bound zinc ions mainly localized in vesicles interspersed in the cytoplasm (zincosomes [22]), that act as reservoir for metabolic needs. The question of how cells handle these labile pools of free zinc ions has gained interest in the last decade, propelled among other things by the unique quality of these ions to be traceable by several histochemical/cytochemical techniques. The stable, protein bound zinc pools, on the other hand, can only be quantitatively measured by techniques that demand prior destruction of tissues and cells.

Taking advantage of two independent techniques to image intracellular zinc ions, we describe in this work a vesicular compartment in yeast cells containing labile zinc. Such zinc-enriched vesicles are depleted more rapidly in Zrc1 and Cot1 mutants in zinc deficient medium, suggesting that a dynamic relationship exists between vacuolar and vesicular zinc accumulation.

## Materials and methods

*Yeast strains and growth conditions*. Media used for yeast cultures were YPD (2% yeast extract, 4% peptone, 2% glucose) and synthetic-defined (SD) medium (0.67% yeast nitrogen base without aminoacids, 2% glucose, and the necessary auxotrophic requirements). SD medium was rendered zinc-limiting by adding EDTA at a final concentration of 1 mM (SDE) [18]. Cell density was determined by measuring the optical density at 600 nm ($OD_{600}$). *S. cerevisiae* strains CM100 (*MATα can1-100 his 3-11 15 leu 2-3 112 trp1-1 ura 3-52*), CM102 (*MATα can1-100 his 3-11 15 leu 2-3 112 trp1-1 ura 3-52*, *zrc1::HIS3*), CM103 (*MATα can1-100 his 3-11 15 leu 2-3 112 trp1-1 ura 3-52*, *cot1::URA3*), CM104 (*MATα can1-100 his 3-11 15 leu 2-3 112 trp1-1 ura 3-52*, *zrc1::HIS3 cot1::URA3*) [18], and DEY1531 (*MATa*, *ade 6*, *can1*, *his3*, *leu2*, *trp1*, *ura3*, *end4::LEU2*) [23] were a kind gift of Dr. D. Eide, University of Missouri.

*Zinquin staining and zinc–selenium autometallography ($ZnSe^{AMG}$)*. Cells pre-grown in SD medium were inoculated at an initial $OD_{600}$ of 0.1 either in fresh SD or in SDE (SD + 1 mM EDTA). Following overnight growth, cells were washed in 1× phosphate-buffered saline (PBS) and transferred to SD medium with or without the addition of $ZnSO_4$. For Zinquin staining, cells were fixed in 3.7% formaldehyde (Sigma Italia, Milan, Italy) for 1 h at 30 °C, washed in 1× PBS, and incubated for 1 h at RT in 25 μM ethyl-(2-methyl-8-*p*-toluenesulfonamido-6-quin-olyloxy) acetate (Zinquin, kindly provided by Dr. P. Zalewski, University of Adelaide, Australia), freshly diluted in 1× PBS. Cells were subsequently washed in 1× PBS to remove extracellular Zinquin, mounted on poly-lysine coated glass slides (Sigma Italia, Milan), and viewed on a Zeiss Axioskop II fluorescence microscope under UV light. For $ZnSe^{AMG}$ staining, cells were exposed to 0.2% sodium selenite ($Na_2SeO_3$) for 1 h at 30 °C, washed in 1× PBS, fixed in 2% glutaraldehyde for 30 min. at RT, washed again in 1× PBS, and exposed to AMG development for 45 min. After resuspension in the developer the tubes were placed at RT in the dark [24]. Images were taken with Nomarski optics mounted on a Zeiss Axioskop II microscope.

*Fluorescent staining of vacuoles and mitochondria*. Cells were treated with the vital stain FM4-64 (Molecular Probes, Eugene, Oregon) as described [25]. Briefly, cells grown until late log phase were concentrated in fresh SD medium to $OD_{600}$ = 40. FM4-64 (40 μM) was added and the cells were incubated for 1 h at 30 °C, centrifuged and resuspended in fresh SD, and then visualized by fluorescence microscopy using the TRITC filter. For mitochondrial staining, cells were treated with the dye Syto18 (Molecular Probes, Eugene, Oregon) according to the protocol by the manufacturer.

## Results

### *Intracellular labile zinc distribution in yeast cells*

To visualize the intracellular labile zinc pool we used the fluorescent probe Zinquin, which specifically binds zinc ions at nanomolar concentrations and fluoresces upon excitation with UV light [2]. The experiments were performed in the wild type strain, as well as in single and double deletion mutants in the vacuolar zinc transport

proteins Zrc1 and Cot1. The lack of standard protocols to image zinc in yeast cells prompted us to setup an appropriate procedure for Zinquin staining, described in Materials and methods. In cells grown in minimal medium without added zinc, the intracellular labile zinc pool appeared to localize to small punctuate cytoplasmic vesicles (Fig. 1A), similar to the zincosomes described in mammalian cells [22]. This pattern did not change following the addition of 100 μM $ZnSO_4$ to the culture medium for 20 min (Fig. 1B), while overnight growth in SDE medium (SD + 1 mM EDTA) led to complete disappearance of the Zinquin-positive vesicles (Fig. 1C). No differences were observed when comparing wild type cells to single and double mutants under any of the above-described conditions (data not shown).

*Yeast zincosome formation and disappearance*

To further characterize the vesicular compartment containing labile zinc we sought to define the kinetics of its appearance following zinc uptake by zinc-depleted yeast cells. Since transcription of the Zrt1 transporter is upregulated by the zinc-dependent transcription factor Zap1 under conditions of zinc depletion [20], cells were grown overnight in zinc-limiting medium (SDE) to induce the Zap1 regulon (zinc depletion) and stained with Zinquin before and after switching the cell cultures back to zinc containing medium. When transferred for 2 h to SD medium, which contains micromolar concentrations of zinc ions, all strains displayed homogeneous distribution of Zinquin stained vesicles (Fig. 2A). Zinc loading of cells appeared to be a very rapid process in all strains, visible during the first 5 min of zinc repletion (data not shown).

We then switched the cell cultures again to zinc-limiting medium and performed Zinquin staining after 5 min, 30 min, and 2 h, to determine the kinetics of disappearance of the zincosomal compartment which is completely absent following overnight growth in this medium (Fig. 1C). The results in Fig. 2A show that in the wild type strain the zincosomal compartment does not undergo qualitative changes even after 2 h of growth in SDE, while in the mutant strains some cells lack Zinquin positive vesicles after 30 min and most of them are negative by 2 h of growth in SDE. Cell growth was monitored at regular intervals throughout the experiment and is shown in Fig. 2B. While the wild type and the Δ*cot*1 strain enter the logarithmic growth phase upon shifting to zinc containing medium, the Δ*zrc*1 strain undergoes a growth arrest under the same condition, which is even more pronounced in the double mutant Δ*zrc*1Δ*cot*1. This effect on growth is probably due to the zinc shock resulting from sudden zinc uptake following depletion, as a functional Zrc1 protein was shown to be essential in protecting cells from zinc shock [20]. In both mutant strains growth arrest is slowly reverted by the subsequent shift to SDE medium.

*Yeast zincosomes are distinct from vacuoles and mitochondria*

As mentioned in the introduction, the yeast vacuole has been postulated to play a key role in metal ion storage [16]. This organelle is morphologically distinct from other membrane-delimited compartments and can be easily visualized by light microscopy, as well as by specific fluorescent dyes. Double labeling of zinc bodies and the vacuoles was obtained utilizing, in addition to Zinquin, the vital stain FM4-64 which labels the vacuolar membrane [25]. Zinc-loaded vesicles (Fig. 3A) are clearly distinct from vacuolar membranes (Fig. 3B) being interspersed throughout the cytoplasm in wild type cells. Moreover, double staining of yeast cells with Zinquin and with the mitochondrial marker dye Syto18 shows that zinc-enriched cytoplasmic vesicles are distinct from mitochondria (Figs. 3C and D). The same results as those shown in Fig. 3 were obtained when staining Δ*zrc*1 and Δ*cot*1 single and double mutant strains with FM4-64 and Syto18, in addition to Zinquin (data not shown).

*Autometallographic imaging of labile zinc in yeast cells*

To compare the results of Zinquin staining with those obtained employing an independent technique, we visualized intracellular labile zinc pools by autometallography

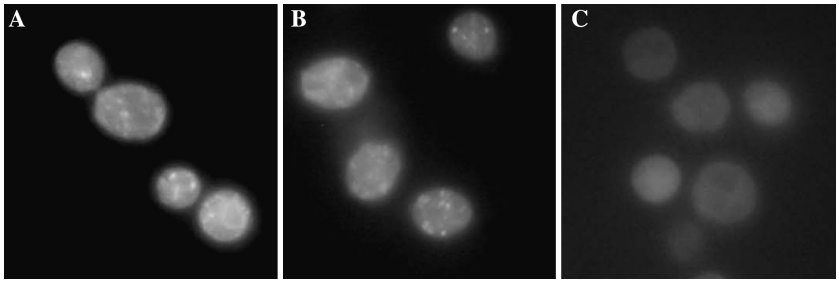


Fig. 1. Zinquin fluorescent staining of intracellular labile zinc. Zinquin staining of wild type yeast cells grown in: minimal SD medium without added Zn (A); SD medium supplemented with 100 μM $ZnSO_4$ for 20 min (B); and zinc-limiting medium (SDE) overnight (C).

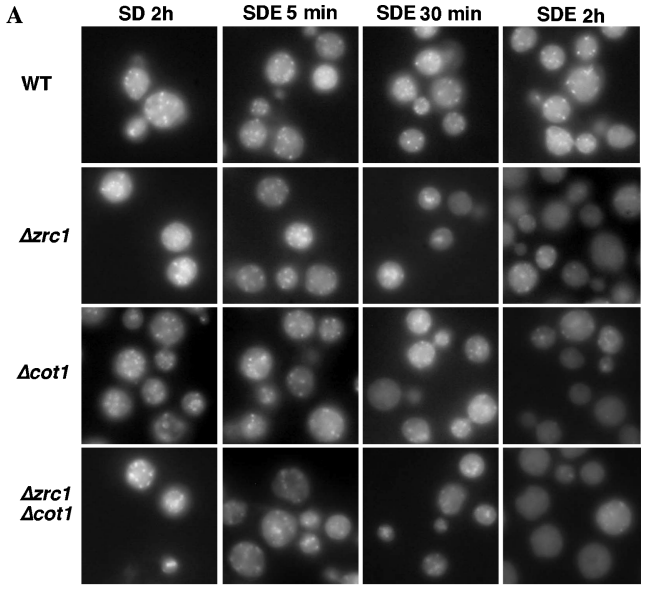


Fig. 2. Zinquin staining of yeast cells during growth in media containing different zinc concentrations. (A) Zinquin staining. The relevant genotypes of the yeast strains are indicated on the left. Each column represents staining of cells withdrawn from the culture at the indicated time points. (B) Growth curves of the four strains under the described culture conditions. Cells were grown overnight in SDE, starting at $t = 0$, then switched to SD for 2 h (arrow) and to SDE for additional 4 h. $OD_{600}$ of the cultures was measured at the beginning and at the end of an overnight growth, then every 30 min during growth in SD medium and every 1 h during subsequent growth in SDE. The arrows indicate switching to SD and SDE media. Strains are labeled as follows: WT, open circles; Δ*zrc*1, filled triangles; Δ*cot*1, filled squares; and Δ*zrc*1Δ*cot*1, filled circles.

(AMG). This method relies on in vivo chemical binding of the zinc ion pool to form zinc selenide nanocrystals, followed by silver enhancement of the nanocrystals with AMG [26]. Until now the $ZnSe^{AMG}$ technique has never been used to trace zinc ions in yeast cells. Therefore, zinc loading of yeast cells was performed using the depletion/repletion protocol used in the Zinquin staining experiments. Cells were subsequently exposed to 0.2% sodium selenite for different time intervals (15, 30, 60, and 120 min), followed by fixation and

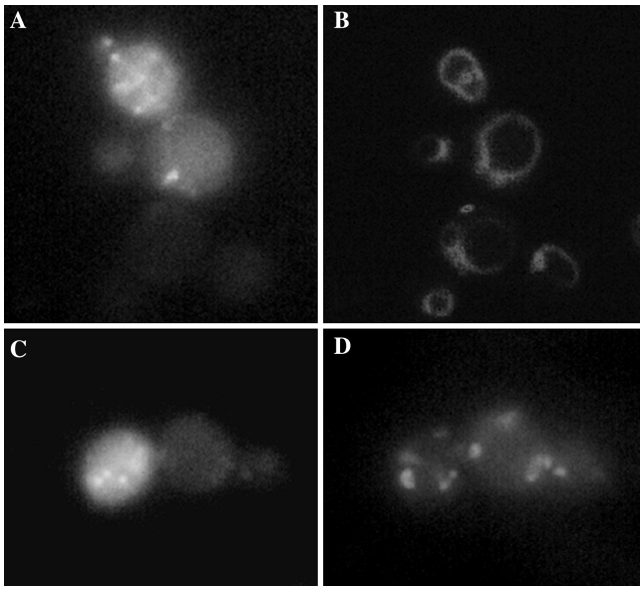


Fig. 3. Double fluorescence staining of yeast zincosomes and organelle-specific dyes. WT cells grown to logarithmic phase in SD medium were double stained with Zinquin (A) and with the vacuolar-specific fluorescent dye FM4-64 (B), or with Zinquin (C) and with the mitochondrial fluorescent stain Syto18 (D).

developing. Fig. 4 shows the results of AMG staining of wild type cells exposed to sodium selenite for 1 h (panel A). As controls, cells were either handled without exposure to sodium selenite (panel B) or subjected to treatment with the zinc chelator sodium diethyldithiocarbamate (DEDTC, panel C) before exposure to selenite. The $ZnSe^{AMG}$ staining shown in panel A revealed the presence of silver-enhanced zinc–selenium nanocrystals interspersed throughout the cytoplasm. The vacuole, clearly visible through Nomarski optics, was not stained.

### *Formation of the zincosomal compartment is not dependent on endocytosis*

To determine whether endocytosis might be responsible for zincosome formation we employed the Δ*end4* mutant strain which is completely blocked in endocytosis [23]. Cells were grown in zinc-limiting medium overnight, switched to SD medium for 2 h, and subsequently returned to zinc-limiting medium as described for Fig. 2. Cell growth was monitored throughout the experiment by measuring the $OD_{600}$ and parallel samples were withdrawn for Zinquin staining at regular time intervals. The results in Fig. 5 (upper row) show that neither formation nor disappearance of the zincosomal compartment is affected by a block in endocytosis. The growth curve of the Δ*end4* strain is slightly lower than that of the wild type, but it displays the same slope, unlike what was previously observed for the Δ*zrc1* mutants (Fig. 2B). Moreover, Zinquin

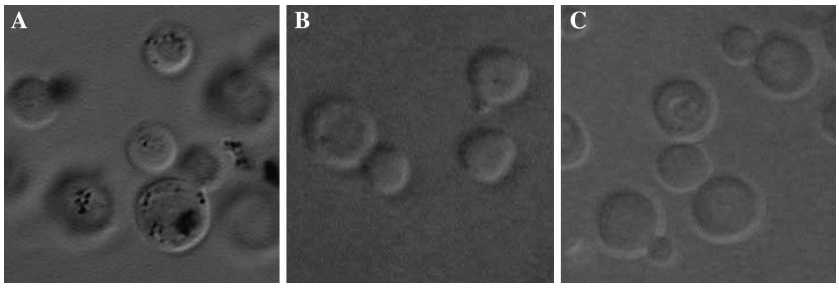


Fig. 4. Autometallographic detection of intracellular free zinc. WT yeast cells processed for AMG in the presence (A) or in the absence (B) of sodium selenite, or following 1 h treatment with the zinc chelator sodium diethyldithiocarbamate (DEDTC) (C).

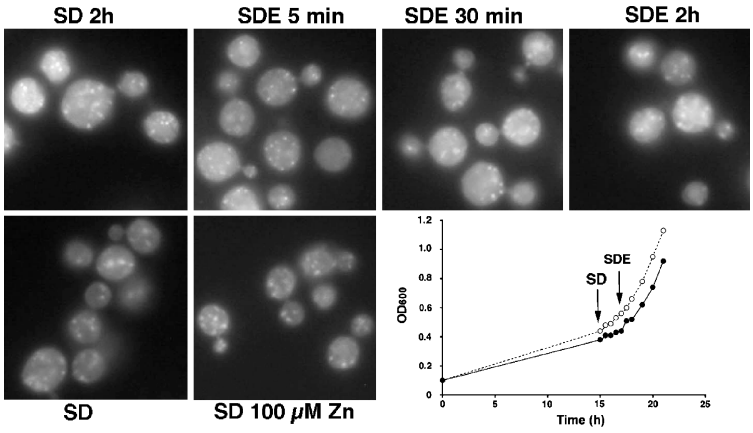


Fig. 5. Zinquin fluorescence in the end4 mutant strain grown in media with different zinc contents. Yeast Δ*end*4 mutant cells were grown as in the legend to Fig. 2. WT, open circles; Δ*end*4, filled circles. Samples were withdrawn from the culture and stained with Zinquin at the indicated time points. The bottom two panels show Zinquin staining of cells grown to logarithmic phase either in SD or in SD supplemented with 100 μM $ZnSO_4$, without previous zinc depletion.

positive vesicles were evenly distributed in cells containing the *END4* deletion grown in minimal SD media supplemented with 100 μM $ZnSO_4$ (Fig. 5, lower panels).

## Discussion

Several transporter proteins have been identified that contribute to maintenance of zinc homeostasis in eukaryotic cells. They are most numerous in mammals, some with overlapping tissue specificities and intracellular localizations [6]. From an evolutionary point of view they all appear to have arisen from duplication and independent evolution of few distinct genes. In *S. cerevisiae* the ancestors of the mammalian ZnT family members are most likely the Zrc1 and Cot1 proteins, sharing both primary sequence and structural similarities.

Taking advantage of the zinc-specific fluorescent probe Zinquin, a widely used reagent to visualize intracellular zinc pools in mammalian cells [27], and of a modification of the zinc–selenium autometallography technique [24], we have visualized the dynamic pools of free Zn ions in wild type and mutant strains of *S. cerevisiae* exposed to different concentrations of zinc in the growth media.

It is the first time that ZnSe autometallography has been applied for zinc tracing in yeast, while Zinquin has been used in a prior report in this organism. In that work, however, staining was only found in transporter mutant cells [14].

Our findings indicate the presence of a distinct, zinc-enriched vesicular compartment containing free Zn ions both in wild type and mutant yeast cells under conditions of normal growth. This labile zinc containing compartment, which resembles the zincosomes of mammalian cells, is detected in yeast cells with two independent zinc imaging techniques, namely Zinquin staining and $ZnSe^{AMG}$. The principal features of this compartment are as follows: under normal growth conditions in minimal medium no qualitative differences are detected between wild type cells and single or double Δ*zrc1*, Δ*cot*1 mutants. Overnight growth in zinc-limiting medium leads to complete disappearance of Zinquin positive vesicles in all strains, while a subsequent shift of depleted cells to a culture medium containing micromolar concentrations of Zn ions results

in rapid appearance of Zinquin-positive vesicles, which start to be detectable during the first 5 min following repletion. Formation of zinc-loaded vesicles does not involve endocytosis, as it occurs the same way also in a Δ*end4* strain that is completely blocked in the endocytic pathway. All strains used in this study are fully capable of tight transcriptional regulation of zinc transporters. Therefore, zinc depletion strongly induces Zap1 that transcriptionally activates the genes responsible for zinc uptake and storage (*ZRT1-3* and *ZRC1*) [28], suggesting a dependence of zincosome formation on the Zap1-inducible uptake proteins Zrt 1 and 2.

While yeast zincosome formation occurs very rapidly, disappearance of this compartment when cells are shifted to zinc-limiting medium is a slow process. Our results show persistence of the zincosomal compartment in wild type cells up to 2 h following zinc depletion, while in the vacuolar transporter mutants the loss of Zinquin staining is already visible after 30 min and it occurs in the majority of cells after 2 h. These results suggest that Zn ions accumulate transiently in this compartment, from which they might be easily mobilized for metabolic needs upon nutritional deficiency. No fluorescence was ever observed in the vacuole, which was previously implicated as the major intracellular organelle responsible for detoxification of potentially toxic metal ions, including zinc ([16] and references therein). Our results do not argue against a key role for the vacuole in zinc storage, which was elegantly shown by other authors [17], but rather provide an experimental demonstration of a rapidly appearing vesicular compartment in wild type cells in response to a sudden increase in Zap1 dependent zinc intake. It is possible that Zinquin binding as well as formation of zinc–selenium nanocrystals are both impaired in the vacuole by the acidic pH, which is actively maintained inside this compartment, as no zinc-specific staining could be detected with either technique. Our results indicate that the vacuolar transporters Zrc1 and Cot1 are indirectly involved in the persistence of the Zinquin positive vesicular compartment, with a postulated mechanism depicted in Fig. 6: wild type cells efficiently accumulate zinc in the vacuole (panel A), unlike single and double deletion mutants in the *ZRC1* and *COT1* genes (panel B). Under zinc sufficient conditions wild type cells accumulate labile zinc both in the vacuole and in the zincosomal compartment, while the mutants are impaired in vacuolar zinc intake, and display properly loaded zincosomes. Therefore, when shifted to zinc-limited growth conditions, wild type cells can use both compartments to supply zinc for metabolic needs, while in the mutants only zincosomal labile zinc can be exchanged and this results in a more rapid depletion of zinc bodies.

The yeast Zinquin/AMG positive compartment is analogous to that described in mammalian cells as zinc-enriched vesicles (ZEN) [29], zinc containing vesicles [30] or zincosomes [22]. The existence of such compartment was postulated by other authors based on studies performed in yeast vacuolar transporter mutants [18]. Their findings suggested that Zn ions taken up from the extracellular environment enter a labile cytoplasmic pool, sensed by the transcription factor Zap1, from which they could be vehicled to target sites. Excess zinc could be moved from this cytoplasmic pool to the vacuole, where it was no longer sensed by Zap1, it could not exert toxic effects and it could be stored for later use. The Zinquin stained vesicles that we describe appear to have all the features of such a shuttle compartment, as their presence reflects the balance between Zn usage by the cellular machinery and vacuolar storage to prevent toxicity. We have previously observed rapid disappearance of the labile zinc pools also in Zn-depleted mammalian tissue culture cells [31]. Therefore, the vesicular, Zn-containing compartment might represent an extremely important, evolutionarily conserved defence system to buffer the onset of transient conditions of mild nutritional zinc deficiency and to prevent toxicity.

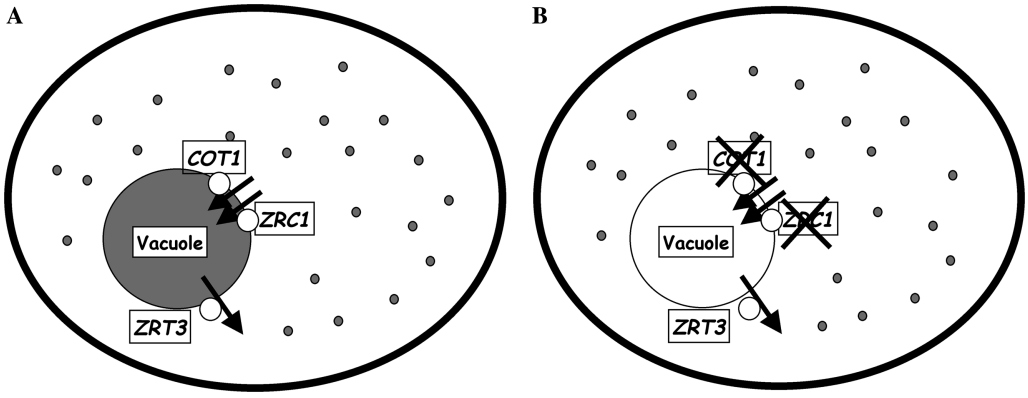


Fig. 6. Schematic model for the role of the zincosomal compartment in yeast. (A) Wild type cell. (B) Mutants in vacuolar zinc accumulation.

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