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Calcium signaling and copper toxicity in *Saccharomyces cerevisiae* cells

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Abstract To respond to metal surpluses, cells have developed intricate ways of defense against the excessive metallic ions. To understand the ways in which cells sense the presence of toxic concentration in the environment, the role of Ca^{2+} in mediating the cell response to high Cu^{2+} was investigated in *Saccharomyces cerevisiae* cells. It was found that the cell exposure to high Cu^{2+} was accompanied by elevations in cytosolic Ca^{2+} with patterns that were influenced not only by Cu^{2+} concentration but also by the oxidative state of the cell. When Ca^{2+} channel deletion mutants were used, it was revealed that the main contributor to the cytosolic Ca^{2+} pool under Cu^{2+} stress was the vacuolar Ca^{2+} channel, Yvc1, also activated by the Cch1-mediated Ca^{2+} influx. Using yeast mutants defective in the Cu^{2+} transport across the plasma membrane, it was found that the Cu^{2+}-dependent Ca^{2+} elevation could correlate not only with the accumulated metal, but also with the overall oxidative status. Moreover, it was revealed that Cu^{2+} and H_2O_2 acted in synergy to induce Ca^{2+}-mediated responses to external stress.

Keywords Copper · Calcium signaling · *Saccharomyces cerevisiae* · Aequorin · H_2O_2

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Introduction

Heavy metal stress is an important environmental burden caused by numerous anthropomorphic activities such as smelting, mining, industrial manufacturing, sewage and industrial waste, metal-containing pesticides and fertilizers, transportation, etc. (He et al. 2005; Tchounwou et al. 2012). Many heavy metals accumulate in living organisms, which may be subsequently transferred to human body via the food chain, leading to a variety of diseases (Järup 2003; Boyd 2010; Gall et al. 2015). Heavy metals are tricky pollutants because they are natural components of the earth crust, and unlike the organic pollutants, they are non-degradable (Wuana and Okieimen 2011). Of special interest are the metals essential for life (Cu, Co, Fe, Mn, Ni, Zn) which are necessary in minute amounts and for which cells have developed intricate mechanisms of uptake, intracellular traffic, buffering, and storage. Nevertheless, even the essential metals become toxic when the environmental levels get higher than the physiological threshold, due to excessive accumulation which leads to rapid and non-specific binding to biomolecules (Khan et al. 2015). For this reason, an immediate response to the sudden changes in heavy metal concentrations is essential for cell survival and adaptation.

The living organisms have developed various mechanisms to respond to environmental insults, and one of the conserved ways to transmit an external signal into the eukaryotic cell is by inducing transient elevations in Ca^{2+} concentrations within the cytosol ($[Ca^{2+}]_{cyt}$). Acting as a second messenger, Ca^{2+} triggers a variety of cascade responses by temporarily activating Ca^{2+}-binding components of signaling pathways which can lead either to adaptation to the environmental changes or to cell death (Bootman et al. 2012).

In a previous study, we investigated the involvement of Ca^{2+} in the response to high Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+},

Zn^{2+}, Cd^{2+}, or Hg^{2+} using *Saccharomyces cerevisiae* as model. This study revealed that the yeast cells responded through sharp increases in $[Ca^{2+}]_{cyt}$ when exposed to high Cd^{2+}, and to a lesser extent to Cu^{2+}, but *not* to Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, or Hg^{2+} (Ruta et al. 2014). In the present study, we focused on investigating the role of Ca^{2+} in mediating the cell response to high concentrations of external Cu^{2+} and we found that the cell exposure to high Cu^{2+} determined broad and prolonged $[Ca^{2+}]_{cyt}$ waves which showed a different pattern from the $[Ca^{2+}]_{cyt}$ pulses induced by high Cd^{2+}.

Copper is one of the most important essential transition metals, and a variety of enzymes require copper as a cofactor for electron transfer reactions (De Freitas et al. 2003). Nevertheless, when in excess, copper is very toxic in the free form because of its ability to produce free radicals when cycling between oxidized Cu^{2+} and reduced Cu^{+1}. Copper uptake, buffering, and traffic in *S. cerevisiae* have been extensively studied and reviewed (Nevitt et al. 2012). Under high concentration conditions, Cu^{2+} is transported by the low-affinity plasma membrane transporters Fet4 (Hassett et al. 2000) and Smf1 (Cohen et al. 2000), which can also transport Mn^{2+}, Fe^{2+}, and Zn^{2+} to fulfill the cellular demand for these cations. Additionally, Pho84, a high-affinity inorganic phosphate transporter, was found to act also as a low-affinity transporter for divalent cations, including Cu^{2+} (Jensen et al. 2003). The Cu^{2+} ions are also transported by the cell surface Fet3/Ftr1 high-affinity iron uptake system which also acts as a Cu^{1+} oxidase (Fet3) and Cu^{2+} transporter (Ftr1), being induced by high copper (Labbé et al. 1999; Gross et al. 2000).

There are numerous reports indicating that yeasts use Ca^{2+}-mediated signaling to respond to a variety of environmental stimuli (Batiza et al. 1996; Kanzaki et al. 1999; Locke et al. 2000; Matsumoto et al. 2002; Pinontoan et al. 2002; Viladevall et al. 2004; Popa et al. 2010; Rao et al. 2010; Courchesne et al. 2011; Roberts et al. 2012; Ruta et al. 2014; Rigamonti et al. 2015). Ca^{2+} acts as a second messenger in response to certain stimuli, a case when its concentration in the cytosol ($[Ca^{2+}]_{cyt}$) increases abruptly; in yeast, this can be a consequence of calcium influx via the Cch1/Mid1 Ca^{2+} channel on the plasma membrane (Batiza et al. 1996; Catterall 2000; Matsumoto et al. 2002), release of vacuolar Ca^{2+} into the cytosol through the vacuole-located Ca^{2+} channel Yvc1 (Palmer et al. 2001; Denis and Cyert 2002), or both (Popa et al. 2010; Khan et al. 2015). Once the message is delivered, the normal very low level of $[Ca^{2+}]_{cyt}$ is restored through the action of Ca^{2+} pumps and exchangers, reviewed by Cunningham (2011).

In the present study, the Ca^{2+}-dependent response to surplus Cu^{2+} in the yeast environment was investigated.

Materials and methods

Yeast strains, yeast manipulation, plasmids, and growth media

The *S. cerevisiae* strains used in this study were isogenic to the "wild-type" (WT) parental strain BY4741 (*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) (Brachmann et al 1998). The knock-out mutants used were *cch1Δ*, *cnb1Δ*, *mid1Δ*, *yvc1Δ*, *fet4Δ*, *smf1Δ*, *pho84Δ*, *fet3Δ*, *ftr1Δ*, and *yap1Δ*. All strains were purchased from EUROSCARF (www.euroscarf.de). Cell storage, growth, and manipulation were done as described by Sherman et al. (1986), using yeast extract–polypeptone–dextrose (YPD) or in synthetic complete dextrose (SD) media lacking specific amino acids when selective conditions were imposed. For solid media, 2 % agar was used. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich.

In vivo monitoring of $[Ca^{2+}]_{cyt}$

Monitoring of $[Ca^{2+}]_{cyt}$ changes in cells exposed to external stimuli was done using an apo-aequorin complementary DNA (cDNA) expression system (Nakajima-Shimada et al. 1991). In this study, yeast strains were transformed with the multicopy plasmid pYX212-*cytAEQ* harboring the apo-aequorin cDNA under the control of a constitutive yeast promoter (Tisi et al. 2002). Yeast transformation was performed by a modified lithium acetate method (Schiestl and Gietz 1989). Transformed cells were maintained on SD-Ura selective medium and prepared for Ca^{2+}-dependent luminescence detection as described (Tisi et al. 2015) with slight modifications. Overnight pre-cultures of cells expressing apo-aequorin were diluted to density 5×10^6 cells/mL with fresh SD-Ura supplemented with 10 μM $CuCl_2$ and incubated with shaking (200 rpm) at 28 °C for four additional hours. At this concentration, the supplemental $CuCl_2$ was completely non-toxic, but it suppressed the expression of the *CTR1* gene which encodes the high-affinity transporter for Cu (Dancis et al. 1994). Cells were harvested by centrifugation and resuspended in SD-Ura containing 10 μM $CuCl_2$ (10^8 cells/mL). To reconstitute functional aequorin, native coelenterazine was added to the cell suspension (from a methanol stock, 50-μM final concentration) and the cells were incubated for 1 h at 28 °C in the dark. Cells were treated with coelenterazine sequentially, maintaining a constant time of incubation before addition of stressors. The excess coelenterazine was washed away by centrifugation with 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES)–Tris buffer, pH 6.5, prepared with Ca^{2+}-free reagents and deionized water. The cells (approximately 10^7 cells/determination) were finally resuspended in 0.1 M MES–Tris buffer, pH 6.5, containing 1 mM $CaCl_2$ and transferred to the luminometer tube. To evaluate the requirement of extracellular

Ca^{2+}contribution, the coelenterazine-treated cells were washed three times with 1 M MES–Tris buffer, pH 6.5, containing 20 mM $MgCl_2$ and re-suspended in 0.1 M MES–Tris buffer, pH 6.5, containing 10 μM (1,2-bis(o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) (BAPTA, cell-impermeant Ca^{2+} chelator). A cellular luminescence baseline was determined for each strain by approximately 1 min of recordings at 1/s intervals. Thirty seconds after ensuring a stable signal, chemicals tested were injected from sterile stocks to give the final concentrations indicated, and the Ca^{2+}-dependent light emission was monitored in a single tube luminometer (Turner Biosystems, 20^n/20). The light emission was measured at 1 s intervals for at least 10 min after the stimulus and reported as relative luminescence units/second (RLUs/s). To ensure that the total reconstituted aequorin was not limiting in our assay, at the end of each experiment, aequorin expression and activity were checked by lysing cells with 1 % Triton X-100; only the cells with considerable residual luminescence were considered. Relative luminescence emission was normalized to an aequorin content giving a total light emission of 10^6 RLUs in 10 min after lysing cells with 1 % Triton X-100. The relative maximum luminescence (RLM) was the average of the RLUs flanking the maximum value (ten values on each side) relatively to the average luminescence baseline recorded before cells were exposed to Cu^{2+}.

Assessment of cell growth using spot assay

Overnight pre-cultures were washed and shifted to fresh medium (to approximately 10^6 cells/mL) then grown for 4 h at 28 °C under strong agitation (200 rpm). The exponentially growing cells were serially diluted in sterile water and stamped on agar plates containing various chemicals using a pin replicator (approximately 4 μL/spot). Plates were photographed after 3–4 days of incubation at 28 °C. Chemicals were added to the agar media from sterile stocks, after autoclaving. To mimic calcium depletion, the medium was supplemented with increasing concentrations of the Ca^{2+} chelator ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA).

Intracellular copper assay

Copper loading of the cells was done as follows. Overnight pre-cultures were diluted in fresh YPD to density 10^6 cells/mL. The cells were incubated with shaking for four additional hours at 28 °C before $CuCl_2$ was added from sterile stocks. For metal accumulation assay, the cells were harvested at various times by centrifugation and washed three times with ice-cold solutions containing 1 mM MES–Tris buffer, pH 6.0, and 5 mM ethylenediaminetetraacetic acid (EDTA, tetrasodium salt) for a better removal of the cell wall-bound copper. All centrifugation (1 min, 5000 rpm) was done at 4 °C. Cells were finally suspended in deionized water (10^9 cells/mL) and used for metal assay. The samples were digested for 24 h with nitric acid of ultrapure grade (Merck, Germany) and stabilized in Tris/HCl buffer (pH 8). Apart from cell digestion, HNO_3 ensures the complete oxidation of Cu^{1+} to Cu^{2+}. The copper released by cell digestion was determined colorimetrically with the chromogenic reagent bis(cyclohexanone) oxaldihydrazone (Cuprizone) as described (Marczenko and Balcerzak 2000) and normalized to cell total protein as nanomoles copper per milligram cell protein. Cellular total protein was determined by the method described by Bradford (1976) using a Shimadzu UV–vis spectrophotometer (UV mini-1240).

Reproducibility of the results and statistics

All experiments were repeated at least three times, and only those with trends that were fully consistent among the independent experiments were considered. Values in graphs are means ± standard deviation (SD). For aequorin luminescence determinations, traces represent the mean ± SD from three independent experiments performed on different days ($n = 3$). For copper accumulation experiments, the values were expressed as the mean ± SD of triplicate determinations made on 2 days ($n = 6$). For visual results (photographs), one representative example is shown.

Student's *t* test was used for the statistical analysis of each strain in control conditions compared with Cu^{2+} treatment. A $p < 0.01$ was deemed indicative of a statistically significant difference for these tests. For copper accumulation, the analysis of mutant strains compared with the wild type was performed using a one-way analysis of variance followed by Dunnett's test for multiple comparisons. A $p < 0.05$ was deemed indicative of a statistically significant difference for these tests. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results and discussion

Yeast cells exposed to high Cu^{2+} respond through slow but transient increase in $[Ca^{2+}]_{cyt}$

In a previous study, we showed that the presence of high Cd^{2+} in the environment induced sudden $[Ca^{2+}]_{cyt}$ elevations in normal *S. cerevisiae* cells. Other divalent metal ions tested showed no effect with the exception of Cu^{2+}, whose presence in the growth media induced broad elevations of $[Ca^{2+}]_{cyt}$ (Ruta et al. 2014). To determine whether the cell response to high Cu^{2+} was mediated by Ca^{2+} in yeast cells, we made use of the Ca^{2+}-induced luminescence of a photoprotein, aequorin, a system suitable for detecting transient modifications in the $[Ca^{2+}]_{cyt}$ (Nakajima-Shimada et al. 1991). For this purpose, yeast

cells were transformed with a plasmid harboring the cDNA of the luminescent Ca^{2+} reporter apo-aequorin under the control of a constitutive promoter, which afforded abundant transgenic protein within the cytosol (Tisi et al. 2002). Transgenic parental (wild type (WT)) cells expressing apo-aequorin were pre-treated with the cofactor coelenterazine to reconstitute functional aequorin, and then, the cells were exposed to Cu^{2+} shocks directly in the luminometer tube. By testing various concentrations, it was noticed that the cells responded to external Cu^{2+} ions through slow, but transient luminescence peaks which indicated similar elevations in the $[Ca^{2+}]_{cyt}$ (Fig. 1a). It was noted that while the wild-type cells exposed to high Cd^{2+} had required no more than 100 s to restore the low level of $[Ca^{2+}]_{cyt}$ (Ruta et al. 2014), the cells exposed to high Cu^{2+} needed longer time to recover after the Cu^{2+} shock by restoring the normal levels of $[Ca^{2+}]_{cyt}$ (Fig. 1a). The maximum luminescence recorded increased with Cu^{2+} concentration (Fig. 1a), to reach a plateau at concentrations higher than 0.5 mM (Fig. 1b). Inversely, the time required to achieve the maximum luminescence decreased when increasing the concentration of external Cu^{2+} (Fig. 1c). For Cu^{2+} concentrations between 0.1 and 0.5 mM, the time to reach the maximum luminescence was higher than 200 s (Fig. 1a), approximately ten times slower than the response to Cd^{2+}, for which the luminescence peak was reached after approximately 20 s following the exposure to Cd^{2+} shock (Ruta et al. 2014). The luminescence traces recorded for Cu^{2+} were broad even when the cells were exposed to concentrations as high as 10 mM (data not shown), indicating that high Cu^{2+} induced $[Ca^{2+}]_{cyt}$ elevations, but the cell response was different from the response to high Cd^{2+}.

Ca^{2+}-mediated response to high Cu^{2+} depends predominantly on internal Ca^{2+} stores

To further characterize the Ca^{2+} response to exogenous Cu^{2+}, we examined whether the Cu^{2+}-dependent Ca^{2+} fluxes had external or internal source. In the wild-type cells expressing functional aequorin, the Cu^{2+} exposure induced broad and transient luminescence peaks caused by the increase in the cytosolic Ca^{2+} (Fig. 2a, full line). The peak intensity was attenuated when measurements were done in Ca^{2+}-free resuspension buffer (Fig. 2a, dotted line), suggesting that the Cu^{2+}-dependent increase in $[Ca^{2+}]_{cyt}$ depended on both external and internal Ca^{2+} stores. Under stress conditions, the increase in $[Ca^{2+}]_{cyt}$ is mainly the result of the plasma membrane Ca^{2+} channel Cch1/Mid1 activity (Batiza et al. 1996; Matsumoto et al. 2002; Rao et al. 2010) and/or of the vacuole-located Ca^{2+} channel Yvc1 (Palmer et al. 2001; Denis and Cyert 2002). To determine whether the Cu^{2+}-mediated release of $[Ca^{2+}]_{cyt}$ occurs through these channels, we measured the Cu^{2+}-induced luminescence of knock-out *cch1Δ*, *mid1Δ*, or *yvc1Δ* mutant cells expressing the apo-aequorin cDNA. It was noticed that the cells lacking Cch1 showed a lower increase in $[Ca^{2+}]_{cyt}$ when exposed to Cu^{2+} surplus (Fig. 2b, full line), indicating that Cch1 is required to achieve the $[Ca^{2+}]_{cyt}$ maximum peak. The luminescence trace did not change significantly in *cch1Δ* cells resuspended in Ca^{2+}-free buffer (Fig. 2b, dotted line), suggesting that the Ca^{2+} ions that come from outside the cell enter mainly through the Cch1/Mid1 channel and that Cch1 integrity is essential for Ca^{2+} entry under high Cu^{2+}. Nevertheless, as an increase in $[Ca^{2+}]_{cyt}$, could still be recorded in *cch1Δ* cells, other ways of Ca^{2+} release are likely to exist under Cu^{2+} stress, when probably the Ca^{2+} internal stores are also mobilized. In contrast to *cch1Δ* cells, the *mid1Δ* cells responded to high Cu^{2+} similarly to the wild-type cells (Fig. 2c, full line) exhibiting an attenuation of the Ca^{2+}-dependent luminescence in Ca^{2+}-free buffer (Fig. 2c, dotted line). This observation indicated that if the Ca^{2+} ions entered the cell via the Cch1/Mid1 channel, Cch1 would be sufficient for inducing $[Ca^{2+}]_{cyt}$ elevations in response to Cu^{2+}. As the luminescence trace was broader in *mid1Δ* than in the wild-type cells, it is highly probable that Mid1 is necessary mainly for *tuning* the Ca^{2+} influx through Cch1 in response to high Cu^{2+}. On the other hand, in aequorin-expressing *yvc1Δ* cells, lacking the vacuole-located Ca^{2+} channel Yvc1p, the Cu^{2+}-dependent $[Ca^{2+}]_{cyt}$ pulse was almost undetectable (Fig. 2d), suggesting that Yvc1p is the main contributor to the $[Ca^{2+}]_{cyt}$ pool under Cu^{2+} stress. It was reported that the release of vacuolar Ca^{2+} via Yvc1p is stimulated by the Ca^{2+} ions which enter the cytosol from outside or are released from the vacuole by Yvc1p *itself* in a positive feedback (Palmer et al. 2001; Denis and Cyert 2002); therefore, it is possible that Yvc1p *massively* releases Ca^{2+} into the cytosol only when the Cu^{2+}-induced Ca^{2+}, which enters the cell via the Cch1p channel, reaches a critical threshold. This would explain why the cells lacking the Cch1 (but having intact Yvc1) responded through less strong, but nonetheless significant $[Ca^{2+}]_{cyt}$ elevations. It was demonstrated that the release of Ca^{2+} from intracellular stores stimulates the extracellular Ca^{2+} influx, a process known as capacitative calcium entry (Locke et al. 2000), which is probably blocked in *cch1Δ* cells. Inversely, the release of vacuolar Ca^{2+} via Yvc1p can be stimulated by small amounts of Ca^{2+} from outside the cell as well as by the Ca^{2+} released from the vacuole by Yvc1p itself, in a positive feedback called Ca^{2+}-induced Ca^{2+} release (CICR) (Palmer et al. 2001; Zhou et al. 2003; Su et al. 2009a, b), thus explaining

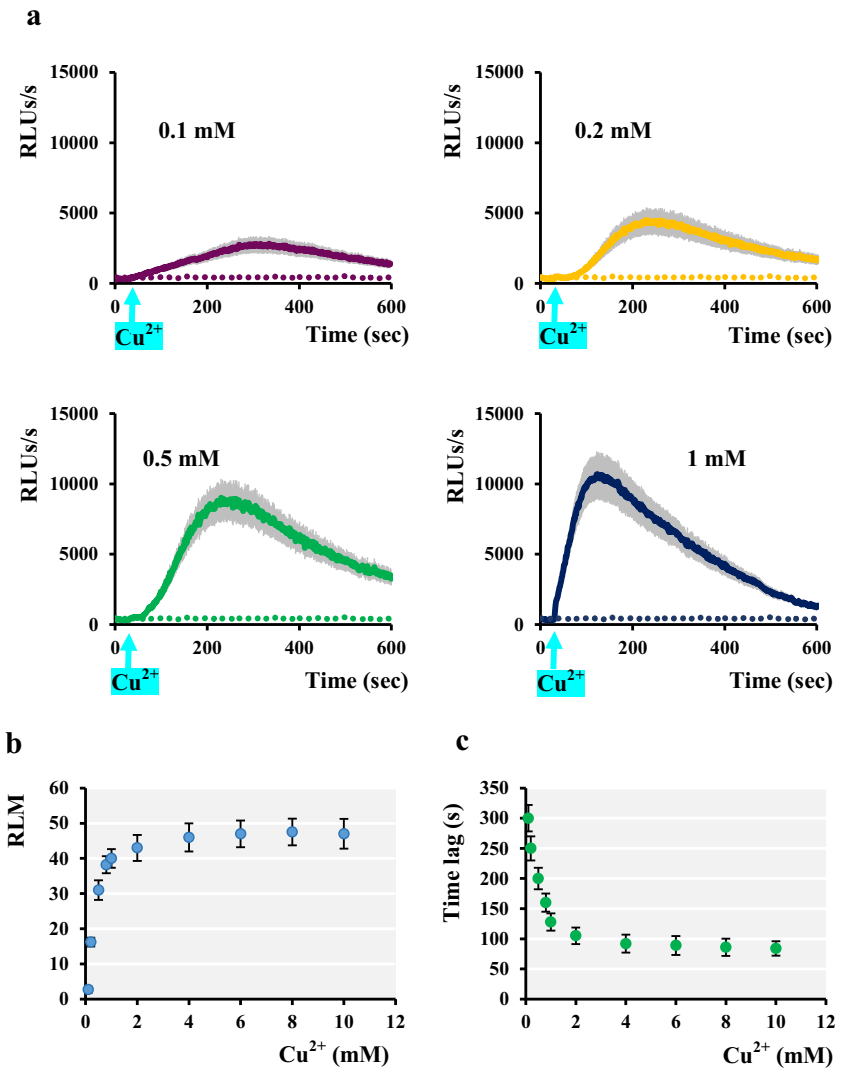

Fig. 1 Changes in the $[Ca^{2+}]_{cyt}$ upon exposure to exogenous Cu^{2+}. Wild-type cells BY4147 were transformed with the plasmid pYX212-*cytAEQ*, and the cells expressing coelenterazine-reconstituted aequorin were exposed to various concentrations of Cu^{2+} directly in the luminometer tube as described in "Materials and methods" section. Cu^{2+}-induced Ca^{2+}-dependent aequorin luminescence was recorded on samples of approximately 10^7 cells and normalized to an aequorin content giving a total light emission of 10^6 RLUs in 10 min after lysing cells with 1 % Triton X-100. **a** Effect of Cu^{2+} concentration upon Ca^{2+}-dependent luminescence. Traces showing Ca^{2+}-dependent aequorin luminescence in response to increasing Cu^{2+} concentrations: 0.1 mM (*purple*), 0.2 mM (*yellow*), 0.5 mM (*green*), and 1 mM (*blue*). The *arrow* indicates the time when Cu^{2+} (in the form of $CuCl_2$ stock solution) was added. *Dotted lines* represent the luminescence traces recorded in cells transformed with the control vector (pYX212). The luminescence traces represent the mean ± SD from independent experiments performed on three different days. **b** Effect of Cu^{2+} concentration on the maximum intensity of the Ca^{2+}-dependent aequorin luminescence. The relative maximum luminescence (RLM) was the average of the RLUs flanking the maximum value recorded (10 values on each side) divided by the average luminescence recorded before cells were exposed to Cu^{2+}. **c** Time required by aequorin-expressing cells to attain maximum Ca^{2+}-dependent aequorin luminescence following exposure to Cu^{2+}. In **b**, **c** values are mean ± SD of three determinations made on two distinct experiments ($n = 6$)

the almost complete lack of $[Ca^{2+}]_{cyt}$ increase in cells devoid of Yvc1, but having a functional Cch1/Mid1 system.

Although different in intensity, the Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ elevations in wild-type, *cch1Δ*, *mid1Δ*, or *yvc1Δ* cells followed a pattern similar to the $[Ca^{2+}]_{cyt}$ elevations induced in these strains by H_2O_2 (Popa et al. 2010). This observation prompted the idea that Cu^{2+} may indirectly induce $[Ca^{2+}]_{cyt}$ elevations by generating oxidative stress.

Mutants with defects in Ca^{2+} homeostasis exhibit various Cu^{2+} tolerance phenotypes

As Cu^{2+} stress was signaled within the wild-type cells via elevations in the $[Ca^{2+}]_{cyt}$, the next step was to check the tolerance to high Cu^{2+} of the mutants defective in Ca^{2+} transport and homeostasis (Fig. 2e). It was noticed that *cch1Δ* sensitivity to Cu^{2+} was slightly lower than that of the wild type, while and *yvc1Δ* cells were Cu^{2+}-tolerant (Fig. 2e).

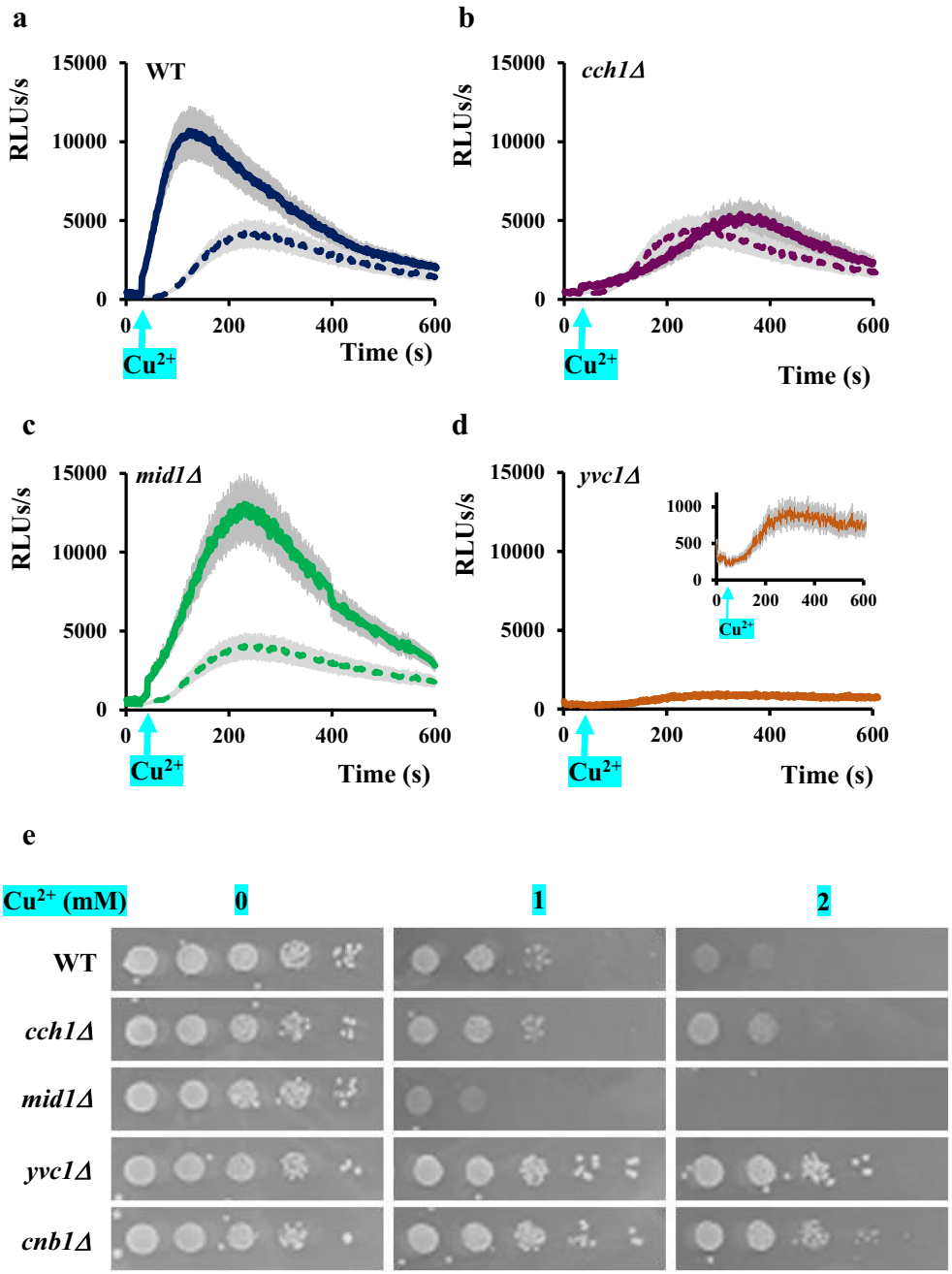

Fig. 2 Effect of mutations affecting Ca^{2+} transport upon $[Ca^{2+}]_{cyt}$ elevations in response to Cu^{2+}. Isogenic strains expressing coelenterazine-reconstituted cytAEQ were exposed to Cu^{2+} (1-mM final concentration) directly in the luminometer tube as described in "Materials and methods" section. *Dotted lines* represent the traces showing Ca^{2+}-dependent aequorin luminescence of cells resuspended in Ca^{2+}-free buffer. The *arrows* indicate the addition of Cu^{2+}. The luminescence traces represent the mean ± SD of independent experiments performed on three different days. **a** Wild-type strain. **b** Null-mutant *cch1Δ* strain. **c** Null-mutant *mid1Δ* strain. **d** Null-mutant *yvc1Δ* strain. *Inset*: as main figure, but magnified. **e** Effect of Cu^{2+} on the growth of mutants with defects in Ca^{2+} transport and homeostasis. Exponentially growing yeast cells from the isogenic strains WT, *cch1Δ*, *mid1Δ*, *yvc1Δ*, and *cnb1Δ* were 10-fold serially diluted (from 10^7 cells/mL, *left*) and were spotted (approximately 4 μL) on SD plates supplemented with $CuCl_2$. Cells were photographed after 3–4-day incubation at 28 °C. The experiments were repeated three times and the results were similar. One representative set of plates is shown

The *mid1Δ* cells were clearly more sensitive than the wild type; thus, the sensitivity to Cu^{2+} paralleled the sensitivity to H_2O_2 (Popa et al. 2010). In terms of Cu^{2+} uptake, the strains were not significantly different (data not shown), indicating that the differences in the tolerance to Cu^{2+} are not the result of different Cu^{2+} accumulation but are rather caused by the different ways in which the cells signal the presence of high Cu^{2+}.

The sudden increase in $[Ca^{2+}]_{cyt}$ under environmental conditions forms the basis of Ca^{2+} action as a second messenger. Once in the cytosol, the Ca^{2+} ions bind to the universal Ca^{2+} sensor protein calmodulin, which in turn can bind and activate calcineurin. Calcineurin is the protein phosphatase required for yeast to adapt to a variety of environmental stresses (Cyert 2003) by regulating calcium homeostasis at both transcriptional (via the transcription factor Crz1p) and post-transcriptional levels (for review, Cunningham 2011). Since Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ elevations may induce calcineurin activation, the effect of high Cu^{2+} on *cnb1Δ* cells, defective in the regulatory subunit of calcineurin, was tested. It was found that the *cnb1Δ* cells exhibited Cu^{2+} tolerance similar to *yvc1Δ* cells (Fig. 2e), suggesting that high Cu^{2+} may activate calcineurin-dependent pathways which are detrimental to

cell survival under high Cu^{2+}. This phenotype also paralleled the tolerance to H_2O_2 of *cnb1Δ* cells (Popa et al. 2010).

The yeast mutants with defects in the low-affinity Cu^{2+} uptake exhibit attenuated Cu^{2+}-dependent $[Ca^{2+}]_{cyt}$ elevations

Copper is a redox-very active metal, and in yeast treated with copper, reactive oxygen species are generated via Fenton/Haber–Weiss pathways both in the reaction medium and inside the cell (Liang and Zhou 2007). As such, the question which arose was whether Cu^{2+} was more active in generating a $[Ca^{2+}]_{cyt}$ pulse from outside or after entering the cytosol. To find an answer to this question, yeast mutants with defects in Cu^{2+} transport across the plasma membrane under high concentration conditions were further investigated. Under higher-than-normal concentrations, three low-affinity transporters were reported to transport Cu^{2+} across plasma membrane: Fet4, Smf1, and Pho84 (Cohen et al. 2000; Hassett et al. 2000; Jensen et al. 2003). Under normal to low concentrations, Cu^{2+} is transported across the yeast plasma membrane by the high-affinity transporter Ctr1p. *CTR1* transcription is strongly regulated by Cu^{2+} availability, and it is completely abolished at Cu^{2+} as low as 10 μM (Dancis et al. 1994). As *CTR1* is not active under Cu^{2+} surplus and to avoid interference of with the low-affinity transporters, the *CTR1* transcription was inhibited by growing the cells in media containing 10 μM $CuCl_2$, a concentration which was completely non-toxic to the yeast cells.

Cells defective in Fet4, Smf1, and Pho84 transporters are more tolerant to high Cu^{2+} (data not shown) apparently due to decreased Cu^{2+} accumulation (Cohen et al. 2000; Hassett et al. 2000; Jensen et al. 2003). The Cu^{2+} uptake of knock-out mutants *fet4Δ*, *smf1Δ*, and *pho84Δ* was recorded in the first 10 min of Cu^{2+} exposure (time corresponding to the average duration of the Cu^{2+}-dependent $[Ca^{2+}]_{cyt}$ elevation), and it was found that in all three mutants, Cu^{2+} accumulated less than in the wild-type cells (Fig. 3a).

To see if the lower Cu^{2+} accumulation modifies the Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ elevations, the Ca^{2+}-dependent luminescence in cells expressing transgenic aequorin in response to Cu^{2+} was recorded. It was noted that *fet4Δ* cells, having the lowest level of Cu^{2+} accumulation in the first 10 min of Cu^{2+} exposure, also exhibited lower Cu^{2+}-dependent $[Ca^{2+}]_{cyt}$ elevations (Fig. 3b). The *smf1Δ* and *pho84Δ* cells also accumulated less Cu^{2+}, but the Cu^{2+}-dependent $[Ca^{2+}]_{cyt}$ elevations were stronger compared to *fet4Δ* cells, but still weaker than in the wild-type cells (Fig. 3c, d). This observation suggested that Cu^{2+} ions must enter the cell to exert their full action on inducing $[Ca^{2+}]_{cyt}$ elevation.

The yeast *yap1Δ* knock-out mutant is tolerant to Cu^{2+}

The different tolerance to Cu^{2+} of WT, *cch1Δ*, *mid1Δ*, *yvc1Δ*, and *cnb1Δ* cells paralleled the tolerance of these mutants to H_2O_2 (Popa et al. 2010), suggesting that Cu^{2+} might induce $[Ca^{2+}]_{cyt}$ indirectly, by generating reactive oxygen species. In yeast, Cu^{2+} is a potent superoxide (ubiquitous in all the aerobic cells) scavenger, easily accepting one electron from this anion radical to generate H_2O_2 (Lapinskas et al. 1995). If this were the case, mutants with altered tolerance to H_2O_2 might behave differently when exposed to high Cu^{2+}. We therefore tested the tolerance to Cu^{2+} of a H_2O_2-hypersensitive mutant, *yap1Δ*. This mutant lacks Yap1, the extensively studied transcription factor which regulates the response to oxidative and Cd^{2+} stress (Wu et al. 1993; Kuge and Jones 1994); cells lacking a functional Yap1 are hypersensitive to both H_2O_2 and Cd^{2+}. Surprisingly, it was found that the *yap1Δ* cells were slightly more tolerant to Cu^{2+} than the wild-type cells (Fig. 4a), an observation which further supported the idea that Ca^{2+}-mediated response to high Cu^{2+} follows a different mechanism from that involved in the tolerance to Cd^{2+}. When recording the $[Ca^{2+}]_{cyt}$ in *yap1Δ* cells expressing functional aequorin, it was seen that exposure to high Cu^{2+} induced a sudden and sharp peak of Ca^{2+}-dependent luminescence which lasted only a few seconds before the Ca^{2+} levels were restored to normal (Fig. 4b). This observation suggested that sudden and short pulses of $[Ca^{2+}]_{cyt}$ may be benefic, orienting the cell toward adaptation pathways. In *yap1Δ* cells, the oxidative state is altered due to low expression of the genes regulated by Yap1; thus, the Cu^{2+}-related oxidative stress would add up to generate an overall shock transduced in the sharp $[Ca^{2+}]_{cyt}$ elevations. It was shown before that strong and brief $[Ca^{2+}]_{cyt}$ pulses can be correlated with adaptation to external stimuli (Ruta et al. 2014), explaining why *YAP1* deletion results in gained tolerance to high Cu^{2+}. In *yap1Δ* cells, the Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ elevation depended mainly on the Ca^{2+} internal stores, since exposing the cells to Cu^{2+} in Ca^{2+}-free resuspension buffer did not result in significant attenuation of Ca^{2+}-dependent cell luminescence (Fig. 4c). This observation suggested that the oxidative state inside the *yap1Δ* boosted the Cu^{2+} action by triggering the Ca^{2+} release from the internal stores. In this line of evidence, the Ca^{2+}-dependent luminescence of aequorin-expressing *yap1Δ* was considerably attenuated by cell pre-incubation with *N*-acetyl-cysteine (NAC), a cell-permeant antioxidant which elevates the intracellular reduced glutathione levels (Fig. 4d).

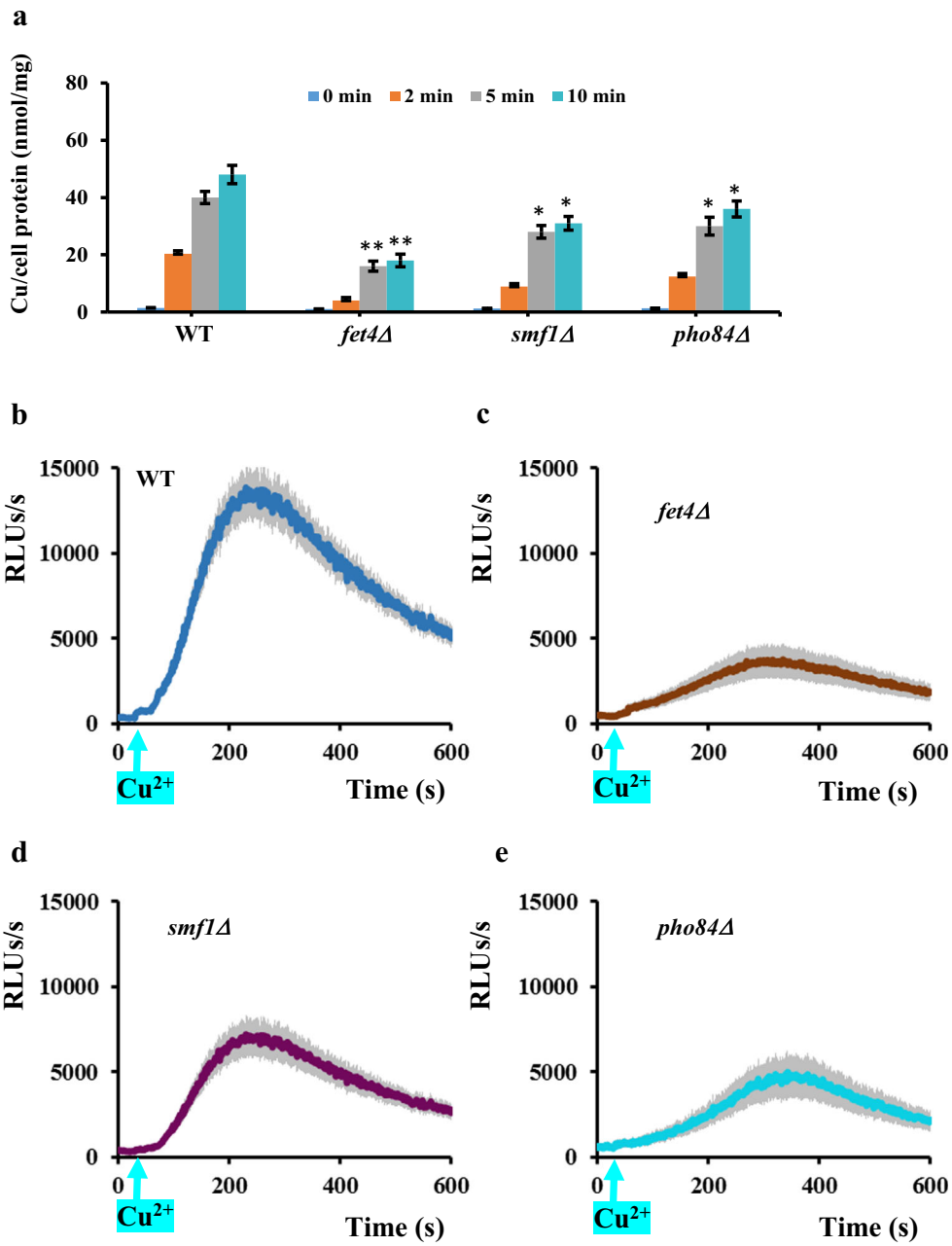

Fig. 3 Effect of mutations affecting low-affinity Cu^{2+} uptake upon $[Ca^{2+}]_{cyt}$ elevations in response to high Cu^{2+}. **a** Cu^{2+} accumulation. Exponentially growing yeast cells were shifted to SD medium supplemented with 1 mM $CuCl_2$ and were incubated with shaking (200 rpm) at 28 °C for 5 or 10 min before cells were harvested for determination of Cu^{2+} accumulation, as described in "Materials and methods" section. Values are mean ± SD of three determinations made on two distinct experiments ($n = 6$). $^*p < 0.05$, $^{**}p < 0.01$, significantly lower than the control strain. **b**–**e** Cu^{2+}-induced mobilization of $[Ca^{2+}]_{cyt}$ in yeast cells defective in low-affinity Cu^{2+} transport across plasma membrane. The yeast cells expressing coelenterazine-reconstituted *cyt*AEQ were exposed to Cu^{2+} directly in the luminometer tube. The *arrows* indicate the moment when 1 mM $CuCl_2$ was added from a sterile stock. **b** Wild-type strain. **c** Null-mutant *fet4Δ* strain. **d** Null-mutant *smf1Δ* strain. **e** Null-mutant *pho84Δ* strain. Strains were pre-grown in SD-Ura supplemented with 10 μM $CuCl_2$ for inhibition of the high-affinity transporter Ctr1. The luminescence traces represent the mean ± SD from independent experiments performed on three different days

The yeast mutants *fet3Δ* and *ftr1Δ* exhibit increased tolerance to H_2O_2

As the study progressed, it became clear that there is a connection between the Ca^{2+}-mediated response to high Cu^{2+} and the oxidative state of the cell. Apart from the low-affinity transporters Fet4, Smf1, and Pho84, Cu^{2+} is also transported by the Ftr1/Fet3 complex, in which Fet3 oxidizes the deleterious Cu^{1+} to the less toxic Cu^{2+} at the cell surface, the Cu^{2+} ions being subsequently transported into the cell by the Ftr1 (Shi et al. 2003; Stoj and Kosman 2003). As the Ftr1/Fet3 complex is induced by high Cu^{2+} (Gross et al. 2000), the behavior of cells defective in either of the two components was investigated further.

First, we tested the effect of exogenous oxidative stress generated by H_2O_2 exposure upon the growth of mutants with defective Cu^{2+} transport across the plasma membrane. It was found that while *fet3Δ*, *smf1Δ*, and *pho84Δ* showed similar sensitivity to exogenous H_2O_2 as the wild type, *fet3Δ*, and *ftr1Δ* were clearly more tolerant (Fig. 5a). The mutants' behavior flipped when the medium was supplemented with the cell-permeant antioxidant *N*-acetyl-cysteine (NAC, Fig. 5a), indicating that a more reduced environment is deleterious to the cells defective in the Fet3/Ftr1 system. It is largely

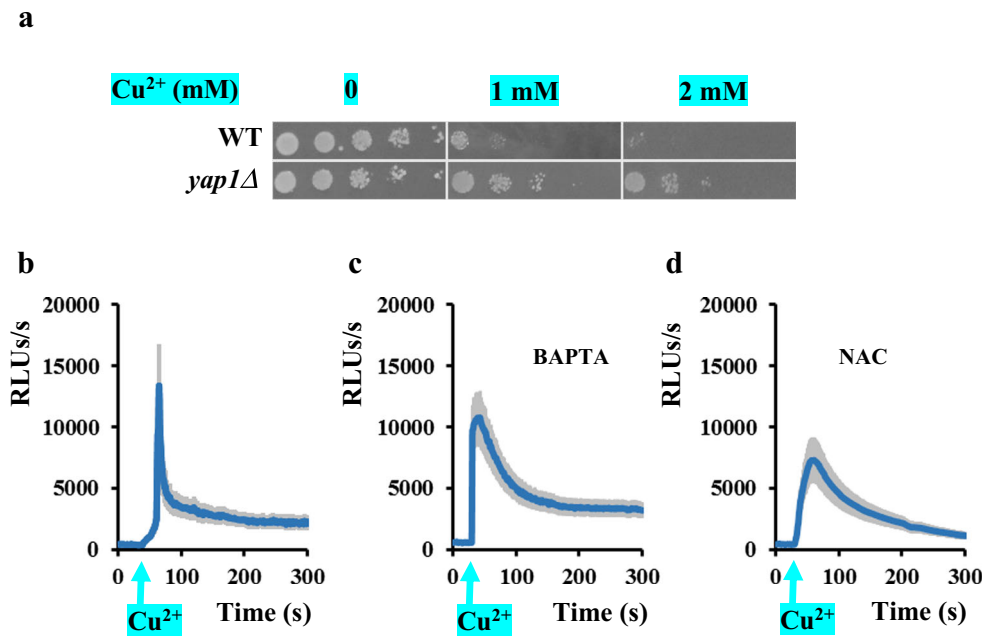

Fig. 4 Effect of Cu^{2+} on *yap1Δ* cells. **a** *YAP1* deletion results in Cu^{2+} tolerance. Exponentially growing cells from the isogenic strains WT and *yap1Δ* were 10-fold serially diluted (from 10^7 cells/mL, *left*) and were spotted (approximately 4 μL) on SD plates supplemented with the indicated concentrations of $CuCl_2$. Cells were photographed after 3-day incubation at 28 °C. The experiments were repeated three times, and the results were similar. One representative set of plates is shown. **b**–**d** Changes in the $[Ca^{2+}]_{cyt}$ in *yap1Δ* cells upon exposure to Cu^{2+}. The *yap1Δ* cells expressing coelenterazine-reconstituted aequorin were exposed to 1 mM Cu^{2+} directly in the luminometer tube, as described in the "Materials and methods" section. The *arrow* indicates the time when Cu^{2+} (in the form of $CuCl_2$ stock solution) was added. **b** No pre-treatment. **c** Cells pre-grown in SD supplemented with Ca^{2+} chelator BAPTA (1 mM) and finally resuspended in Ca^{2+}-free buffer. **d** Cells pre-grown in SD supplemented with the cell-permeant antioxidant *N*-acetyl-cysteine (NAC, 5 mM). The luminescence traces represent the mean ± SD from independent experiments performed on three different days

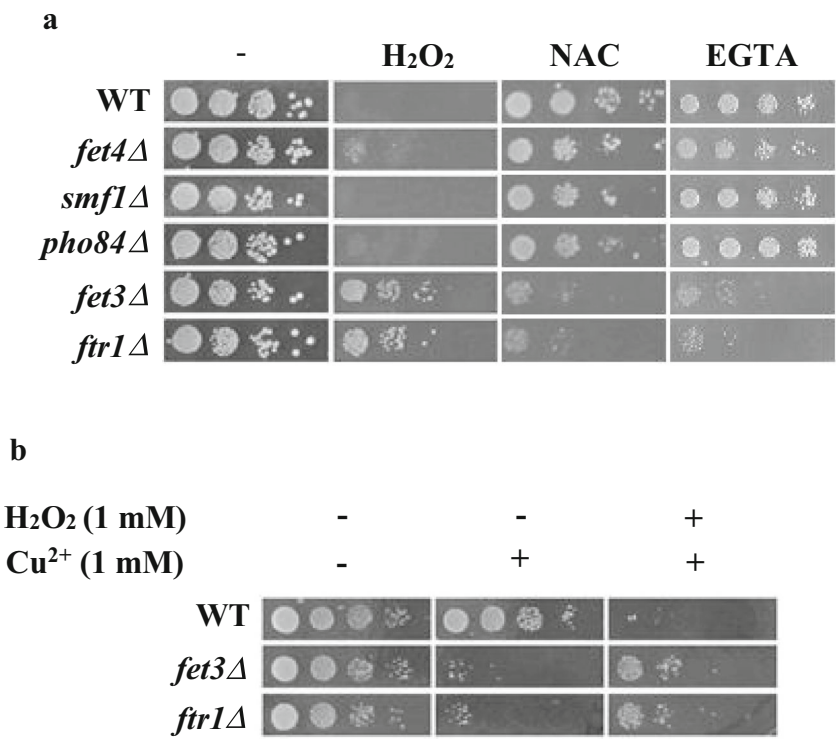

Fig. 5 H_2O_2 alleviates the Cu^{2+} sensitivity of yeast cells defective in the complex Fet3/Ftr1. Exponentially growing cells from the isogenic wild-type and knock-out strains were 10-fold serially diluted (from 10^7 cells/mL, *left*) and were spotted (approximately 4 μL) on SD plates supplemented with the indicated chemicals. Cells were photographed after 3-day incubation at 28 °C. The experiments were repeated three times and the results were similar. One representative set of plates is shown. **a** Effect of exogenous oxidative stress (H_2O_2, 4 mM), antioxidants (*N*-acetyl-cysteine, NAC, 10 mM), or Ca^{2+} depletion (EGTA, 15 mM) on yeast mutants defective in Cu^{2+} transport across plasma membrane. **b** Effect of exogenous H_2O_2 on the Cu^{2+} sensitivity of yeast cells defective in Fet3/Ftr1 complex

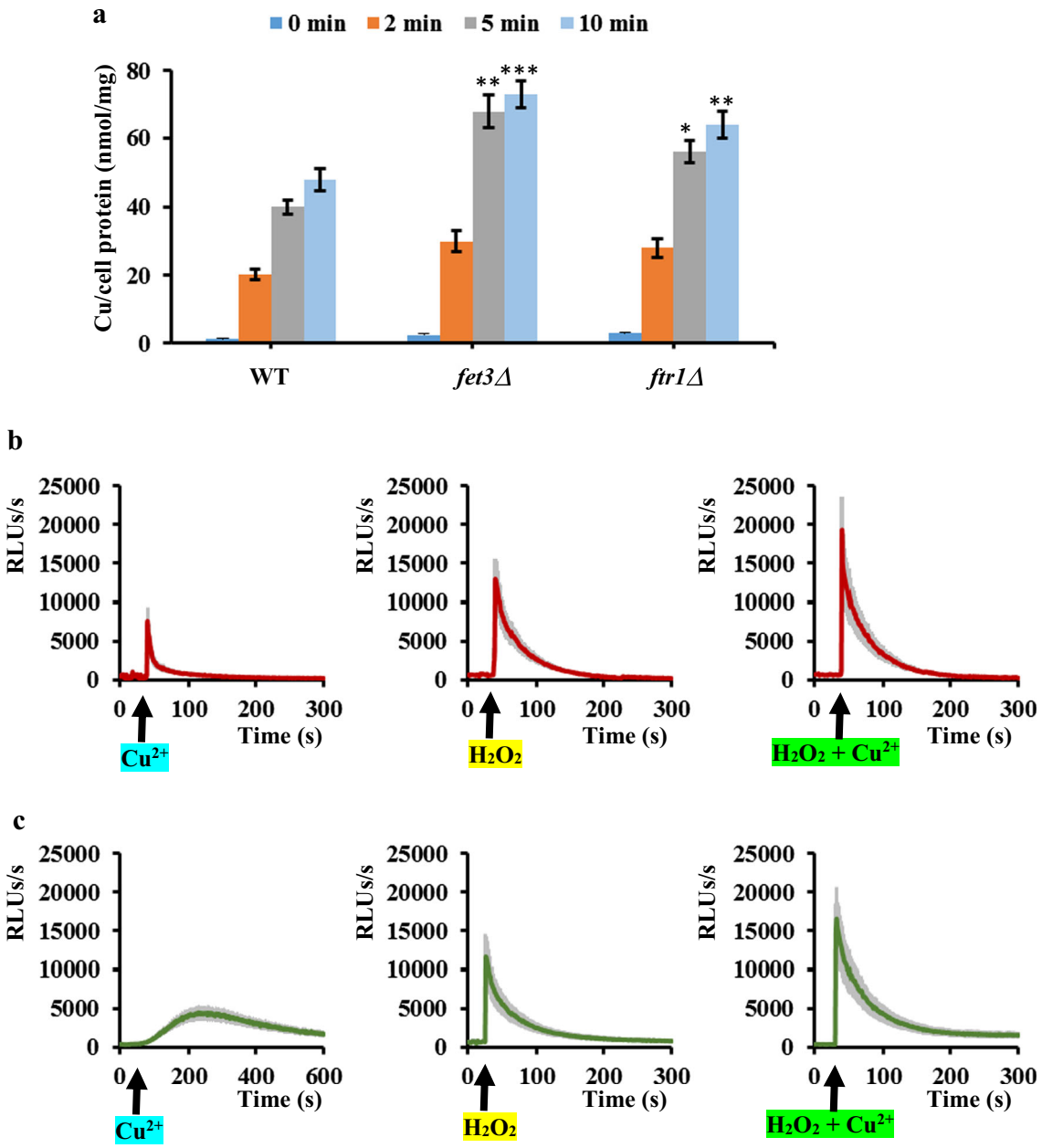

Fig. 6 Cu^{2+}-induced mobilization of $[Ca^{2+}]_{cyt}$ in cells defective in the complex Fet3/Ftr1 is augmented by H_2O_2. **a** Cu^{2+} accumulation. Exponentially growing yeast cells were shifted to SD medium supplemented with 1 mM $CuCl_2$ and were incubated with shaking (200 rpm) at 28 °C for 5 or 10 min before cells were harvested for determination of Cu^{2+} accumulation, as described in "Materials and methods" section. Values are mean ± SD of three determinations made on two distinct experiments ($n = 6$). $^{**}p < 0.01$, $^{***}p < 0.001$, significantly lower than the control strain. **b**, **c** The *fet3Δ* or *ftr1Δ* cells expressing coelenterazine-reconstituted aequorin were exposed to millimolar Cu^{2+}, H_2O_2, or ($Cu^{2+} + H_2O_2$) directly in the luminometer tube as described in the "Materials and methods" section (final concentration, 1 mM for both chemicals. Traces showing Ca^{2+}-dependent aequorin luminescence of **b** *fet3Δ* and **c** *ftr1Δ*. The *arrow* indicates the time when the chemicals were added. The luminescence traces represent the mean ± SD from independent experiments performed on three different days

believed that the growth defects of *fet3Δ* and *ftr1Δ* mutants are the result of the inability to efficiently oxidize Cu^{1+} to Cu^{2+} at the cell surface (Shi et al. 2003), a defect which can be either alleviated by exogenous oxidants (in this case H_2O_2) or augmented by reducing agents (in this case NAC). In this line of evidence, it was noted that the sensitivity to high Cu^{2+} of *fet3Δ* and *ftr1Δ* was mitigated by the presence of exogenous H_2O_2 (Fig. 5b). Numerous studies indicate that copper toxicity is not given by the amount accumulated, but rather by the amount of osmotically free ions, the Cu^{+1} ions being the most deleterious (Shi et al. 2003; Stoj and Kosman 2003).

Under such circumstances, the mitigation of copper toxicity by H_2O_2 in *fet3Δ* and *ftr1Δ* mutants may be, at least in part, the result of H_2O_2-related Cu^{+1} oxidation at the cell surface.

Cu^{2+} and H_2O_2 synergistically induce $[Ca^{2+}]_{cyt}$ elevations in cells defective in the Fet3/Ftr1 system

The tolerance to Cu^{2+} gained by the H_2O_2-sensitive *yap1Δ* cells, as well as the tolerance to H_2O_2 gained by the Cu^{2+}-sensitive *fet3Δ* or *ftr1Δ* cells, suggested a synergism between Cu^{2+} and H_2O_2 in signaling the stress conditions.

We further investigated the cumulative effect of H_2O_2 and Cu^{2+} on inducing $[Ca^{2+}]_{cyt}$ elevations in cells defective in the Fet3/Ftr1 system. The *fet3Δ* cells accumulated more Cu^{2+} (Fig. 6a) than the wild-type cells, resulting in sudden and more transient elevations in $[Ca^{2+}]_{cyt}$, with maxima reached after approximately 20 s from Cu^{2+} exposure; a more rapid restoration of the low levels of $[Ca^{2+}]_{cyt}$ was also noticed in *fet3Δ* cells (Fig. 6b, left). On the other hand, the *ftr1Δ* cells accumulated less Cu^{2+} than the *fet3Δ* cells within the first 10 min of Cu^{2+} exposure (Fig. 6a), causing a broadening of the $[Ca^{2+}]_{cyt}$ trace (Fig. 6c, left). It was difficult to assess that the role of the Cu^{2+}-dependent $[Ca^{2+}]_{cyt}$ elevation was under high Cu^{2+} in *fet3Δ* and *ftr1Δ* mutants. Knocking out the *YVC1* gene under *fet3Δ* and *ftr1Δ* background did not significantly alter the cell behavior under Cu^{2+} stress (data not shown); nevertheless, the *fet3Δ* and *ftr1Δ* cells were sensitive to the calcium chelator EGTA (Fig. 5c, right), indicating that these mutations required available Ca^{2+} for growth and probably for signaling of the stress conditions.

The alleviation of copper toxicity by H_2O_2 in the *fet3Δ* and *ftr1Δ* mutants (Fig. 5b) may be result of Cu^{+1} oxidation at the cell surface to the less toxic Cu^{2+} form. Nevertheless, besides oxidizing the cuprous ions, H_2O_2 also had the ability to induce $[Ca^{2+}]_{cyt}$ waves in *fet3Δ* (Fig. 6b, middle) and *ftr1Δ* (Fig. 6c, middle). Moreover, H_2O_2 and Cu^{2+} showed a synergistic effect on inducing stronger and sharper $[Ca^{2+}]_{cyt}$ pulses in both *fet3Δ* (Fig. 6b, right) and *ftr1Δ* (Fig. 6c, right), thus increasing the cell chances of survival.

Conclusions

In a previous study (Ruta et al. 2014), it was demonstrated that yeast cells use Ca^{2+} to signal the presence of high concentrations of Cd^{2+} in the environment. In the present study, we investigated the Ca^{2+}-mediated response to high Cu^{2+} and we found that Cd^{2+} and Cu^{2+} interact with yeast cells differently, yielding different Ca^{2+}-mediated responses. While Cd^{2+} induced sudden $[Ca^{2+}]_{cyt}$ elevations as a result of direct interaction with cell surface, the Cu^{2+} effect upon cell was more likely indirect. It was determined that contrary to Cd^{2+}, the cell exposure to high Cu^{2+} determined broad and prolonged $[Ca^{2+}]_{cyt}$ elevations which lasted approximately 600 s in wild-type cells. Clearly, Cch1 played a role in Ca^{2+} entry under Cu^{2+} stress, but mainly to potentiate the release of Ca^{2+} from the vacuole via the Yvc1 channel. As in the case of other stress conditions (Pinontoan et al. 2002; Ene et al. 2015; Rigamonti et al. 2015), Cch1 activation by Cu^{2+} was not significantly dependent on Mid1, as it had been reported for Cch1 activation by H_2O_2 (Popa et al. 2010). Since Cu^{2+} can generate reactive oxygen species (H_2O_2, for instance, by reacting with the superoxide anion radical), it is highly probable that the effect of Cu^{2+} on Ca^{2+} release is indirect, through generation of reactive oxygen species which trigger the Ca^{2+} release into the cytosol. In this line of evidence, other divalent cations (Co^{2+}, Ni^{2+}, Zn^{2+}) failed to elicit Ca^{2+}-dependent responses (Ruta et al. 2014). On the other hand, other redox-active metals such as Mn^{2+} or Fe^{3+} were also inactive in inducing $[Ca^{2+}]_{cyt}$ waves (Ruta et al. 2014; data not shown), probably because these metals are less redox reactive than the Cu^{2+}/Cu^{+1} couple is under aerobic conditions (Shi et al. 2003).

Generation of broad $[Ca^{2+}]_{cyt}$ elevations did not improve the tolerance to high Cu^{2+}; the longer Ca^{2+} lingered in the cytosol, the more sensitive the cells became, such as was the case of *mid1Δ* cells (Fig. 2c, e). The elevations of $[Ca^{2+}]_{cyt}$ in response to high Cu^{2+} probably resulted in calcineurin activation, which in turn activated processes deleterious for cell survival under high Cu^{2+}. This would explain why cells defective in calcineurin activity, such as the *cnb1Δ* cells, gain tolerance to high Cu^{2+}. In this line of evidence, the absence of $[Ca^{2+}]_{cyt}$ elevation in *yvc1Δ* cells as response to high Cu^{2+} (Fig. 2d) keeps the calcineurin inactive and the *yvc1Δ* cells Cu^{2+}-tolerant (Fig. 2e). It was shown that calcineurin activity is reduced by Nmt1-dependent myristoylation of the regulatory subunit Cnb1 in response to submaximal Ca^{2+} signals in order to prevent constitutive phosphatase activity (Connolly and Kingsbury 2012). This would explain why *cch1Δ* and *mid1Δ* mutants would have opposite tolerance to high Cu^{2+}: in *cch1Δ*, the low-intensity Ca^{2+} signal may render calcineurin inactive and the cells slightly more tolerant to Cu^{2+}. In this way, the regulation of Cu^{2+} tolerance appears as a complex and finely tunable process depending both on the broadness and on the intensity of the Ca^{2+} signal. In either direction, the adaptation to high Cu^{2+} was favored when the oxidative state of the cell shifted to pro-oxidant (for instance, by deleting *YAP1* gene or by adding H_2O_2 to the growth medium). In this case, the cells gained tolerance to high Cu^{2+} not only by limiting the amount of available Cu^{1+}, but also by potentiating the cell ability to respond to high Cu^{2+} through rapid and sharp $[Ca^{2+}]_{cyt}$ pulses, leading the cells toward the adaptation alternative.

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