Role of the yeast multidrug transporter Qdr2 in cation homeostasis and the oxidative stress response.

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Abstract:

We have identified QDR2 in a screening for genes able to confer tolerance to sodium and/or lithium stress upon overexpression. Qdr2 is a multidrug transporter of the major facilitator superfamily, originally described for its ability to transport the antimalarial drug quinidine and the herbicide barban. In order to identify its physiological substrate, we have screened for phenotypes dependent on QDR2 and found that Qdr2 is able to transport monovalent and divalent cations with poor selectivity, as shown by growth tests and the determination of internal cation content. Moreover, strains overexpressing or lacking QDR2 also exhibit phenotypes when reactive oxygen species producing agents, such as hydrogen peroxide or menadione, were added to the growth medium. We have also found that the presence of copper and hydrogen peroxide repress the expression of QDR2. In addition, the copper uptake of a qdr2 mutant strain is similar to a wild type, but the extrusion is clearly impaired. Based on our results, we propose that free divalent copper is the main physiological substrate of Qdr2. As copper is a substrate for several redox reactions that occur within the cytoplasm, this function in copper homeostasis explains its role in the oxidative stress response.
Introduction:

The yeast overexpression approach has been a powerful technique to identify the genes defining the molecular mechanisms underlying ion homeostasis in yeast (reviewed in Arino et al., 2010). This technique has allowed the identification of the HAL genes, that comprise regulators of potassium transport such as HAL1 (Gaxiola et al., 1992) and HAL3 (Ferrando et al., 1995), the HAL4 and HAL5 protein kinases (Mulet et al., 1999), targets of ion toxicity as HAL2 (Murguia et al., 1995) and the HAL6-10 transcription factors (Mendizabal et al., 1998), among them the calcineurin dependent transcription factor CRZ1/HAL8/TCN1 (Matheos et al., 1997; Statopoulos and Cyert, 1997). This technique has proven to be very powerful to identify genes encoding for soluble proteins, but has been less successful in identifying genes encoding transporters or membrane proteins in general. This could be due to some technical problems as genes encoding membrane proteins are usually under-represented in cDNA or genomic libraries (our unpublished observations). The main transporters determining ion homeostasis in Saccharomyces cerevisiae are the proton pump ATPase Pma1 (Serrano et al., 1986), responsible for the creation of the proton gradient, and the high affinity potassium transport system encoded by the TRK1 and TRK2 genes (Gaber et al., 1988).

This system is responsible of maintaining the internal content of potassium around 100-200 mM independently of the potassium concentration in the medium, and therefore is the main consumer of the membrane potential generated by Pma1 (Madrid et al., 1998).

Sodium and lithium are toxic for Saccharomyces cerevisiae. The main protein responsible for extrusion of these toxic cations from the cytoplasm is Ena1 (Haro et al., 1991). In addition, the plasma membrane sodium/proton antiporter Nha1 (Prior et al., 1996; Kinclova-Zimmermannova et al., 2006) participates in sodium extrusion at acidic pH and the sodium/proton antiporter Nhox1 localized in the prevacuolar compartment is the major transporter involved in sodium compartmentalization (Nass and Rao, 1998).

Even with this apparent negative selection against membrane proteins, in a screening for yeast genes able to confer salt tolerance upon overexpression, we have identified QDR2, a multidrug resistance gene belonging to the major facilitator superfamily (MFS) (Gofféau et al., 1997). We isolated QDR2 in our screening based on its ability to improve growth under sodium stress. MFS transporters are ubiquitously present in eukaryote and bacterial genomes, and can function as proton-gradient coupled antiporters, uniporters or symporters (Pao et al., 1998). In most cases the multidrug resistance family encodes transport systems which drive the extrusion of hydrophobic molecules, most of them not present in the natural environment of the organism. The Qdr2 protein is localized in the plasma membrane and sequence prediction indicates that it contains 12 transmembrane segments. The QDR2 gene belongs to the DHA1 family and is not conserved in related yeasts such as Ashbya gossypii or Kluuyveromices lactis (Gbelska et al., 2006). Qdr2 was originally identified for its ability to confer tolerance to the antimalarial drug quinidine and the herbicide barban (Vargas et al., 2004). A later report indicated that Qdr2 can also transport the anticancer agents cisplatin and bleomicin (Tenreiro et al., 2005). None of these molecules are present in the environment, so the physiological function of Qdr2 remains to be determined. It has been proposed that MFS transporters could also participate in ion homeostasis.

Specifically, it has been proposed that some MFS proteins may contribute to sodium extrusion (Krulwich et al., 2005). Qdr2 has also been proposed to have a role in potassium homeostasis (Vargas et al., 2007). In addition, the four identified substrates for Qdr2 are positively charged at physiological pH, suggesting that the physiological
role of Qdr2 may be related to cation homeostasis. In these report, we present evidence
that Qdr2 is able to transport monovalent and divalent cations, including transition
metals, among them, copper. In the environment copper is usually found as Cu$$^{2+}$$, owing
mainly to the fact that Cu$$^{+}$$ is very insoluble and is oxidized by O$_2$, and thus, its
bioavailability is low. Extracellular copper is reduced by the Ftr1/2 iron reductase
system, then Cu$$^+$$ is transported to the cytoplasm by Ctr1 (Puig and Thiele, 2002).
Copper is an essential micronutrient for yeast, as it is incorporated in the metallic core
of antioxidant enzymes, such as Sod1, and is also present in some subunits of the
mitochondrial cytochrome c oxidase (reviewed in Bleackley and MacGillivray, 2011).
Another feature of copper is that the redox pair of Cu$$^+$$ and Cu$$^{2+}$$, ranging from +0,2 to
+0,8 is extremely useful for biological reactions (Frausto da Silva and Williams, 2001).
but, on the other hand, these redox reactions can lead to the formation of hydroxyl
radicals through the Fenton reaction (Valko et al., 2005). Copper homeostasis should be
tightly controlled, as it can be very toxic due to unspecific binding to sulphur, oxygen
and imidazole ligands (Culotta, 2010). Our data indicates that Qdr2 extrudes divalent
copper. This is, to date, the first description of a yeast protein able to extrude copper.
Previous reports have shown that Qdr2 is able to transport non-physiological substrates,
or potassium under very particular conditions. Here we propose that copper is the main
physiological substrate of Qdr2. As copper is a substrate for some deleterious redox
reactions that can occur inside the cell, this role in copper homeostasis also relates Qdr2
to redox homeostasis.
Materials and Methods:

Yeast strains and culture conditions:
Standard methods for yeast culture and manipulation were used (Guthrie and Fink, 1991). The BY4741 strains lacking QDR2 or QDR1 were obtained from the Euroscarf collection (Frankfurt, Germany). YPD medium contained 2% glucose, 2% peptone, and 1% yeast extract. SD medium (synthetic minimal medium) contained 2% glucose, 0.7% yeast nitrogen base (Difco) without amino acids, 50 mM succinic acid adjusted to pH 5.5 with Tris, and the amino acids, purine and pyrimidine bases required by the strains. Growth assays were performed on solid media by spotting serial dilutions of saturated cultures onto plates with the indicated composition. The indicated salts were added at the indicated concentration in each case, with the exception of H₂O₂, and menadione, that were added after autoclaving.

Isolation of QDR2 and plasmid construction:
The screen for tolerance to sodium and lithium has been described previously (Mulet et al., 1999). QDR2 was isolated from the genomic clone PM54 as a Bgl II fragment that contained the full ORF YIL121w, comprising 1107 bp before the start codon and 299 bp after the stop codon, and subcloned into the Bam HI site of YEp351 (2 µm origin, LEU2 marker) (Hill et al., 1986), provisionally named HAL11, but renamed QDR2 after the publication of (Vargas et al., 2005).

For analysis of QDR2 expression using the Lac-Z reporter gene, we amplified 611 bp of the promoter region of QDR2 with Primer Prom QDR2D upstream (5'-CTC AAG CTT TCC CAC ATG ACG TGC AG; Hind III site underlined) and Primer Prom QDR2R downstream (5'-CCC AAG CTT GCC ATC GTT GCA GTAC; EcoR I site underlined), digested and ligated into the Hind III site of plasmid pYIp355 (ampicillin resistance in bacteria and URA3 complementation in yeast; Myers et al, 1986). The resulting plasmid was named JM214.

Measurement of Intracellular cation concentrations:
Cells were grown in YPD to an absorbance at 660 nm of 0.6 to 0.7, centrifuged for 5 min at 1.900 X g, resuspended at the same concentration in YPD containing the indicated chemical at the indicated concentration and incubated at 30 ºC for 90 minutes. Aliquots were taken, centrifuged in plastic tubes for 5 min at 2.000 rpm and 4 ºC and washed twice with 10 ml of ice cold solution of 20 mM MgCl₂. The cell pellets were resuspended in 0.5 ml of 20 mM MgCl₂. Ions were extracted by heating the cells for 15 min at 95 ºC. After centrifugation, aliquots of the supernatant were analyzed with an atomic absorption spectrometer (SensAA) in flame emission mode. For the copper extrusion assays strains were incubated with the indicated amounts of copper for 120 minutes. At that point aliquots were taken to determine the copper content at time 0 and the rest of the culture was washed twice with 20 mM MgCl₂ and transferred to fresh YPD medium. Aliquots were taken at the indicated times and treated as explained previously. Copper was measured in a plasma emission spectrophotometer (Shimadzu).

β-Galactosidase assays
Plasmid JM214, digested with Nco I, was integrated by homologous recombination in the URA3 locus of the BY4741 yeast strain. Three independent colonies were used for
analysis. Cultures were incubated for 1.5 h after addition of the mentioned chemical. β-Galactosidase activity was measured in permeated cells as described previously (Rios et al., 1997). Units of activity were normalized to cell density.
Results:

Qdr2 confers tolerance to sodium and lithium

We have screened for yeast genes able to confer tolerance upon overexpression. In the past, this strategy has been useful to identify determinants for ion homeostasis, such as the HAL genes (Arino et al., 2010, and references within). This technique has also been useful to screen for genes from other organisms, such as plants (Mulet et al., 2004; Serrano et al., 2003). Despite the amount of published data, some identified genes remain uncharacterized. We screened 200,000 independent colonies of yeast transformed with an episomal plasmid containing Saccharomyces cerevisiae genomic fragments. A fragment containing QDR2 was isolated from four independent clones for its ability to confer tolerance to lithium and sodium. Only QDR2 (YIL121w) was complete in the 4 different clones. This gene shares 70% homology with QDR1 (YIL120w), which was also present in some of the isolated clones. Therefore, we subcloned both and compared their ability to confer tolerance to sodium or lithium upon overexpression. Only QDR2 was responsible for the salt tolerance phenotype, as overexpression of QDR1 did not confer sodium or lithium tolerance (Fig. 1). The original screening was performed in the RS16 genetic background (Gaxiola et al., 1992). In order to assess whether the phenotype was reproducible in different genetic backgrounds, we transformed different yeast strains with the plasmid overexpressing QDR2. We could reproduce the observed tolerance to sodium and lithium in W303-1A (data not shown) and in BY4741 (Brachmann et al., 1998) (Fig. 2). The P-type ATPase ENA1 is the main transporter responsible for sodium and lithium extrusion from the cytoplasm in S. cerevisiae (Haro et al., 1991). This gene belongs to a family composed by three or four members (depending on the strain) located in tandem in the yeast genome. In order to determine whether the observed sodium and lithium tolerance could be due to an indirect effect on ENA1, we transformed a SKY697 strain (Ferrando et al., 1995) which has a complete deletion of the four ENA genes. We could also observe tolerance in this genetic background (data not shown). In addition overexpression of QDR2 had no effect on ENA1 expression under normal conditions or after induction with sodium or lithium (data not shown), so the sodium and lithium phenotype is independent of the main extrusion pump for sodium and lithium, Ena1.

Qdr2 transports lithium, but is not essential for monovalent cation homeostasis

After confirming that the salt tolerance phenotype was reproducible in different strains and independent of ENA1, we investigated whether QDR2 function was essential for tolerance to monovalent toxic cations by investigating the phenotypes of a qdr2 strain. A qdr2 strain showed a very weak sensitivity phenotype when grown in the presence of monovalent toxic cations (Fig. 2A). We also analyzed the qdr1 mutant strain under the same conditions, but growth was similar to the wild type control strain in all conditions assayed (data not shown). We also tried to understand the mechanism of tolerance determined by QDR2. The most obvious explanation for the observed tolerance is that Qdr2 is transporting toxic cations outside the cell. We grew different strains in medium containing LiCl and our results indicate that cells lacking QDR2 accumulate more lithium and cells overexpressing QDR2 accumulate less than control cells, indicating that Qdr2 is transporting lithium (Fig. 2B).
Qdr2 has a role in divalent cation homeostasis.

In order to investigate the spectrum of cations transported by Qdr2, we tested other toxic cations and we found phenotypes related to transition metals such as nickel, manganese and copper. Overexpression of \textit{QDR2} confers tolerance to Ni\(^{2+}\) and Mn\(^{2+}\) (Fig. 3A), although we could not observe any sensitivity in the mutant strain. Ion content analysis showed small differences (data not shown). We could not observe any clear phenotype upon overexpression of \textit{QDR2} in copper containing medium, but the \textit{qdr2} mutant strain was very sensitive to this cation. This result suggests that Qdr2 has a role in divalent cation extrusion (Fig. 3A). We also investigated copper content after a 90’ incubation. Internal content between wild type and the strain overexpressing \textit{QDR2} was similar, confirming the observed phenotype that overexpression of \textit{QDR2} does not confer tolerance, but the mutant strain accumulated about 50% more than copper than the wild type (Fig. 2B).

We further investigated whether Qdr2 could contribute to homeostasis of essential divalent cations, such as calcium or magnesium. We did not observe any difference in growth in the presence of excess magnesium or calcium, or differences in internal content (data not shown). These results do not discard that Qdr2 could have a role in conditions with limiting calcium or magnesium. For this purpose, we compared the growth of different strains in the presence of the divalent cation chelators Ethylene diamine tetra-acetic acid (EDTA) or ethylene glycol tetra-acetic acid (EGTA). Under these conditions the mutant strains showed better growth that wild type or strains overexpressing \textit{QDR2}, suggesting that Qdr2 could take part in calcium or magnesium extrusion (Fig 3C).

Qdr2 can transport divalent cations inside the cell.

Interestingly, when we investigated the spectrum of divalent cations transported by Qdr2, we found that some transition metals produced different results. We found that strains defective for \textit{qdr2} were slightly tolerant to cadmium and cobalt. We did not observe any phenotype upon overexpression of \textit{QDR2} (Fig. 4A). We performed most of our experiments in rich media (YPD) that does not select for the plasmid. Under normal conditions YEp351, a 2 micron derivative yeast episomal plasmid used in this study is very stable (Hill \textit{et al.}, 1986). However, when this plasmid contains a gene whose expression has some deleterious effect, a negative selection can occur, such that strains that have lost the plasmid or express less of the inserted gene are selected. To test whether the lack of phenotype in strains overexpressing \textit{QDR2} was due to a negative selection, we used minimal SD media without leucine, to prevent the growth of yeast colonies without plasmid. Under these conditions strains overexpressing \textit{QDR2} grew less than control strains in the presence of cobalt, indicating that \textit{QDR2} overexpression is deleterious under these growth conditions (Fig. 4B). To assess whether this effect could be related to transport or whether it is an indirect effect, we measured the accumulation of this cation in cells grown in the presence of cobalt. We observed that the \textit{qdr2} mutant accumulates less cobalt that wild type control cells (Fig. 4C).
**QDR2 expression is repressed by copper and by hydrogen peroxide**

In order to investigate the regulation of *QDR2*, we constructed a plasmid containing the Lac-Z reporter gene (Myers *et al.*, 1986) expressed under the control of the *QDR2* promoter. We have shown that Qdr2 is involved in monovalent and divalent cation homeostasis, with poor selectivity. So first we tested changes in expression after treatments with different toxic cations, but we did not observe any significant differences, with the exception of copper, where we could observe an approximately 10 fold repression (Fig. 5B). This observation suggests that the physiological role of *QDR2* is deleterious in the presence of copper, an apparent discrepancy with the fact that a qdr2 mutant strain is very sensitive to copper. Monovalent copper is insoluble, so copper is present in the medium as a divalent cation. Divalent copper is reduced in the extracellular matrix by the Cu-Fe reductase Fre1. Monovalent copper is then transported inside the cell by the high affinity transporters Ctr1 and Ctr2 (Dancis *et al.*, 1994). Besides being a micronutrient, intracellular Cu\(^{2+}\) pools must be tightly controlled, as an excess of this cation can lead to toxicity through the formation of oxygen radicals via de Fenton reaction (Valko *et al.*, 2005). Specifically, Cu\(^{2+}\) can react with hydrogen peroxide to produce the hydroxyl radical and Cu\(^{2+}\). Alternatively, Cu\(^{2+}\) could react with the superoxide anion via the Haber-Weiss reaction to form molecular oxygen and Cu\(^{+}\). The involvement of copper cations in these classical biominorganics chemistry reactions could provide a hint to understand the physiological role of Qdr2. In the presence of hydrogen peroxide Cu\(^{+}\) will produce hydroxyl radicals, deleterious for the cell, and thus compromising H\(_2\)O\(_2\) detoxification by catalases or glutathione peroxidases. If Qdr2 is extruding Cu\(^{2+}\) from the cell, this could increase the rate of Fenton reaction by eliminating one of the products. If this hypothesis is correct, we would predict that Qdr2 would be deleterious in the presence of H\(_2\)O\(_2\). As indicated in Fig. 5A, qdr2 strain grows better than the wild type control strain and *QDR2* expression is repressed in the presence of H\(_2\)O\(_2\) (Fig. 5B). Intracellular Cu\(^{2+}\) can also induce the formation of molecular oxygen via the Haber-Weiss reaction, using the superoxide anion as a substrate. If Qdr2 is extruding Cu\(^{2+}\) from the cytoplasm, the deleterious effect of Haber-Weiss reaction will be diminished, as Qdr2 will eliminate the substrate from the cytoplasm. We used menadione as a superoxide generator (Castro *et al.*, 2008) and found that overexpression of *QDR2* confers tolerance to menadione (Fig. 5A), and we did not observe a significant decrease in expression of *QDR2* upon a treatment with menadione (Fig. 5B).

**Extrusion of copper depends on QDR2**

We have found that copper is the only cation that regulates *QDR2* expression and that a qdr2 mutant strain is sensitive to copper. These results suggest that cytoplasmic Cu\(^{2+}\) should be the most relevant physiological substrate of Qdr2. To confirm this hypothesis we have measured copper uptake and copper extrusion in qdr2 mutants. A qdr2 mutant accumulates more copper than its parental wild type, but uptake at short times is undistinguishable, suggesting that uptake rate is similar and the difference is the extrusion rate (Fig. 6A). To confirm this hypothesis we evaluated the copper extrusion in qdr2 cells. We incubated wild type cells with 12.5 mM CuSO\(_4\) and qdr2 mutant with 10 mM in order to attain a similar level of intracellular copper at time 0. Wild type cells could extrude copper, but this extrusion was impaired in qdr2 cells (Fig. 6B).
Discussion:

Qdr2 is a plasma membrane protein which belongs to the Major Facilitator Superfamily, a family described as H+/chemical transport proteins (Goffau et al., 1997). Qdr2 was originally characterized based on its ability to transport quinidine, cisplatine, bleomicin and barban (Vargas et al., 2004; Tenreiro et al., 2005). None of these substrates is physiological, nor is present in the natural environment of Saccharomyces cerevisiae. Thus, it is unlikely that the main function of Qdr2 is related to any of these molecules.

Qdr2 has also been related to potassium transport (Vargas et al., 2007). The transporters that have a prominent role in potassium homeostasis in yeast have been well-studied (reviewed in Arino et al., 2010) and it is clear that the contribution of Qdr2 to this process in yeast cells is very minor and only apparent in the absence of the major potassium transporters Trk1 and Trk2. There are reports indicating that members of the MFS can act as H+/Na+ antiporters (Krulwich et al., 2005). Taken together, this published data indicates that the substrate selectivity of Qdr2p is low, but none of the published evidence indicates what is likely to be the physiological substrate of Qdr2. In this report we try to bring some light to this question.

We have identified QDR2 in a screening for genes able to confer tolerance to salt stress upon overexpression. This phenotype is reproducible in different genetic backgrounds and pleiotropic to toxic monovalent cations such as sodium, lithium or cesium. In addition, our data also indicate that Qdr2 is participating in the homeostasis of divalent cations, such as manganese, nickel and copper. The presence of EDTA or EGTA in the medium is deleterious for the cell because of its ability to sequester divalent cations, among them, the essential oligoelements calcium and magnesium. Deletion of the QDR2 gene confers a growth advantage under these conditions, indicating that it could also be involved in the efflux transport of these essential cations. Interestingly strains overexpressing QDR2 or mutants for qdr2 behave in a different way when cobalt is present in the growth medium. We observe changes in cobalt accumulation dependent on the genetic dosage of QDR2, indicating that cobalt can enter the cell in a QDR2-dependent manner. It is difficult to assume that the physiological role of QDR2 could involve the transport of divalent cations in both directions, so probably cobalt induces some kind of change in Qdr2 structure or even an inactivation or a deregulation of the protein, but the pore could be used by cobalt to enter the cell in an unspecific way taking advantage of the electrochemical gradient.

An important hint to understand the physiological function of Qdr2 was provided by investigating the phenotype under oxidative stress conditions. We have shown that Qdr2 activity is deleterious in the presence of hydrogen peroxide. Deletion of QDR2 confers tolerance to oxidative stress, and this effect seems to be physiological, as the expression of the reporter gene Lac Z driven by the QDR2 promoter indicated that treatment with hydrogen peroxide induces a 10 fold decrease in the expression level of QDR2. We only observed a similar phenotype upon treatment with copper. Cu^{2+} enters the cell as Cu^{+}. The presence of Cu^{+} as a free cation in the cytoplasm is very limited and the window between copper starvation and copper excess is very narrow (Wegner et al., 2011). Accordingly, copper homeostasis must be tightly regulated, as deregulation of copper homeostasis can lead to toxicity. Free Cu^{+} can participate in the Fenton reaction. An increase in the rate of this reaction by the presence of Cu^{+} and H_{2}O_{2} in the cytoplasm increases the amount of hydroxyl radicals, and competes with the detoxification
mechanisms driven by enzymes such as catalases. If Qdr2 is extruding one of the products of the reaction ($\text{Cu}^{2+}$) this would increase the reaction rate, and therefore, increase the toxicity (Valko et al., 2005). Therefore if $\text{Cu}^{2+}$, and probably other divalent cations are the physiological substrates of Qdr2, it is logical that under these conditions a decrease in its expression would enhance the oxidative stress response. On the other hand, and further confirming this hypothesis, the effect of the overexpression of QDR2 is the opposite when menadione is added to the medium. Once in the yeast cytoplasm, menadione can induce the production of several reactive oxygen species (Castro et al., 2008), among them the superoxide anion $\text{O}_2^{-}$. Extrusion of $\text{Cu}^{2+}$ by Qdr2 would impair the Haber-Weiss reaction by eliminating one of the substrates from the medium, and thus impair the production of molecular oxygen as a result of the mentioned reaction. As shown in Fig. 5A overexpression of QDR2 confers tolerance to menadione. Considering copper homeostasis together with oxidative stress explains the apparent discrepancy between the observations that Qdr2 transports copper whereas addition of copper blocks its expression. Qdr2 appears to act as a $\text{Cu}^{2+}$ extrusion system under normal conditions. An increase of copper or an increase of $\text{H}_2\text{O}_2$ blocks its expression, presumably to avoid the deleterious effects of the Fenton reaction and the production of hydroxyl radicals. We have confirmed this by determining copper uptake and copper extrusion in qdr2 mutants. While copper uptake in the qdr2 mutant is similar that of a wild type, extrusion is impaired in this mutant, pointing out that Qdr2p is extruding copper in vivo (fig. 6). Another fact supporting this model is that QDR2 overexpression has no growth phenotype in copper medium. Copper content is also similar to a wild type (Fig. 3B), copper uptake and extrusion kinetic of strains overexpressing QDR2 is also similar to a wild type (data not shown). This suggests that under copper stress an increase of the protein could be deleterious so there are mechanisms (mainly transcriptional) preventing an increase of QDR2 activity under these conditions. Using the model proposed in Figure 7, we can explain the phenotypes observed with cadmium. We could not detect any change in cadmium content depending on QDR2, but the deletion of QDR2 conferred tolerance to this metal. Cadmium is a strong oxidant. As mentioned above, Qdr2 can compete with the oxidative stress response through its effect on copper homeostasis, favouring the production of hydroxyl radicals. So the observed phenotypes with cadmium would be an indirect effect and not the result of a direct transport of this cation. Therefore, based on the results presented in this report, we propose that the physiological role of Qdr2 is the extrusion of $\text{Cu}^{2+}$ originated from the oxidation of $\text{Cu}^{+}$ in the cytoplasm. This is the first description of a yeast protein able to extrude copper from the cytoplasm. The P-type ATPase Ccc2 is able to transport copper to internal compartments (Yuan et al., 1997). The P-type plasma membrane ATPase Pca1 was originally suggested to be responsible of copper extrusion (Rad et al., 1994), but later reports indicated that Pca1 transports cadmium rather than copper (Shiraiishi et al., 2000). Pca1 binds copper with high affinity, but is not active in copper ion transport, so the main contributions of Pca1 to copper homeostasis would be the chelation and sequestration of copper ions (Adle et al., 2007). In addition, previous reports have shown that Qdr2 is able to transport non-physiological substrates or potassium under very specific conditions. Here we propose that copper is the main physiological substrate of Qdr2. As copper is a substrate for some deleterious redox reactions that can occur in the cytoplasm such as the Fenton reaction this role in copper homeostasis explains the oxidative stress related phenotypes that we have observed in qdr2 mutants.
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Figure legends:

Figure 1. Overexpression of QDR2 confers tolerance to monovalent toxic cations. Cultures of the strains transformed with the empty episomal plasmid (RS16), with the plasmid containing one of the genomic fragments originally isolated in the screening (PM54), and with the episomal plasmid containing QDR2 and its promoter and terminator sequence (QDR2), and with the episomal plasmid containing QDR1 with the promoter and terminator sequence (QDR1), were grown in selective SD medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentration of sodium or lithium. Growth was recorded after 4 days.

Figure 2. Tolerance conferred by overexpression of QDR2 is independent of the genetic background and correlates with the internal ion content. (A) Cultures of the BY4741 strain (wt), the BY4741 strain overexpressing QDR2 (YEpQDR2) or BY4741 with a complete deletion in the QDR2 gene (qdr2) were grown in selective medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentration of sodium, lithium or cesium and growth was recorded after 4 days. (B) QDR2 affects lithium accumulation. The indicated strains were grown overnight in YPD and transferred to fresh YPD in the presence of 0.4 M LiCl. After 90 minutes, cells were collected and internal lithium content was determined. Results are the averages of six determinations and the error bar represents standard deviations.

Figure 3. Qdr2 can transport divalent cations. (A) Cultures of the wild type control strain (wt), and strains overexpressing QDR2 (YEpQDR2) or lacking the QDR2 gene (qdr2) were grown in selective medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentration of manganese, nickel or copper and growth was recorded after 4 days. (B) The indicated strains were grown overnight in YPD and transferred to fresh YPD with the presence of 12.5 mM CuSO4. After 90 min. cells were collected and internal copper content was determined. Results are the averages of six determinations, and the error bar represents standard deviations. (C) Cultures of the wild type control strain (wt), and strains overexpressing QDR2 (YEpQDR2) or lacking the QDR2 gene (qdr2) were grown in selective medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentration of EDTA or EGTA. Growth was recorded after 4 days.

Figure 4. Qdr2 can participate in the uptake of cadmium and cobalt. (A) Cultures of the strains transformed with the empty plasmid (wt), overexpressing QDR2 (YEpQDR2) or lacking the QDR2 gene (qdr2) were grown in selective medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentrations of cadmium or cobalt. Growth was recorded after 4 days. (B) Overexpression of QDR2 is deleterious in the presence of cobalt. Cultures of the strains transformed with the empty plasmid (wt) or overexpressing QDR2 (YEpQDR2) were grown in selective medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto SD medium containing the indicated concentration of cobalt and growth was recorded after 4 days. (C) A qdr2 mutant accumulates less cobalt. The indicated strains were grown overnight in YPD and transferred to fresh YPD with the presence of 5 mM CoCl2. After 90 minutes, cells were
collected and internal cobalt content was determined. Results are the average of six independent determinations. The error bar represents standard deviations.

**Figure 5.** Qdr2 affects tolerance to oxidative stress. (A) Cultures of the wild type strain (wt), and strains overexpressing QDR2 (YEpQDR2) or with a complete deletion of the QDR2 gene (qdr2) were grown in selective medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentration of H2O2 or menadione. Growth was recorded after 4 days. (B) Expression of QDR2 is inhibited by copper and H2O2. Strains were incubated for 30 minutes with 12.5 mM CuSO4, 2 mM H2O2 and 175 μM menadione. Results are the average of six independent determinations. The error bar represents standard deviations.

**Figure 6.** Qdr2 extrudes copper. (A) Copper uptake kinetics of qdr2 is similar to a wild type strain. Cultures of the wild type strain (wt), and with a complete deletion of the QDR2 gene (qdr2) were grown in YPD, at time 0 12.5 mM of CuSO4 was added. Aliquots were extracted at the indicated times and copper content was determined. (B) Copper extrusion kinetics is defective in a qdr2 mutant. Strains were incubated for 90 minutes, wild type with 12.5 mM CuSO4 and qdr2 with 10 mM CuSO4. At time 0 cells were washed and transferred to fresh YPD medium. Aliquots were extracted at the indicated times and copper content was determined. Results are the average of three independent determinations. The error bar represents standard deviations.

**Figure 7.** Proposed model for the role of Qdr2 in copper homeostasis and oxidative stress. The proposed function of Qdr2 is the extrusion of Cu2+, that can be produced as a result of the Fenton reaction between Cu2+ and H2O2.
References:


