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* (Murguía *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; Stathopoulos 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 Nhx1 localized in the prevacuolar compartment is the major transporter involved in sodium compartimentalization (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) (Goffeau *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 gossipii* or *Kluyveromices 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 bioavalibility 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 H_2O_2, 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; *Hin*d III site underlined) and Primer Prom *QDR2R* downstream (5'-CCC AAG CTT GCC ATC GTT GCA GTAC; *Eco*R I site underlined), digested and ligated into the *Hin*d 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 $MgCl_2$. The cell pellets were resuspended in 0.5 ml of 20 mM $MgCl_2$. 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 $MgCl_2$ 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 *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 *QDR2* in copper containing medium, but the *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 *QDR2* was similar, confirming the observed phenotype that overexpression of *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 *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 *qdr2* were slightly tolerant to cadmium and cobalt. We did not observe any phenotype upon overexpression of *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 *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 *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 *QDR2* grow less than control strains in the presence of cobalt, indicating that *QDR2* overexpression is deleterious under these growth conditions (Fig. 4B). To asses 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 *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^{+} 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^{+} 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 bioinorganic 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_2O_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_2O_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_2O_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 Falicitator Superfamily, a family described as H^+/chemical transport proteins (Goffeau *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_2O_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 (Cu^{2+}) this would increase the reaction rate, and therefore, increase the toxicity (Valko *et al.*, 2005). Therefore if 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 $O_2^{\cdot-}$. Extrusion of 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 Cu^{2+} extrusion system under normal conditions. An increase of copper or an increase of H_2O_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 Cu^{2+} originated from the oxidation of 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 (Shiraisi *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 homeostais 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* (YEp*QDR2*) 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 overexpresing *QDR2* (YEp*QDR2*) 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 $CuSO_4$. 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* (YEp*QDR2*) 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* (YEp*QDR2*) 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 $CoCl_2$. 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* (YEp*QDR2*) 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 H_2O_2 or menadione. Growth was recorded after 4 days. (B) Expression of *QDR2* is inhibited by copper and H_2O_2. Strains were incubated for 30 minutes with 12.5 mM $CuSO_4$, 2 mM H_2O_2 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 $CuSO_4$ 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 $CuSO_4$ and *qdr2* with 10 mM $CuSO_4$. 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 Cu^{2+}, that can be produced as a result of the Fenton reaction between Cu^{2+} and H_2O_2.

References:

Adle, D.J., Sinani, D., Kim, H. & Lee, J. (2007) A cadmium-transporting P1B-type ATPase in yeast *Saccharomyces cerevisiae*. J Biol Chem **282:** 947-55.

Arino, J., Ramos, J. & Sychrova, H. (2010) Alkali metal cation transport and homeostasis in yeasts. Microbiol. Mol. Biol. Rev. **74:** 95-120.

Bleackley, M.R. & MacGillivray, R.T. (2011) Transition metal homeostasis: from yeast to human disease. Biometals **24:** 785-809.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P. & Boeke, J.D. (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115-132.

Castro, F.A., Mariani, D., Panek, A.D., Eleutherio, E.C. & Pereira, M.D. (2008) Cytotoxicity mechanism of two naphthoquinones (menadione and plumbagin) in *Saccharomyces cerevisiae*. PLoS. One. **3:** e3999.

Culotta, V. (2010) Cell biology of copper. J. Biol. Inorg. Chem. 15: 1-2.

Dancis, A., Haile, D., Yuan, D.S. & Klausner, R.D. (1994) The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. J. Biol. Chem. **269:** 25660-25667.

Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J. & Klausner, R.D. (1994) Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. Cell **76:** 393-402.

Ferrando, A., Kron, S.J., Rios, G., Fink, G.R. & Serrano, R. (1995) Regulation of cation transport in *Saccharomyces cerevisiae* by the salt tolerance gene *HAL3*. Mol. Cell Biol. **15:** 5470-5481.

Frausto d Silva, J. & Williams RJP. (2001) Copper: extracytoplasmic oxidases and matrix formation. In: The biological chemistry of the elements (Frausto d Silva, J. & Williams RJP). Oxford University Press, Oxford.

Gaber, R.F., Styles, C.A. & Fink, G.R. (1988) *TRK1* encodes a plasma membrane protein required for high-affinity potassium transport in *Saccharomyces cerevisiae*. Mol. Cell Biol. **8:** 2848-2859.

Gaxiola, R., de Larrinoa, I.F., Villalba, J.M. & Serrano, R. (1992) A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. EMBO J. **11:** 3157-3164.

Gbelska, Y., Krijger, J.J. & Breunig, K.D. (2006) Evolution of gene families: the multidrug resistance transporter genes in five related yeast species. FEMS Yeast Res. **6:** 345-355.

Goffeau, A., Park, J., Paulsen, I.T., Jonniaux, J.L., Dinh, T., Mordant, P. & Saier, M.H., Jr. (1997) Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of yeast open reading frames with the major facilitator superfamily. Yeast **13:** 43-54.

Guthrie, C. & Fink G.R. (1991) Guide to yeast genetics and molecular biology. Academic Press Inc, N.Y., New York.

Haro, R., Garciadeblas, B. & Rodriguez-Navarro, A. (1991) A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. **291:** 189-191.

Hill, J.E., Myers, A.M., Koerner, T.J. & Tzagoloff, A. (1986) Yeast/E. coli shuttle vectors with multiple unique restriction sites. Yeast **2:** 163-167.

Kinclova-Zimmermannova, O., Gaskova, D., Sychrova, H. (2006) The Na^+,K^+/H^+ - antiporter Nha1 influences the plasma membrane potential of *Saccharomyces cerevisiae*. FEMS Yeast Res. **6:** 792-800.

Krulwich, T.A., Lewinson, O., Padan, E. & Bibi, E. (2005) Do physiological roles foster persistence of drug/multidrug-efflux transporters? A case study. Nat. Rev. Microbiol. **3:** 566-572.

Madrid, R., Gomez, M.J., Ramos, J. & Rodriguez-Navarro, A. (1998) Ectopic potassium uptake in trk1 trk2 mutants of Saccharomyces cerevisiae correlates with a highly hyperpolarized membrane potential. J. Biol. Chem. **273:** 14838-14844.

Matheos, D.P., Kingsbury, T.J., Ahsan, U.S. & Cunningham, K.W. (1997) Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in Saccharomyces cerevisiae. Genes Dev. **11:** 3445-3458.

Mendizabal, I., Rios, G., Mulet, J.M., Serrano, R. & de Larrinoa, I.F. (1998) Yeast putative transcription factors involved in salt tolerance. FEBS Lett. **425:** 323-328.

Mulet, J.M., Leube, M.P., Kron, S.J., Rios, G., Fink, G.R. & Serrano, R. (1999) A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. Mol. Cell Biol. **19:** 3328-3337.

Mulet, J.M., Alejandro, S., Romero, C. & Serrano, R. (2004) The trehalose pathway and intracellular glucose phosphates as modulators of potassium transport and general cation homeostasis in yeast. Yeast **21:** 569-582.

Mulet, J.M., Alemany, B., Ros, R., Calvete, J.J. & Serrano, R. (2004) Expression of a plant serine O-acetyltransferase in Saccharomyces cerevisiae confers osmotic

tolerance and creates an alternative pathway for cysteine biosynthesis. Yeast **21:** 303-312.

Murguia, J.R., Belles, J.M. & Serrano, R. (1995) A salt-sensitive 3'(2'),5'-bisphosphate nucleotidase involved in sulfate activation. Science **267:** 232-234.

Myers, A.M., Tzagoloff, A., Kinney, D.M. & Lusty, C.J. (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions. Gene **45:** 299-310.

Nass, R. & Rao, R. (1998) Novel localization of a Na^+/H^+ exchanger in a late endosomal compartment of yeast. Implications for vacuole biogenesis. J. Biol. Chem. **273:** 21054-21060.

Pao, S.S., Paulsen, I.T. & Saier, M.H., Jr. (1998) Major facilitator superfamily. Microbiol. Mol. Biol. Rev. **62:** 1-34.

Prior, C., Potier, S., Souciet, J.L. & Sychrova, H. (1996) Characterization of the *NHA1* gene encoding a Na^+/H^+-antiporter of the yeast Saccharomyces cerevisiae. FEBS Lett. **387:** 89-93.

Puig, S., Rees, E.M. & Thiele, D.J. (2002) The ABCDs of periplasmic copper trafficking. Structure. **10:** 1292-1295.

Rad, M.R, Kirchrath, L. & Hollenberg, C.P. (1994) A putative P-type Cu^{2+} -transporting ATPase gene on chromosome II of *Saccharomyces cerevisiae*. Yeast **10:**1217-1225

Rios, G., Ferrando, A. & Serrano, R. (1997) Mechanisms of salt tolerance conferred by overexpression of the *HAL1* gene in *Saccharomyces cerevisiae*. Yeast **13:** 515-528.

Serrano, R., Kielland-Brandt, M.C. & Fink, G.R. (1986) Yeast plasma membrane ATPase is essential for growth and has homology with $(Na^+ + K^+)$, K^+- and Ca^{2+}-ATPases. Nature **319:** 689-693.

Serrano, R., Montesinos, C., Gaxiola, R., Ríos, G., Forment, J., Leube, M., Mulet, J.M., Naranjo, M.A., Roldán, M., Vicente, O., Kanhonou, R.A., Rausell, A. & Ros, R. 2003. Functional genomics of salt tolerance: the yeast overexpression approach. Acta Hort. (ISHS) **609:** 31-38.

Shiraishi, E., Inouhe. M., Joho, M. & Tohoyama, H. (2000) The cadmium-resistant gene, CAD2, which is a mutated putative copper-transporter gene (PCA1), controls the intracellular cadmium-level in the yeast *S. cerevisiae*. Curr Genet **37:** 79-86.

Stathopoulos, A.M. & Cyert, M.S. (1997) Calcineurin acts through the *CRZ1/TCN1*-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11:** 3432-3444.

Tenreiro, S., Vargas, R.C., Teixeira, M.C., Magnani, C. & Sa-Correia, I. (2005) The yeast multidrug transporter Qdr3 (Ybr043c): localization and role as a

1 determinant of resistance to quinidine, barban, cisplatin, and bleomycin.
2 Biochem. Biophys. Res. Commun. **327:** 952-959.

3 Valko, M., Morris, H. & Cronin, M.T. (2005) Metals, toxicity and oxidative stress.
4 Curr. Med. Chem. **12:** 1161-1208.

5 Vargas, R.C., Tenreiro, S., Teixeira, M.C., Fernandes, A.R. & Sa-Correia, I. (2004)
6 Saccharomyces cerevisiae multidrug transporter Qdr2p (Yil121wp): localization
7 and function as a quinidine resistance determinant. Antimicrob. Agents
8 Chemother. **48:** 2531-2537.

9 Vargas, R.C., Garcia-Salcedo, R., Tenreiro, S., Teixeira, M.C., Fernandes, A.R., Ramos,
10 J. & Sa-Correia, I. (2007) *Saccharomyces cerevisiae* multidrug resistance transporter
11 Qdr2 is implicated in potassium uptake, providing a physiological advantage to
12 quinidine-stressed cells. Eukaryot. Cell **6:** 134-142.

13 Wegner, S.V., Sun, F., Hernandez, N. & He, C. (2011) The tightly regulated copper
14 window in yeast. Chem. Commun. (Camb.) **47:** 2571-2573.

15 Yuan, D.S., Dancis, A. & Klausner, R.D. (1997) Restriction of copper export in
16 Saccharomyces cerevisiae to a late Golgi or post-Golgi compartment in the
17 secretory pathway. J Biol Chem **272:** 25787-25793.