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2 uptake in the budding yeast *Saccharomyces cerevisiae*

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16

17 Keywords: Yeast, radionuclide, uranium, metal, incorporation, accumulation, mutants

18

19 Highlights

- 20 • Living yeast *Saccharomyces cerevisiae* is able to take up U
- 21 • Availability of a metabolizable substrate stimulates U uptake
- 22 • Calcium, iron and copper inhibit U uptake
- 23 • Strains deleted in Mid1/Cch1 calcium channel and Ftr1 iron permease are  
24 affected in U uptake
- 25 • Expression of *MID1* gene in *mid1*Δ strain restore wild type levels of U uptake

26

27 **Abstract**

28 Uranium (U) is a naturally-occurring radionuclide toxic for living organisms that can  
29 take it up. To date, the mechanisms of U uptake are far from being understood. Here,  
30 we used the yeast *Saccharomyces cerevisiae* as a unicellular eukaryote model to  
31 identify U assimilation pathways. Thus, we have identified, for the first time, transport  
32 machineries capable of transporting U in a living organism. First, we evidenced a  
33 metabolism-dependent U transport in yeast. Then, competition experiments with  
34 essential metals allowed us to identify calcium, iron and copper entry pathways as  
35 potential routes for U uptake. The analysis of various metal transport mutants revealed  
36 that *mid1Δ*, *cch1Δ* and *ftr1Δ* mutants, affected in calcium (Mid1/Cch1 channel) and  
37 Fe(III) (Ftr1/Fet3 complex) transport, respectively, exhibited highly reduced U uptake  
38 rates and accumulation, demonstrating the implication of these import systems in U  
39 uptake. Finally, expression of the *Mid1* gene into the *mid1Δ* mutant restored U uptake  
40 levels of the wild type strain, underscoring the central role of the Mid1/Cch1 calcium  
41 channel in U absorption process in yeast. Our results also open up the opportunity for  
42 rapid screening of U-transporter candidates by functional expression in yeast, before  
43 their validation in more complex higher eukaryote model systems.

44

## 45 **Introduction**

46 Uranium (U) is a naturally-occurring trace metal element and radionuclide ubiquitous  
47 in the Earth crust. It is primarily redistributed in the environment by anthropogenic  
48 activities related to U mining and milling industries, civil and nuclear activities, and  
49 extensive enrichment of agricultural soils with phosphate fertilizers, which may be  
50 significantly contaminated with U. The accumulation of U in soil, water and air can pose  
51 potential risks to ecosystems, agrosystems, and ultimately human health through food  
52 chain contamination, as the radionuclide has both chemical and radiological effects.  
53 Natural U is of low radiotoxicity due to its isotopic composition (>99%  $^{238}\text{U}$ ) but the  
54 uranyl cation ( $\text{UO}_2^{2+}$ ) that is prevalent in oxidizing environments is highly chemotoxic  
55 (Gao et al., 2019; Ribera et al., 1996). Since U is not an essential element, its uptake  
56 and intracellular trafficking depends on existing metal transporters or channels of broad  
57 metal selectivity. In particular, cation transporters offer potential transport pathways for  
58 toxic metals. These transport systems may differ from a living organism to another.  
59 Even if U is known for a long time to interfere with iron, manganese, phosphate and  
60 calcium homeostasis in microorganisms, plants and animals, and despite significant  
61 efforts to decipher the mechanisms that contribute to the absorption of the radionuclide  
62 from the environment, no U entry route in the cell has been identified so far in  
63 prokaryotic or eukaryotic cells (Berthet et al., 2018; Gao et al., 2019; Khare et al., 2020)  
64 and references therein).

65 The yeast *Saccharomyces cerevisiae* has long been used as a powerful eukaryotic cell  
66 model for studying cell transport systems, in particular those related to trace elements  
67 transport and homeostasis (Eide et al., 2005). The usefulness of yeast for genome-  
68 wide studies of nutrient homeostasis markedly increased with the completion of the  
69 *Saccharomyces* Genome Deletion Project that resulted in a collection of mutant strains  
70 disrupted in most of the approximately 6,000 genes in the yeast genome (Winzeler et  
71 al., 1999). This strain collection provides a unique resource for the analysis of gene  
72 function in a eukaryotic cell. Moreover, insights from yeast can be easily translated to  
73 other organisms, making it an efficient and attractive model system. In many instances,  
74 for example, it has been shown that mammalian proteins are capable of functionally  
75 replacing yeast proteins, thereby revealing their remarkable functional conservation.  
76 Thus, yeast has allowed the identification and molecular understanding of human  
77 transporters and has provided insights into diseases that result from defects in ion

78 homeostasis (Cyert and Philpott, 2013; Laliberté and Labbé, 2008; Van Ho et al.,  
79 2002). Functional expression of plant genes in yeast also enabled the identification of  
80 numerous plant ion transporters before their validation and further characterization *in*  
81 *planta*. This includes, but is not restricted to, the wheat potassium transporter HKT1  
82 (Schachtman and Schroeder, 1994), the Arabidopsis iron transporters IRT1 (Eide et  
83 al., 1996), NRAMP1 (Curie et al., 2000), NRAMP3 and NRAMP4 (Thomine et al.,  
84 2000), the Arabidopsis MCA1 and MCA2 calcium channels (Nakagawa et al., 2007;  
85 Yamanaka et al., 2010) and the Arabidopsis copper transporter COPT1 (Kushnir,  
86 1995). Finally, a systematic approach for transforming yeast into metal  
87 hyperaccumulators was recently designed (Sun et al., 2019). This work demonstrated  
88 that yeast can be engineered to hyperaccumulate metals by overexpressing and  
89 evolving native metal transporters and engineering mechanisms for metal  
90 detoxification, allowing to consider the use of this organism as an integral tool for waste  
91 treatment processes and recycling.

92 In this study, we used *S. cerevisiae* to identify pathways for U entry into an eukaryotic  
93 cell. U has a strong propensity of biosorption to dead or living yeast cell wall, a process  
94 that do not depend on temperature and that has been studied in a bioremediation  
95 context (Lu et al., 2013; Shen et al., 2018). Very few studies, however, report on U  
96 uptake by yeast cells (Kolhe et al., 2020; Ohnuki et al., 2005; Volesky and May-Phillips,  
97 1995). Here, we developed an efficient and sensitive assay for measuring U uptake in  
98 yeast that discriminates U adsorbed on cell surface from internalized U. Uranium  
99 uptake was shown to depend on cell metabolism. By using a combination of  
100 competition experiments with metal ions and the analysis of metal transport deficient  
101 mutants, we showed that calcium, iron and copper assimilation pathways are important  
102 routes for U uptake. More precisely, we demonstrated that the high-affinity calcium  
103 channel Mid1/Cch1 and the high-affinity iron transporter Ftr1/Fet3 complex are major  
104 U importers in *S. cerevisiae*. The potential use of yeast mutants for these transport  
105 systems as tools to investigate candidate mammalian and plant U transporters by  
106 functional expression, for future human health, phytoremediation and food chain  
107 management purposes is discussed.

108

109

## 110 **Materials and Methods**

### 111 ***Strains and plasmids***

112 Table I lists all yeast strains employed in this study, which were derived from the wild-  
113 type *S. cerevisiae* strain BY4742 (MAT $\alpha$ , *his3* $\Delta$ 1, *leu2* $\Delta$ 0, *lys2* $\Delta$ 0, *ura3* $\Delta$ 0) (Brachmann  
114 et al., 1998) and were generated by the Genome Deletion Project (Winzeler et al.,  
115 1999). All strains were obtained from EUROSCARF.

116 Low copy number plasmid YCpMID1 [*URA3 MID1 ARS1 CEN4 amp<sup>r</sup>*] (Iida et al., 2004)  
117 was kindly provided by Professor Iida (Tokyo Gakugei University). Empty low copy  
118 number plasmid pRS316 [*URA3 ARSH4 CEN6 amp<sup>r</sup>*] (Sikorski and Hieter, 1989) was  
119 used as control. High copy number plasmids YEp351-FTR1-myc [*LEU2 FTR1-myc 2*  
120  *$\mu$ m-ori amp<sup>r</sup>*], YEp352-FET3-HA [*URA3 FET3-HA 2  $\mu$ m-ori amp<sup>r</sup>*] and corresponding  
121 control empty plasmids YEp351 [*LEU2 2  $\mu$ m-ori amp<sup>r</sup>*] and YEp352 [*URA3 2  $\mu$ m-ori*  
122 *amp<sup>r</sup>*] (Stearman et al., 1996) were kindly provided by Doctor Stearman (Indiana  
123 University). Plasmids were introduced into yeast strains by using conventional lithium  
124 acetate transformation procedure (Kuo and Campbell, 1983). The *Escherichia coli*  
125 strain DH5 $\alpha$  was used for plasmid amplifications.

### 126 ***Growth media and culture conditions***

127 Yeast strains were cultured to late-exponential growth phase at 30°C, under  
128 continuous stirring at 200 rpm, either in YD medium (10 g/l yeast extract, 20 g/l  
129 glucose) or, where indicated, in YG medium (10 g/l yeast extract, 20 ml/l glycerol).  
130 Transformed cells were cultured in modified synthetic media SD/Ca100 or SD/Fe0 (6.8  
131 g/l Yeast Nitrogen Base without amino acids; Foremedium Ltd, containing 100  $\mu$ M  
132 CaCl<sub>2</sub> instead of 900  $\mu$ M CaCl<sub>2</sub> or 0  $\mu$ M FeCl<sub>3</sub> instead of 1  $\mu$ M FeCl<sub>3</sub> in standard  
133 mixture, and 20 g/l glucose), supplemented with appropriate amino acids plus adenine  
134 (by addition of 0.77 g/l of Complete Supplement Mixture Drop-out formulation minus  
135 leucine and/or minus uracil; MPBio). Cells were counted using a LUNA-FL automated  
136 cell counter (Logos Biosystems).

### 137 ***Kinetics of U uptake and accumulation***

138 Cultured yeast cells were harvested by centrifugation at 4000 g for 5 min, washed twice  
139 with water and resuspended in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH  
140 5.5 buffer, at a concentration of 2 X 10<sup>8</sup> cells/ml with or without a carbon source (20

141 mM glucose, or 3% (v/v) glycerol when cells were grown in YG medium). After a 15  
142 min preincubation at 4°C (in a cold room) or 30°C in a Xtemp climatic chamber  
143 (Mechanical Convection Oven XT5116 – IN140) with 30 rpm rotary shaking, uranyl  
144 nitrate ( $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ; >98%; Fluka) was added to 10 ml of the cell suspensions to  
145 the desired final concentration, from convenient stock solutions. At appropriate  
146 intervals, samples (0.5 to 1 ml) were taken and centrifuged at 14 000 g for 2 min. Cell  
147 pellets were then washed twice with 1ml 10 mM sodium carbonate.  $\text{NaN}_3$  inhibitor or  
148 other metals used in competition experiments were added in the uptake assay at the  
149 beginning of the preincubation period. Cell-free controls were conducted using the  
150 same procedure to assure that the amount of U precipitating in the reaction medium or  
151 adsorbed on the tube wall was negligible under our assay conditions, confirming that  
152 U removal resulted exclusively from biotic interaction.

### 153 ***Determination of cell viability***

154 Yeast viability kit based on fluorescein diacetate/propidium iodide staining method was  
155 used to analyze the cell concentration and viability of yeast samples with the  
156 automated fluorescence cell counter LUNA-FL, following the manufacturer's  
157 recommendations (Logos Biosystems). In all experiments performed in this study it  
158 was found that cell viability was not significantly affected at the end of the absorption  
159 kinetics of U (Supplemental Figure 1 A-D).

### 160 ***U quantification by inductively coupled plasma-mass spectrometry (ICP-MS)***

161 Yeast cells were digested at 90°C for 4 hours in 65% (w/v)  $\text{HNO}_3$  (Suprapur, Merck).  
162 Mineralized samples were diluted in 0.5% (v/v)  $\text{HNO}_3$  and analyzed using an iCAP RQ  
163 quadrupole mass instrument (Thermo Fisher Scientific GmbH, Germany) equipped  
164 with a MicroMist U-Series glass concentric nebulizer, a quartz spray chamber cooled  
165 at 3°C, a Qnova quartz torch, a nickel sample cone, a nickel skimmer cone with a high-  
166 sensitivity insert, and an ASX-560 autosampler (Teledyne CETAC Technologies,  
167 USA).  $^{238}\text{U}$  was analyzed using the standard mode. Concentrations were determined  
168 using standard curves prepared from serial dilutions of  $\text{UO}_2(\text{NO}_3)_2$  and corrected using  
169 an internal standard solution containing  $^{172}\text{Yb}$  added online. Data integration was done  
170 using the Qtegra software (Thermo Fisher Scientific GmbH, Germany).

### 171 ***Statistical analyses***

172 Statistical parameters including the definitions and values of n and SDs are reported  
173 in the figures and corresponding figure legends. Non-parametric statistical analysis  
174 was performed on data sets analyzed in Fig. 4, 6 and 7, which typically contain small  
175 sample sizes ( $n < 10$ ) and do not meet the assumptions of parametric tests (normal  
176 distribution and homogeneity of variance, as determined using the Shapiro-Wilk and  
177 Fisher tests, respectively). When reporting significance, multiple non-parametric  
178 comparisons were performed with the Dunnett's many-to-one test using the package  
179 nparcomp (Konietschke et al., 2015) and the R computing environment (R  
180 Development Core Team, 2011). The normal approximation option was used and the  
181 confidence level was set at 99.9 %.

182

## 183 **Results**

### 184 ***S. cerevisiae* is able to take up U**

185 The budding yeast *S. cerevisiae* is known to adsorb large amounts of U at the cell  
186 surface. This massive adsorption of U possibly hides a potential U uptake whose  
187 existence and *a fortiori* mechanisms are still largely unknown. To analyze the capacity  
188 of yeast cells to transport U intracellularly, we measured kinetics of U incorporation by  
189 the strain BY4742 at different U concentrations and two different temperatures. In a  
190 first set of experiments, yeast cells ( $2 \times 10^8$  cells/ml) were challenged with 10  $\mu$ M uranyl  
191 nitrate in 10 mM MES buffer pH 5.5 in the presence of 20 mM glucose for up to 3 h, at  
192 4°C or 30°C. Cellular U content was measured by ICP-MS after two washing steps  
193 with 10 mM sodium carbonate. As shown in Figure 1, more than 70% of U initially  
194 added to the reaction medium was recovered immediately in the unwashed cell pellets  
195 (time 0 corresponds to the time necessary to separate cells from surrounding buffer by  
196 centrifugation after U exposure, *i.e.* less than 5 min). This fast and temperature  
197 independent U binding to yeast cells corresponded to adsorption. Indeed, metal  
198 cations adsorption on yeast cell wall is reported to be unchanged across the  
199 temperature range 4-30°C (Blackwell et al., 1995; White and Gadd, 1987). At 4°C, the  
200 level of biosorbed U remained stable throughout the kinetics. After one washing step  
201 with sodium carbonate most of U adsorbed at the cell surface was washed out and  
202 recovered in the washing solution after centrifugation (Figure 1A). After the second  
203 washing step, almost all adsorbed U was eliminated (U measured in cell pellets did not  
204 exceed the background value throughout the kinetics, demonstrating the efficiency of



205 the washing procedure). This demonstrated that almost no U enters yeast cells at 4°C.  
206 At 30°C, U associated to the cell pellets was only partially washed away with sodium  
207 carbonate. Indeed, a time-dependent increase of U incorporated was observed in  
208 washed cells (starting from nearly 0 immediately upon addition of U), which correlated  
209 with a decrease of U remaining in the incubation medium and in the washing solutions  
210 (Figure 1B). Under these experimental conditions, we measured a rate of 70-100 ng U  
211 (0.29-0.42 nmol) transported per hour by  $10^8$  cells. Altogether, these experiments  
212 showed that yeast cells are able to incorporate intracellularly in a temperature  
213 dependent manner a portion of U added exogenously after a rapid and massive  
214 adsorption at the cell surface.

215

### 216 **U transport in *S. cerevisiae* requires an active metabolism**

217 To learn more about U transport mechanisms in *S. cerevisiae*, the effect of energy  
218 source on U uptake was investigated. To this aim, cultured cells suspended in MES  
219 buffer were challenged at 30°C and 4°C with increasing amounts of uranium nitrate  
220 from 1 to 10  $\mu\text{M}$  either in the presence or absence of glucose for up to 24h. As already  
221 suggested by experiments depicted in Figure 1, a huge difference in U uptake was  
222 observed between 30°C and 4°C (Figure 2). At 30°C, an important dose-dependent U  
223 influx was observed in the presence of glucose. Under these conditions, U uptake was  
224 linear for at least 3h for all U concentrations tested and after 24 h incubation, 30% to  
225 40% of U initially present in the reaction mixture accumulated into the cells (Figure 2).  
226 In the absence of glucose, accumulation levels of U were much lower. Interestingly,  
227 the ratio between the glucose-dependent and the glucose-independent U  
228 accumulation increased with U concentration (ratio around 2 at 1 and 3  $\mu\text{M}$  U (Figure  
229 2A,B); ratio of 6 at 10  $\mu\text{M}$  U (Figure 2C)). U uptake measured at 4°C was very low, but  
230 for the three U concentrations tested, a positive effect of the presence of glucose on U  
231 uptake was also observed. The transport rate of U was assessed in the presence of  
232 glucose at 30°C on 90 min kinetics. As shown in Figure 3, an apparent  $V_{\text{max}}$  of  $145 \pm 5$   
233 ng (0.6 nmol) U incorporated per hour by  $10^8$  cells was reached at saturated  
234 concentrations above 50  $\mu\text{M}$  and an apparent  $K_m$  for U uptake of  $9.0 \pm 1.4$   $\mu\text{M}$  was  
235 calculated from the Michaelis Menten equation (Figure 3). Note that cell viability was  
236 checked throughout the experiment and was found to be largely unaffected regardless  
237 of the tested condition (Supplemental Figure 1A). The stimulation of U uptake in the

238 presence of glucose might be attributable to metabolism-dependent intracellular U  
239 accumulation. The requirement of an active metabolism was also checked using  
240 glycerol, an alternate but non-fermentable carbon source, and sodium azide, an  
241 inhibitor of the mitochondrial respiratory chain. As shown in Figure 4, similar U  
242 transport rates were measured in the presence of glycerol and glucose in the absence  
243 of sodium azide. The latter almost completely abolished U uptake, regardless of the  
244 energy source used in the assay. Altogether, these results demonstrate that U  
245 transport within yeast cells is essentially a metabolism-dependent process that  
246 requires a functional respiratory chain able to produce ATP.

247

### 248 **Fe<sup>2+</sup>, Fe<sup>3+</sup>, Cu<sup>2+</sup> and Ca<sup>2+</sup> compete with U for transport in *S. cerevisiae***

249 As a first step toward the identification of U uptake pathways in yeast, its accumulation  
250 was measured in the presence of various essential metals whose transport systems  
251 are well described. Indeed, since U is not essential for any living organism, its uptake  
252 probably depends, at least in part, on these transport systems that, taken together,  
253 display a broad metal selectivity, including toxic metals (Cyert and Philpott, 2013; Van  
254 Ho et al., 2002). In these competition experiments, U uptake (from 10  $\mu$ M uranyl nitrate)  
255 was challenged with nine essential metals (Fe(III), Cu(II), Fe(II), Ca(II), Mo(VI), Mn(II),  
256 Zn(II) Co(II) and Mg(II)) at concentrations varying from 10  $\mu$ M to 1 mM.

257 As shown in Figure 5, three metals, namely copper, iron and calcium competed with U  
258 uptake. CuSO<sub>4</sub> (Figure 5A) and FeCl<sub>3</sub> (Figure 5B) displayed the strongest  
259 concentration-dependent inhibitory effect. At 10  $\mu$ M, both metals had no or limited  
260 effect on U uptake. At 50  $\mu$ M, they caused a 2- and 3-fold reduction of U accumulation  
261 after 4h, respectively. The inhibitory effect of these two metal ions was even more  
262 marked at 100  $\mu$ M with a 4-fold reduction of U accumulation after 4 h. If we consider  
263 the initial rate of U transport as measured over the first 90 min (see also Figures 2 and  
264 3), it turns out that the low accumulation of U in the presence of Cu(II) and Fe(III) likely  
265 came from a reduction in the initial rate of U uptake. The inhibitory effects of Ca(II)  
266 (Figure 5C) and Fe(II) (Figure 5D) were different from those of Cu(II) and Fe(III) in our  
267 competition assays. They were globally lower and observed at higher concentrations.  
268 Indeed, 1 mM of CaCl<sub>2</sub> or FeSO<sub>4</sub> were required to induce a nearly 2-fold reduction of  
269 U uptake at 4h. Remarkably, Ca(II) and Fe(II) did not seem to significantly affect the

270 rate of U uptake during the first hour of competition (Figure 5 C,D). Finally, Figure 5 E-  
271 I shows that, at all the concentrations tested, MgCl<sub>2</sub>, MnCl<sub>2</sub>, ZnSO<sub>4</sub>, Na<sub>2</sub>MoO<sub>4</sub> and  
272 CoCl<sub>2</sub> had almost no effect on U uptake. Again, cell viability was checked throughout  
273 the experiment and was largely unaltered regardless of the condition tested (85-99%  
274 viability at the end of the experiment, depending on individual cases) (Supplemental  
275 Figure 1C), ruling out the possibility that toxic effects of high concentrations of  
276 competing metals were primarily responsible for the reduced U uptake rates and  
277 accumulation levels. Taken together, our results identified three potential entry  
278 pathways for U in yeast; the calcium, copper and iron uptake routes.

279

### 280 ***S. cerevisiae* iron and calcium transport mutants are affected in U uptake**

281 In order to identify metal transporters involved in U uptake in yeast, a set of mutant  
282 strains isogenic of BY4742 and affected in the uptake of various metals were assayed  
283 for their capacity to accumulate U. The strains *ftr1*Δ and *fet3*Δ are deleted in the genes  
284 coding for the permease Ftr1 and the multi-copper ferrous oxidase Fet3, two  
285 components of the high-affinity iron Fe(III) uptake system (Stearman et al., 1996). The  
286 strain *smf1*Δ is deleted in the gene coding for Smf1 a high-affinity manganese Mn(II)  
287 transporter of the Nramp family that can also transport Fe(II), Cu(II) and other divalent  
288 metal ions into the cytosol (Chen et al., 1999; Cohen et al., 2000). The strain *ctr1*Δ is  
289 deleted in the gene coding for Ctr1 a high-affinity copper transporter that mediates  
290 Cu(I) uptake (Dancis et al., 1994). The strains *cch1*Δ and *mid1*Δ are deleted in the  
291 genes coding for the two components of the high-affinity plasma membrane voltage-  
292 gated calcium channel Cch1, the pore-forming subunit and Mid1, the positive  
293 regulatory subunit (Iida et al., 2004; Iida et al., 2017; Iida et al., 2007; Paidhungat and  
294 Garrett, 1997). Finally, the strain *cot1*Δ is deleted in the gene coding for Cot1, a  
295 transporter induced in iron-deficient yeast that mediates vacuolar accumulation of zinc  
296 and cobalt as well as other metals including toxic ones (Conklin et al., 1992;  
297 MacDiarmid et al., 2000).

298 U uptake kinetics in these seven strains are presented in Figure 6. As shown in Figure  
299 6 A,B,C, U uptake was significantly affected in the *ftr1*Δ, *mid1*Δ and *cch1*Δ strains as  
300 compared to the wild type. Note that in the *ftr1*Δ strain, the initial rate of U uptake was  
301 not altered as compared to the wild type, the inhibitory effect being only visible after 1

302 h incubation. Some inhibition of U uptake was detected in the *ctr1* $\Delta$  and *fet3* $\Delta$  mutants  
303 but it was not statistically significant (Figure 6 D,E). In contrast, no difference in U  
304 uptake was observed in the *smf1* $\Delta$  and *cot1* $\Delta$  mutants as compared to their wild type  
305 counterpart (Figure 6 F,G). Taken together, these data strongly suggest that yeast cells  
306 incorporate U at least through the high-affinity iron transporter Ftr1 and the calcium  
307 channel Cch1/Mid1.

308 Finally, the expression from a low copy plasmid, of wild-type copy of *MID1* gene under  
309 the control of its own promotor in the corresponding mutant strain restored its capacity  
310 to incorporate U to wild-type levels (Figure 7), confirming the role of the Cch1/Mid1  
311 calcium channel in U transport in yeast. We were unable to perform reliable U uptake  
312 experiments using the *ftr1* $\Delta$  mutant bearing high copy number plasmids encoding Ftr1  
313 and Fet3 proteins. Indeed, the co-expression of these recombinant proteins is  
314 controlled by iron deficiency (Stearman et al., 1996) and transformed cells have  
315 difficulties to grow properly on synthetic medium deprived of iron, as compared to  
316 control cells.

317

## 318 **Discussion**

319 In this study, we identified, for the first time, calcium and iron assimilation pathways as  
320 important routes for U uptake into a living cell, using *S. cerevisiae* as a model. This  
321 was made possible, as discussed below, thanks to the development of an efficient  
322 procedure for measuring U transport, under conditions that favor U uptake and that  
323 allow to discriminate the uranyl fraction simply adsorbed on the cells from the  
324 intracellular fraction.

### 325 **Living yeast *S. cerevisiae* is able to accumulate U**

326 Over the last four decades, the interactions of the uranyl ion with microorganisms, and  
327 particularly the yeast *S. cerevisiae*, have been extensively studied because of their  
328 potential use to remediate contaminated waste streams and groundwater (Kolhe et al.,  
329 2020; Lu et al., 2013; Nakajima and Tsuruta, 2002; Strandberg et al., 1981). Non-  
330 pathogenic and easy obtained at low cost from fermentation industry, *S. cerevisiae*  
331 was shown to display a high capacity of U biosorption, a biosorption which consists  
332 essentially of adsorption on the cell wall in the experimental conditions used (Chen et

333 al., 2020; Ohnuki et al., 2005; Volesky and May-Phillips, 1995; Zhang et al., 2020). The  
334 most recent investigations proposed a three-step process for biomineralization of U on  
335 *S. cerevisiae* cell surface: surface adsorption, amorphous precipitation, and  
336 crystallization (Shen et al., 2018; Zheng et al., 2018). U biosorption conditions  
337 described so far, using complex media or unbuffered nutrients-free solutions, living or  
338 dead cell biomass exposed to elevated U concentrations generally higher than 0.4 mM  
339 were optimized for massive U precipitation and/or biomineralization on cell surface. In  
340 these conditions, U internalization was rarely evoked and, if so, it was mainly explained  
341 by the loss of the cell wall and plasma membrane integrity (Ohnuki et al., 2005;  
342 Strandberg et al., 1981; Volesky and May-Phillips, 1995).

343 In our work, U uptake experiments were performed on living cells exposed to low U  
344 concentrations (<100  $\mu\text{M}$ ) to preserve cell viability and to limit U precipitation on cell  
345 surface, in MES buffer adjusted to pH 5.5, which favors  $\text{UO}_2^{2+}$ , the bioavailable form  
346 of U. This buffer was chosen for its low to negligible metal-chelating propensity (Good  
347 et al., 1966) and to prevent pH neutralization of the uptake medium caused by gradual  
348 inorganic phosphate and ammonium ions release from inside the yeast cells upon U  
349 exposure (Shen et al., 2018; Zheng et al., 2018), minimizing uranyl phosphate  
350 precipitation on cell surface. In these conditions, U adsorption on cell surface was  
351 extremely rapid, around 80 % of U present in the medium being adsorbed in a few min.  
352 This observation compares well with data from literature showing equilibrium reached  
353 within 1 to 30 min according to experimental conditions (Faghihian and Peyvandi,  
354 2012; Lu et al., 2013; Nakajima and Tsuruta, 2002; Popa et al., 2003; Volesky and  
355 May-Phillips, 1995). To distinguish U externally adsorbed on cell surface from U  
356 incorporated within the cell, we used two washings with carbonate, one of the highest  
357 affinity ligands for U (Kitano and Oomori, 1971; Poston et al., 1984). In our hands, two  
358 washing steps with 10 mM sodium carbonate allowed complete elimination of U  
359 adsorbed on cell surface, allowing to unmask U incorporated within the cell. This high  
360 washing efficiency was determinant for accurate measurement of uptake kinetics.

### 361 **Efficient U uptake into *S. cerevisiae* cells depends on the availability of a** 362 **metabolizable substrate and necessitates a functional respiratory chain**

363 In this work, we showed that an efficient U uptake requires an active metabolism.  
364 Indeed, net transport was almost null at 4°C regardless of the presence or absence of  
365 externally added glucose in the uptake medium, and low at 30°C in its absence, but it

366 was highly stimulated in its presence. Interestingly, at low U concentrations (up to 3  
367  $\mu\text{M}$  U) glucose-independent U transport was significant, suggesting passive diffusion  
368 contribution to U uptake under these conditions. At 10  $\mu\text{M}$  U, glucose-independent U  
369 uptake was almost negligible. At 30°C and in the presence of glucose, U uptake was  
370 a saturable process, initial rates of U uptake being saturable at concentrations above  
371 50  $\mu\text{M}$ . In *S. cerevisiae* glucose uptake is mainly performed by facilitated diffusion and  
372 is mediated by various uniporters and channels (Boles, 1997; Kruckeberg, 1996;  
373 Reifenberger et al., 1997; Wijsman et al., 2019). Since U and glucose are known to  
374 form complexes under certain conditions (Steudtner et al., 2010) the hypothesis of a  
375 co-transport through hexose transporters could not be excluded at this stage. To test  
376 this hypothesis, we used glycerol, which is not able to form complexes with U, as an  
377 alternative source of carbon in U uptake experiments. Similar stimulation of U uptake  
378 was observed under these conditions, ruling out the possibility of U transport through  
379 hexose transporters. The higher rates of U incorporation in the absence of any energy  
380 source in uptake medium, when yeasts were initially grown in medium containing  
381 glycerol instead of glucose (YG vs YD medium) (Figure 4B), might be explained by  
382 some existing internal storage pool of glycerol. In contrast, in yeast exposed to  
383 fermentable sugars such as glucose, despite high rates of uptake, little or no sugar can  
384 normally be detected within the cell, the glycolytic system removing the sugars as fast  
385 as they enter (Klein et al., 2017).

386 Finally, the use of sodium azide that blocks cell respiration by inhibiting the activity of  
387 mitochondrial cytochrome c oxidase-respiratory chain complex (Keilin, 1936; Leary et  
388 al., 2002), almost completely abolished U uptake in *S. cerevisiae* cells, whatever the  
389 metabolizable substrate source present (glucose or glycerol), demonstrating the  
390 importance of a functional respiratory chain in this process. The unaltered viability (cell  
391 permeability) of azide-treated cells compared to the control precluded the scenario that  
392 the very low U level in these cells resulted from cell death and the release of U to the  
393 medium (Supplemental Figure 1B).

### 394 **Iron, calcium and copper pathways are involved in the absorption of U in *S*** 395 ***cerevisiae***

396 A combination of competition experiments with essential metals and analysis of mutant  
397 strains deleted in genes coding for metal importers allowed us to identify calcium, iron  
398 and copper pathways as potential routes for U uptake in yeast.

399 Uptake of U through the calcium assimilation pathway – In this study, we showed that  
400 in *S. cerevisiae* calcium and U share a common route for the entry into the cell. U(VI)  
401 under its  $\text{UO}_2^{2+}$  cation form resembles  $\text{Ca}^{2+}$ . Uranyl coordination properties having  
402 some similarities with those of calcium, various calcium-binding proteins from different  
403 organisms, including Human, were found to interact strongly with U, among which  
404 calmodulin (Brulfert et al., 2016; Le Clainche and Vita, 2006; Pardoux et al., 2012) and  
405 osteopontin (Creff et al., 2019; Qi et al., 2014; Safi et al., 2013). In biological systems,  
406 U and calcium biosorption relationships are also well documented. In *S. cerevisiae*,  
407  $\text{Ca}^{2+}$  was shown to affect both the initial rate of U biosorption and total amount of U  
408 bound to the cell wall (Strandberg et al., 1981). In plants, it was also found that U  
409 accumulation in plants was directly related to mobile calcium content in soil (Ratnikov  
410 et al., 2020). The interference of U with calcium uptake and homeostasis has been  
411 also reported in numerous studies (El Hayek et al., 2018; Moll et al., 2020; Rajabi et  
412 al., 2021; Straczek et al., 2009; Tawussi et al., 2017; Vanhoudt et al., 2010). The  
413 observed increase in cytoplasmic calcium reported in some cases could represent a  
414 cellular defense mechanism in response to the presence of U (White and Broadley,  
415 2003). Within the cell, U(VI) under its  $\text{UO}_2^{2+}$  cation form can replace  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ,  
416 which can lead to structural changes to cell membranes, enzyme inactivation, and  
417 damage to RNA and DNA (Saenen et al., 2013). Despite all these observations, no  
418 direct evidence for the uptake of U by the calcium assimilation pathway was provided  
419 so far.

420 In *S. cerevisiae*, Mid1 (positive regulatory protein) and Cch1 (pore-forming subunit) are  
421 the two mandatory components of the high affinity voltage-gated calcium channel  
422 (Fischer et al., 1997; Hong et al., 2013; Iida et al., 1994; Iida et al., 2004; Iida et al.,  
423 2017; Iida et al., 2007). The Mid1/Cch1 channel becomes activated by various stimuli  
424 including depolarization, depletion of secretory calcium (Locke et al., 2000),  
425 pheromone stimulation (Paidhungat and Garrett, 1997), hypotonic shock (Batiza et al.,  
426 1996), and alkaline stress (Viladevall et al., 2004) or metallic stress (Gardarin et al.,  
427 2010). Interestingly, the Mid1/Cch1 calcium channel was previously found to contribute  
428 also to  $\text{Cd}^{2+}$  uptake in yeast (Gardarin et al., 2010).

429 In our study, we showed that calcium inhibits U uptake in *S. cerevisiae* in a  
430 concentration-dependent manner, suggesting a common entry pathway for these two  
431 cations. This hypothesis is supported by the fact that  $\text{UO}_2^{2+}$  and  $\text{Ca}^{2+}$  are both divalent

432 cations of similar atomic radius (around 180 pm) and ionic radius (around 80-100 pm).  
433 Indeed, the size and shape of the species handled by channels need to match the size  
434 of the ion selectivity filter cavity for a proper transport (Gouaux and Mackinnon, 2005).  
435 Experimentally, this hypothesis was also supported by the fact that the mutant strains  
436 *mid1Δ* and *cch1Δ* had similar phenotypes *i.e.*, low U uptake activity and accumulation,  
437 consistent with their contribution to a single functional complex, and that the expression  
438 of *MID1* gene into the *mid1Δ* strain restored wild-type levels of U uptake rates (Figure  
439 6B,C). Thus, it is interesting to note the parallel between the kinetics of incorporation  
440 of U in calcium import mutants in our study and those for calcium in equivalent mutant  
441 strains (Iida et al., 1994; Nakagawa et al., 2007). Together, these observations  
442 demonstrated that the main calcium import pathway in yeast is involved in U transport.

443 The incomplete inhibition of U uptake in *mid1Δ* and *cch1Δ* mutant strains or in wild-  
444 type strain challenged with excess calcium suggests that other pathways are involved.  
445 A similar conclusion could be drawn for calcium entry in yeast cells (Cui et al., 2009;  
446 Iida et al., 1994).

447 Uptake of U through the iron assimilation pathways – Our study also suggests that U  
448 might enter the cell through iron transport systems. As for calcium, U and iron crosstalk  
449 is well documented in biological systems, particularly in plants where it was found that  
450 U interferes with iron homeostasis (Berthet et al., 2018; Doustaly et al., 2014; Sarthou  
451 et al., 2020; Vanhoudt et al., 2011). Also, several ferric iron-binding proteins were found  
452 to be able to bind U with high affinity, such as human transferrin, involved in iron  
453 transport to the bloodstream (Vidaud et al., 2007) and ferritin, an iron storage protein,  
454 from the archaea *Picrococcus furiosus*, the crayfish *Procambarus clarkia* or the zebrafish  
455 *Danio rerio* (Cvetkovic et al., 2010; Eb-Levadoux et al., 2017; Xu et al., 2014).

456 In the present study, we provided the first experimental evidence that the iron pathway  
457 can directly contribute to U uptake in yeast. Indeed, we showed that a mutant strain  
458 lacking the ferric iron permease Ftr1 was significantly affected in U uptake. The  
459 presence of functional copper-dependent ferrous oxidase Fet3 being required for the  
460 maturation of Ftr1 and iron transport across the plasma membrane (De Silva et al.,  
461 1995; Hassett et al., 1998a; Stearman et al., 1996) (Figure 8), the rather normal U  
462 uptake observed in the mutant strain *fet3Δ* was unexpected (Figure 6E). However, it  
463 was shown that yeast can compensate for the lack of Fet3 by overexpressing Fet4, a



464 low affinity, low specificity Fe(II) ion transporter. This compensation was not observed  
465 in the mutant strain *ftr1Δ* (Li and Kaplan, 1998). Thus, in Fet3 defective mutants, U  
466 might enter the cell through the Fet4 pathways. In Ftr1 defective mutants, iron enter  
467 the cell mainly *via* Smf1, a broad-spectrum low affinity, low saturable divalent cation  
468 transporter capable of transporting Mn(II), Zn(II) and Fe(II) (Cohen et al., 2000; Portnoy  
469 and Culotta, 2003). Since Smf1 does not seem to be involved in U transport (Figure  
470 6G), this would explain the deficit in U incorporation observed in the *ftr1Δ* mutant.  
471 These observations are consistent with the results of competition assays showing that  
472 a Fe(II) concentration about 10 times higher than that of Fe(III) was necessary to affect  
473 U uptake (Figure 5B,D). Taken together, our results support the idea that *S. cerevisiae*  
474 can incorporate U, through the Ftr1/Fet3 high affinity iron transport system.

475 Uptake of U under copper excess – The negative impact of copper on U uptake by  
476 yeast was unexpected. Indeed, the imported form of copper Cu(I) in yeast has very  
477 different chemical properties compared to U, notably in terms of protein ligands as  
478 deduced from Pearson's (Pearson, 1963) classification. Accordingly, the mutant strain  
479 *ctr1Δ* lacking the high affinity Cu(I) transporter Ctr1 was not affected in U uptake  
480 (Figure 6D). We can exclude that Ctr3, another high affinity copper transporter,  
481 compensate for the lack of Ctr1 since the *Ctr3* gene is inactivated in most laboratory  
482 strains by a TY2 transposon, including S288C, the parental strain of BY4247 used in  
483 our study (Knight et al., 1996). The inhibitory effect of copper on U uptake might be  
484 indirect. For example, it was shown that copper stress induces broad and prolonged  
485 rise in cytosolic calcium through vacuolar and endoplasmic reticulum stored calcium  
486 release (Jo et al., 2008; Ruta et al., 2016) leading to downregulation of the Mid1/Cch1  
487 calcium influx channel (Hong et al., 2010). Thus, in such a scenario, copper effect on  
488 U uptake would be explained by a crosstalk between copper and calcium homeostasis.  
489 Similarly, as described above, copper through its interaction with Fet3 is required for  
490 high affinity iron transport in yeast (Figure 8) (Dancis et al., 1994; Hassett et al., 1998b).  
491 It was shown that exposure to high copper concentrations could indirectly affect the  
492 iron transport machinery by compromising copper loading on Fet3 and subsequent  
493 translocation of the complex to the plasma membrane (Jo et al., 2008). In this scenario,  
494 exposure to copper would indirectly affect U uptake. Finally, at the concentration used  
495 in this study, copper slightly affected cell viability (15% of cells were dead after 3h  
496 incubation in the presence of 100 μM copper; Supplemental Figure 1C). Thus, we

497 cannot exclude that inhibition of U uptake by copper was also related to the pleiotropic  
498 toxic effects of the latter on yeast metabolism (Shanmuganathan et al., 2004).

499

## 500 **Conclusion**

501 In the present work, we have developed an accurate and reliable assay to elucidate U  
502 uptake routes in *S. cerevisiae*. Taken together, our data combining energy source  
503 requirement experiments, competition assays and mutant analyses, showed that U  
504 uptake probably operates through different transport systems, among which the  
505 calcium channel Mid1/Cch1 and the iron transport complex Ftr1/Fet3. This is to our  
506 knowledge, the first identification of U uptake routes in a living cell. Thus, identifying  
507 U-transporters in yeast, represents an important milestone in the fields of radionuclide  
508 toxicology and bioremediation. Particularly, our discoveries make possible rapid and  
509 systematic *in levuro* functional testing of candidate U transporters of many organisms,  
510 regardless of their phylogenetic origins. This can be particularly well suited to identify  
511 transporters from multicellular organisms, like higher plants or mammals, prior more  
512 complex and time-consuming characterization in their natural context. Validation of  
513 yeast-based hypotheses in these organisms would provide new insights into the  
514 chemical toxicity of U in higher eukaryotes and is a prerequisite for a future rational  
515 management of U in polluted soils, in the food chain, and in human health preservation.

516

## 517 **CRedit authorship contribution statement**

518 **Revel B.:** Methodology, Investigation, Formal analysis. **Catty P.:** Conceptualization;  
519 Methodology, Investigation, Formal analysis, Resources, Writing – Review & Editing.  
520 **Ravanel S.:** Conceptualization, Formal analysis, Resources, Writing – Review &  
521 Editing; **Bourguignon J.:** Conceptualization, Supervision, Methodology, Formal  
522 analysis, Resources, Writing – Review & Editing, Funding acquisition. **Alban C.:**  
523 Conceptualization, Supervision, Methodology, Investigation, Formal analysis,  
524 Resources, Writing – Original draft, Writing – Review & Editing.

525

## 526 **Declaration of Competing Interest**

527 The authors declare that they have no known competing financial interests or personal  
528 relationships that could have appeared to influence the work reported in this paper.

529

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535 used in this study.

536

### 537 **Appendix A. Supplementary Material**

538

539

540 Table I: Yeast strains used in this study

Strain	Deleted gene	Genotype	Reference
BY4742	WT	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	Brachmann et al. (1998)
BY <i>ptr1</i> $\Delta$	YER145C	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 ptr1<math>\Delta</math></i>	(Winzeler et al., 1999)
BY <i>fet3</i> $\Delta$	YMR058W	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 fet3<math>\Delta</math></i>	(Winzeler et al., 1999)
BY <i>mid1</i> $\Delta$	YNL291C	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 mid1<math>\Delta</math></i>	(Winzeler et al., 1999)
BY <i>cch1</i> $\Delta$	YGR217W	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 cch1<math>\Delta</math></i>	(Winzeler et al., 1999)
BY <i>smf1</i> $\Delta$	YOL122C	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 smf1<math>\Delta</math></i>	(Winzeler et al., 1999)
BY <i>cot1</i> $\Delta$	YOR316C	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 cot1<math>\Delta</math></i>	(Winzeler et al., 1999)
BY <i>ctr1</i> $\Delta$	YPR124W	MAT $\alpha$ <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 ctr1<math>\Delta</math></i>	(Winzeler et al., 1999)

541

542

543 **Figure legends**

544 **Figure 1:** Effect of sodium carbonate washes on unmasking U taken up by U-exposed  
545 *S. cerevisiae* BY4742 strain cells. Cells grown at late-log phase in YD medium were  
546 suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer  
547 containing 20 mM glucose, and preincubated for 15 min at 4°C (A) or 30°C (B) before  
548 addition of 10  $\mu$ M uranyl nitrate and further incubation at the above-mentioned  
549 temperatures. At the time indicated, 0.5 ml aliquots of the cell suspensions were  
550 withdrawn and processed as detailed in the Materials and Methods section. U  
551 remaining in the incubation medium after centrifugation of the cell suspensions  
552 (“Medium”), in the washing solutions after two washes of pelleted cells with 10 mM  
553 sodium carbonate (“First wash” and “Second wash”, respectively), and in unwashed  
554 and washed cell pellets (“Unwashed cells” and “Washed cells”, respectively), was  
555 quantified by ICP-MS. Dotted line indicates U remaining in the incubation medium of  
556 an acellular control, after centrifugation (“Acellular control”). A result representative of  
557 at least three independent experiments is shown. In all experiments, the U recovery  
558 profiles were very similar, although the absolute values varied from an experiment to  
559 the other.

560 **Figure 2:** Stimulation of U uptake rate by glucose in *S. cerevisiae* BY4742 strain. Cells  
561 grown at late-log phase in YD medium were suspended at a concentration of  $2 \times 10^8$   
562 cells/ml in 10 mM MES, pH 5.5 buffer containing 20 mM glucose (+ Glucose) or not (-  
563 Glucose), and preincubated for 15 min at 4°C or 30°C before addition of 1  $\mu$ M (A), 3  
564  $\mu$ M (B) or 10  $\mu$ M (C) uranyl nitrate and further incubation at the above-mentioned  
565 temperatures. At the time indicated, 0.6 ml aliquots of the cell suspensions were taken  
566 and processed as detailed in the Materials and Methods section. U present in cell  
567 pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. Bars =  
568 SD; n = 3 independent experiments.

569 **Figure 3:** Kinetic parameters determination of U uptake in *S. cerevisiae* BY4742 strain.  
570 Cells grown at late-log phase in YD medium were suspended in 10 mM MES, pH 5.5  
571 buffer containing glucose 20 mM, at a concentration of  $2 \times 10^8$  cells/ml and  
572 preincubated for 15 min at 30°C before addition of 1 to 70  $\mu$ M uranyl nitrate and further  
573 incubation at 30°C. At appropriate intervals (from 0 to 90 min), aliquots of the cell  
574 suspensions were taken and processed as detailed in the Materials and Methods  
575 section. U present in cell pellets washed twice with 10 mM sodium carbonate was

576 quantified by ICP-MS. The initial velocity of U uptake (uptake rate) was then plotted  
577 versus U concentration in the reaction medium. Data (dots) were fitted by nonlinear  
578 regression to the simple Michaelis-Menten equation (curve) using the Kaleidagraph  
579 4.5 program (Synergy Software, Reading, PA). Bars = SD; n = 2 to 9 independent  
580 experiments, for each data point.

581 **Figure 4:** Effect of glycerol as an alternate metabolizable substrate to glucose for U  
582 uptake in *S. cerevisiae* BY4742 strain. Cells grown at late-log phase in YD (A) or YG  
583 (B) medium were suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH  
584 5.5 buffer containing either 20 mM glucose (+ Glucose) or not (- Glucose) (A) or 3%  
585 glycerol (+ Glycerol) or not (- Glycerol) (B) and 10 mM NaN<sub>3</sub> (+ NaN<sub>3</sub>) or not (- NaN<sub>3</sub>),  
586 and preincubated for 15 min at 30°C before addition of 10 μM uranyl nitrate and further  
587 incubation at the same temperature. At the time indicated, 0.6 ml aliquots of the cell  
588 suspensions were taken and processed as detailed in the Materials and Methods  
589 section. U present in cell pellets washed twice with 10 mM sodium carbonate was  
590 quantified by ICP-MS. Bars = SD; n = 3 independent experiments. Statistical  
591 significance for the comparison of all other conditions versus + Glucose (or + Glycerol)  
592 – NaN<sub>3</sub> was determined using a non-parametric Dunnett's test with  $P < 0.001$  (\*).

593 **Figure 5:** Kinetics of U uptake in *S. cerevisiae* BY4742 strain in the presence of  
594 potential essential metal competitors. Cells grown at late-log phase in YD medium were  
595 suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer  
596 containing 20 mM glucose and various concentrations (0 to 1000 μM as indicated) of  
597 CuSO<sub>4</sub> (A), FeCl<sub>3</sub> (B), CaCl<sub>2</sub> (C), FeSO<sub>4</sub> (D), MgSO<sub>4</sub> (E), MnSO<sub>4</sub> (F), ZnSO<sub>4</sub> (G),  
598 Na<sub>2</sub>MoO<sub>4</sub> (H) or CoCl<sub>2</sub> (I). Cell suspensions were then preincubated for 15 min at 30°C  
599 before addition of 10 μM uranyl nitrate and further incubation at the same temperature.  
600 At the time indicated, 0.5 ml aliquots of the cell suspensions were taken and processed  
601 as detailed in the Materials and Methods section. U present in cell pellets washed twice  
602 with 10 mM sodium carbonate was quantified by ICP-MS. Bars = SD when these  
603 values exceed the dimensions of the symbols; n = 2 to 4 independent experiments, as  
604 indicated.

605 **Figure 6:** Kinetics of U uptake in *S. cerevisiae* mutant strains deleted in essential metal  
606 transporters. Yeast strains, *ptr1Δ* (A), *mid1Δ* (B), *cch1Δ* (C), *ctr1Δ* (D), *fet3Δ* (E), *cot1Δ*  
607 (F), *smf1Δ* (G) and their wild type counterpart (BY4742 strain) were grown at late-log  
608 phase in YD medium and then suspended at a concentration of  $2 \times 10^8$  cells/ml in 10

609 mM MES, pH 5.5 buffer containing 20 mM glucose. After a 15 min preincubation at  
610 30°C, 10  $\mu$ M uranyl nitrate was added and cells were further incubated at the same  
611 temperature. At the time indicated, 1 ml aliquots of the cell suspensions were taken  
612 and processed as detailed in the Materials and Methods section. U present in cell  
613 pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. Bars =  
614 SD; n = 2 to 6 independent experiments, as indicated. Statistical significance (mutant  
615 versus wild-type) was determined using a non-parametric Dunnett's test with  $P < 0.001$   
616 (\*).

617 **Figure 7:** Contribution of the calcium channel Cch1/Mid1 to U entry in *S. cerevisiae*  
618 BY4742 strain. The yeast *mid1* $\Delta$  mutant as well as the wild-type parental BY4742 strain  
619 (WT) were transformed with either the empty low copy number plasmid pRS316  
620 (vector; WT/vector and *mid1* $\Delta$ /vector) or YCpMID1 low copy number plasmid (MID1;  
621 *mid1* $\Delta$ /MID1) encoding the yeast *MID1* gene under the control of its own promoter.  
622 Freshly transformed cells were grown at late-log phase in SD/Ca100, plus amino acids  
623 and adenine, minus URA medium and then suspended at a concentration of  $2 \times 10^8$   
624 cells/ml in 10 mM MES, pH 5.5 buffer containing 20 mM glucose. After a 15 min  
625 preincubation at 30°C, 10  $\mu$ M uranyl nitrate was added and cells were further incubated  
626 at the same temperature. At the time indicated, 1 ml aliquots of the cell suspensions  
627 were taken and processed as detailed in the Materials and Methods section. U present  
628 in cell pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS.  
629 Error bars = SD; n = 3 independent transformants. Statistical significance was  
630 determined using a non-parametric Dunnett's test with  $P < 0.001$  (\*). NS = not  
631 significant.

632 **Figure 8:** Recapitulative scheme of U entry routes in *S. cerevisiae*. Our data support  
633 the assertion that the high affinity Mid1/Cch1 calcium channel and the high affinity  
634 Ftr1/Fet3 iron transport complex promote U uptake in *S. cerevisiae*. Mid1/Cch1: the  
635 high affinity calcium channel; Fre1-3: Fre1, Fre2 and Fre3 reductases reduce the Cu(II)  
636 to Cu(I) and Fe(III) to Fe(II); Ctr1: the high affinity Cu(I) transporter; Atx1 chaperonin  
637 allows copper supply to the Golgi device in concert with Ccc2 transporter. In the path  
638 of secretion, copper is incorporated to various metalloproteins including multi-copper  
639 ferrous oxidase Fet3. Once copper incorporated in Fet3, the latter is secreted on the  
640 cell surface together with the permease Ftr1 with which Fet3 is physically associated,  
641 promoting the transport of iron inside the cell (Ftr1/Fet3, the high affinity iron transport

642 complex). Fet4: low affinity and low specificity Fe(II) ion transporter; Smf1: broad-  
643 spectrum low affinity and low saturable divalent cation transporter.

644

## 645 **Supporting information**

646 **Figure S1:** Viability of *S. cerevisiae* strains in the course of the experiments performed  
647 in this study. Viability of yeast cell suspensions (Parental BY4742 wild-type strain and  
648 the related isogenic mutant strains) was determined by the fluorescein  
649 diacetate/propidium iodide staining method using Yeast viability kit and the automated  
650 fluorescence cell counter LUNA-FL (Logos Biosystems) after 4h incubation of cells at  
651 30°C in the presence of 20 mM glucose, uranyl nitrate at the indicated concentrations  
652 (in  $\mu\text{M}$ ), and other molecules (as indicated) in 10 mM MES pH 5.5 buffer. The details  
653 of growth and assay conditions are given in the Materials and Methods section and in  
654 the legend of the figures of the corresponding experiments. (A) Viability of cells from  
655 experiments in Figure 3 (Kinetics parameters determination); (B) Viability of cells from  
656 experiments in Figure 4A (Effect of sodium azide); (C) Viability of cells from  
657 experiments in Figure 5 (Effect of metal competitors). Concentrations of competitors  
658 are given in  $\mu\text{M}$  from 10 to 1000; (D) Viability of cells from experiments in Figure 6  
659 (Mutant strains deleted in essential metal transporters). The results from viable cell  
660 counts are shown as relative values (%) as compared to total counted cells. Error bars  
661 = SD; n = 2 to 6 independent experiments, as indicated in the legend of the referred  
662 figures.

663

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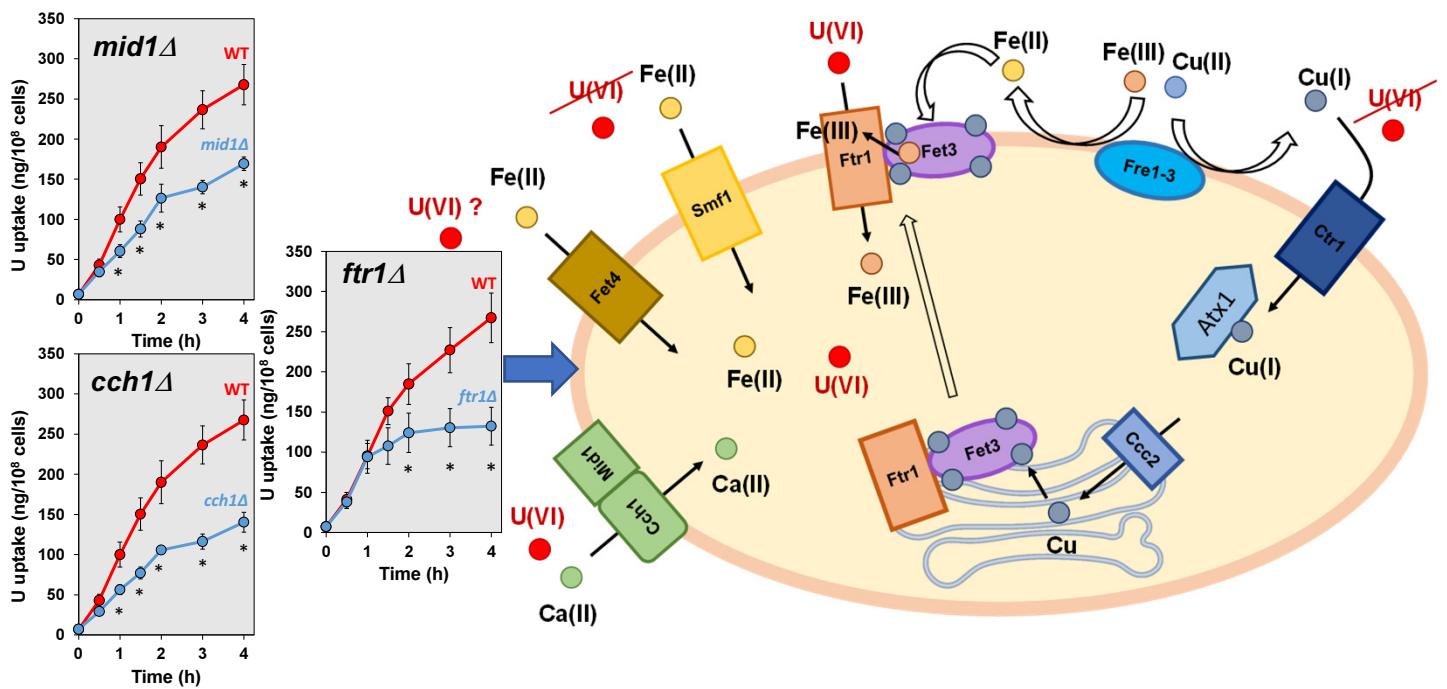
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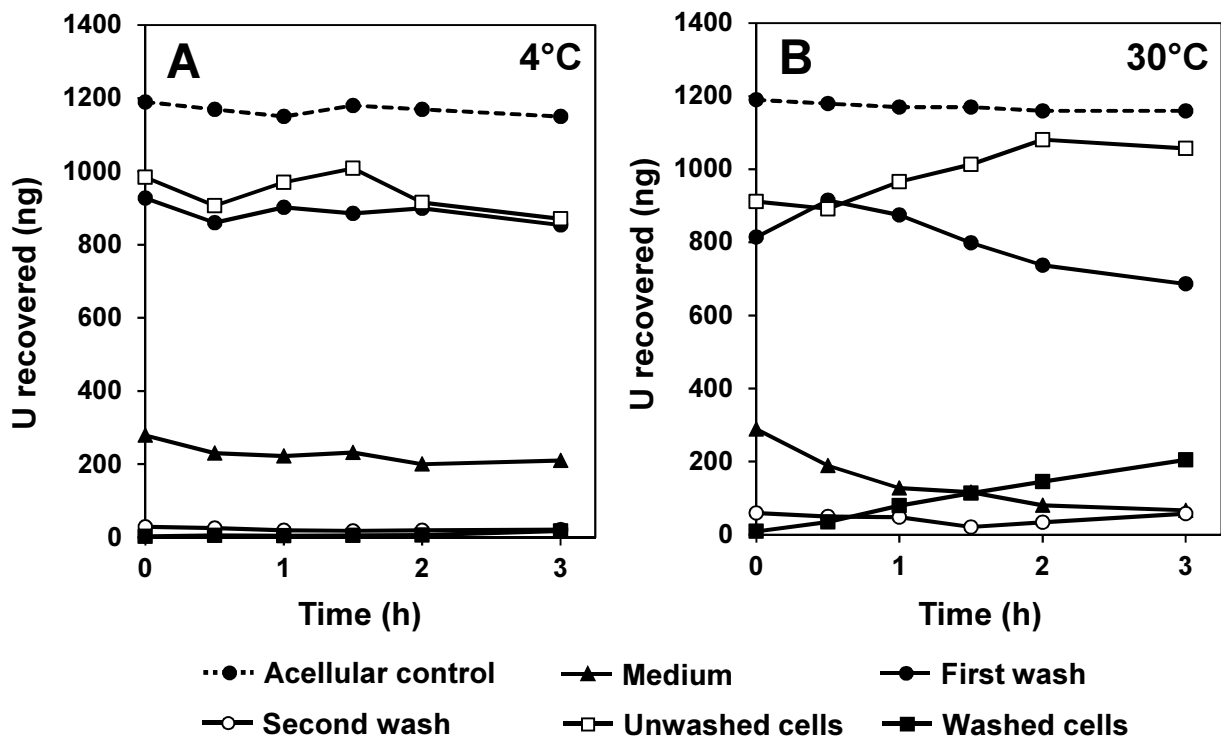
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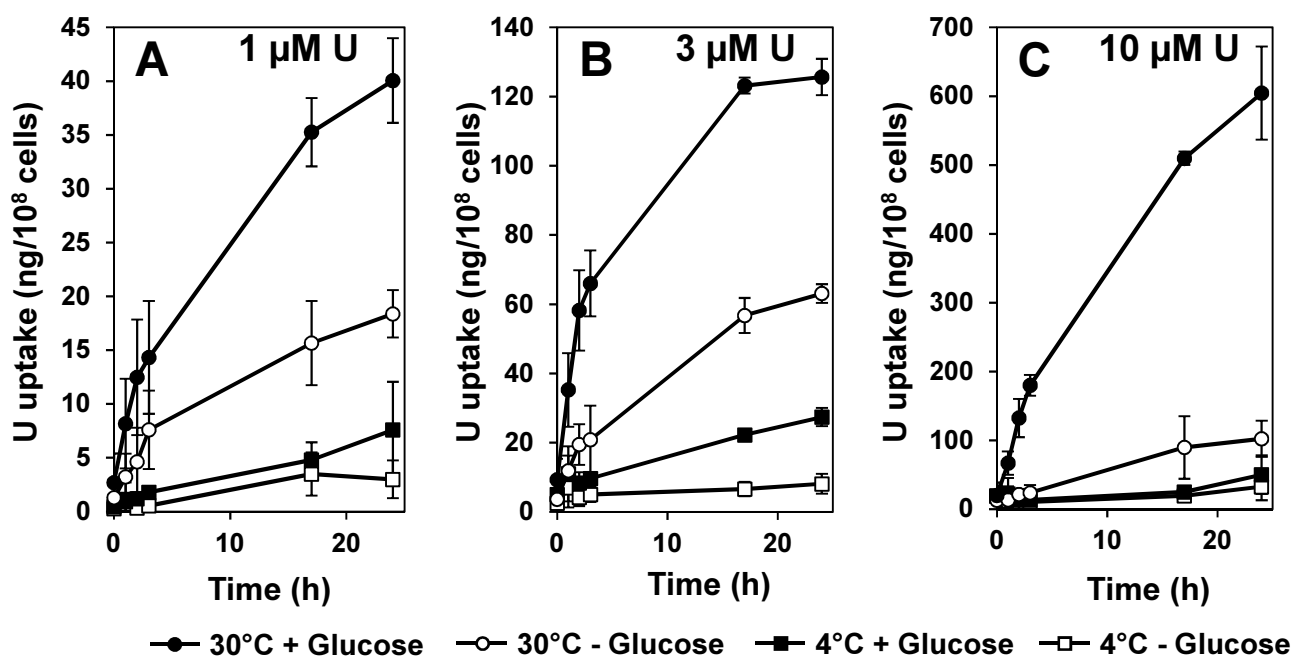
Graphical Abstract



**Figure 1:** Effect of sodium carbonate washes on unmasking U taken up by U-exposed *S. cerevisiae* BY4742 strain cells. Cells grown at late-log phase in YD medium were suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer containing 20 mM glucose, and preincubated for 15 min at 4°C (A) or 30°C (B) before addition of 10  $\mu$ M uranyl nitrate and further incubation at the above-mentioned temperatures. At the time indicated, 0.5 ml aliquots of the cell suspensions were withdrawn and processed as detailed in the Materials and Methods section. U remaining in the incubation medium after centrifugation of the cell suspensions (“Medium”), in the washing solutions after two washes of pelleted cells with 10 mM sodium carbonate (“First wash” and “Second wash”, respectively), and in unwashed and washed cell pellets (“Unwashed cells” and “Washed cells”, respectively), was quantified by ICP-MS. Dotted line indicates U remaining in the incubation medium of an acellular control, after centrifugation (“Acellular control”). A result representative of at least three independent experiments is shown. In all experiments, the U recovery profiles were very similar, although the absolute values varied from an experiment to the other.

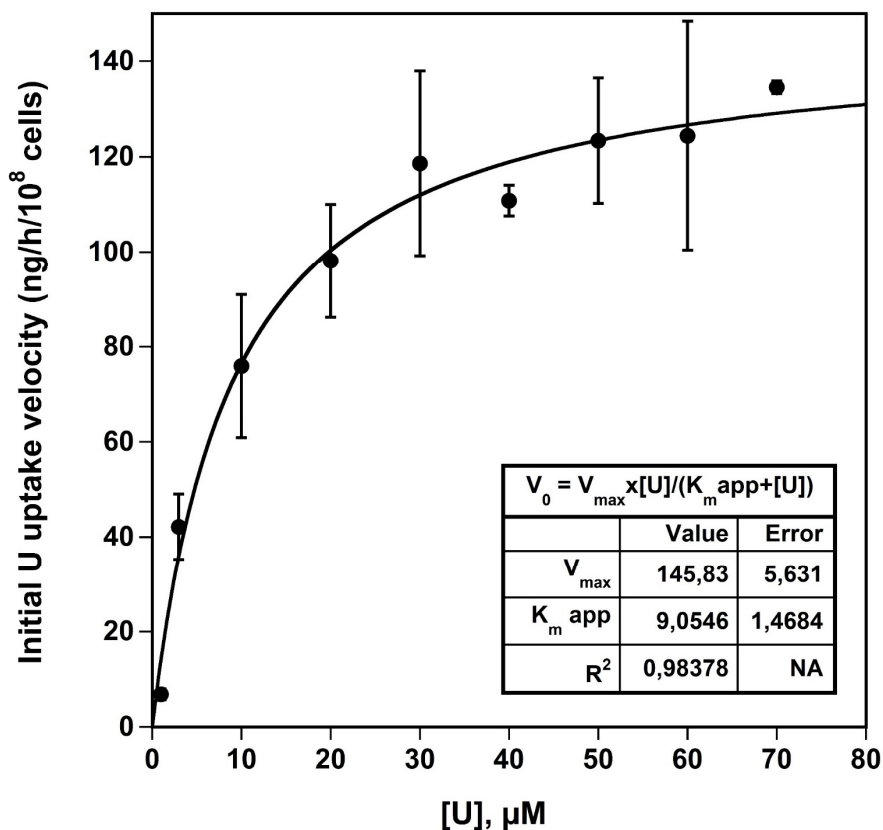
**Figure 1**





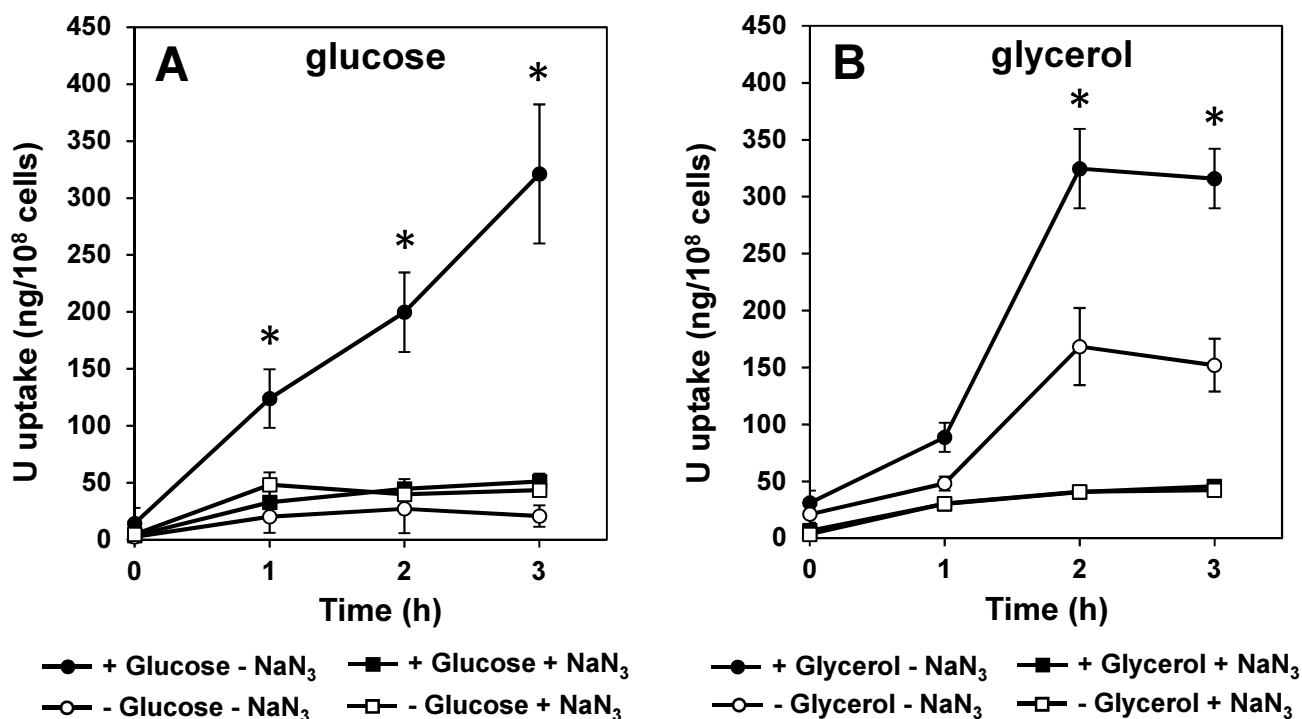
**Figure 2:** Stimulation of U uptake rate by glucose in *S. cerevisiae* BY4742 strain. Cells grown at late-log phase in YD medium were suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer containing 20 mM glucose (+ Glucose) or not (- Glucose), and preincubated for 15 min at 4°C or 30°C before addition of 1 μM (A), 3 μM (B) or 10 μM (C) uranyl nitrate and further incubation at the above-mentioned temperatures. At the time indicated, 0.6 ml aliquots of the cell suspensions were taken and processed as detailed in the Materials and Methods section. U present in cell pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. Bars = SD; n = 3 independent experiments.

**Figure 2**



**Figure 3:** Kinetic parameters determination of U uptake in *S. cerevisiae* BY4742 strain. Cells grown at late-log phase in YD medium were suspended in 10 mM MES, pH 5.5 buffer containing glucose 20 mM, at a concentration of  $2 \times 10^8$  cells/ml and preincubated for 15 min at 30°C before addition of 1 to 70  $\mu\text{M}$  uranyl nitrate and further incubation at 30°C. At appropriate intervals (from 0 to 90 min), aliquots of the cell suspensions were taken and processed as detailed in the Materials and Methods section. U present in cell pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. The initial velocity of U uptake (uptake rate) was then plotted versus U concentration in the reaction medium. Data (dots) were fitted by nonlinear regression to the simple Michaelis-Menten equation (curve) using the Kaleidagraph 4.5 program (Synergy Software, Reading, PA). Bars = SD;  $n = 2$  to 9 independent experiments, for each data point.

**Figure 3**



**Figure 4:** Effect of glycerol as an alternate metabolizable substrate to glucose for U uptake in *S. cerevisiae* BY4742 strain. Cells grown at late-log phase in YD (A) or YG (B) medium were suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer containing either 20 mM glucose (+ Glucose) or not (- Glucose) (A) or 3% glycerol (+ Glycerol) or not (- Glycerol) (B) and 10 mM NaN<sub>3</sub> (+ NaN<sub>3</sub>) or not (- NaN<sub>3</sub>), and preincubated for 15 min at 30°C before addition of 10  $\mu$ M uranyl nitrate and further incubation at the same temperature. At the time indicated, 0.6 ml aliquots of the cell suspensions were taken and processed as detailed in the Materials and Methods section. U present in cell pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. Bars = SD; n = 3 independent experiments. Statistical significance for the comparison of all other conditions versus + Glucose (or + Glycerol) - NaN<sub>3</sub> was determined using a non-parametric Dunnett's test with  $P < 0.001$  (\*).

**Figure 4**

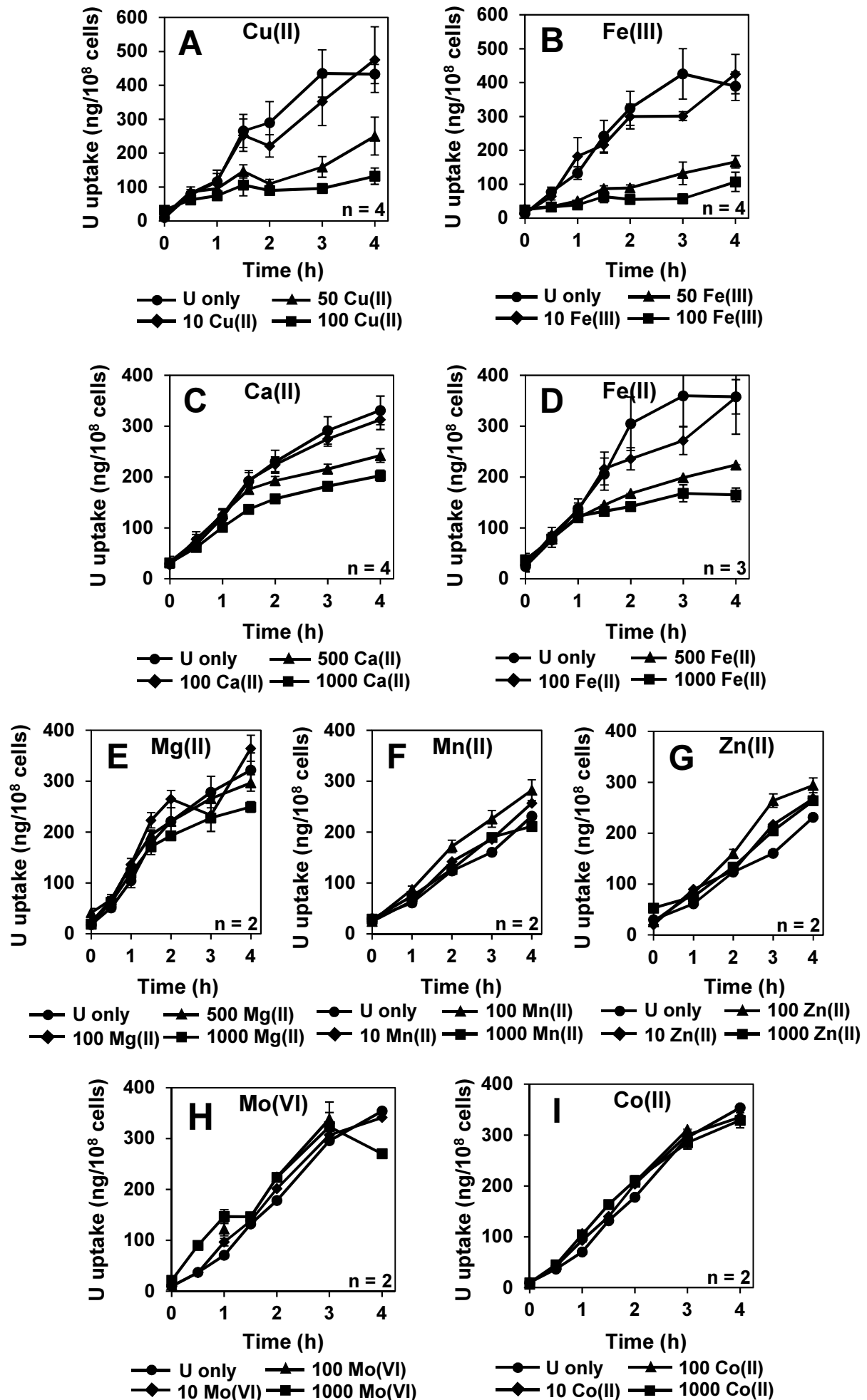
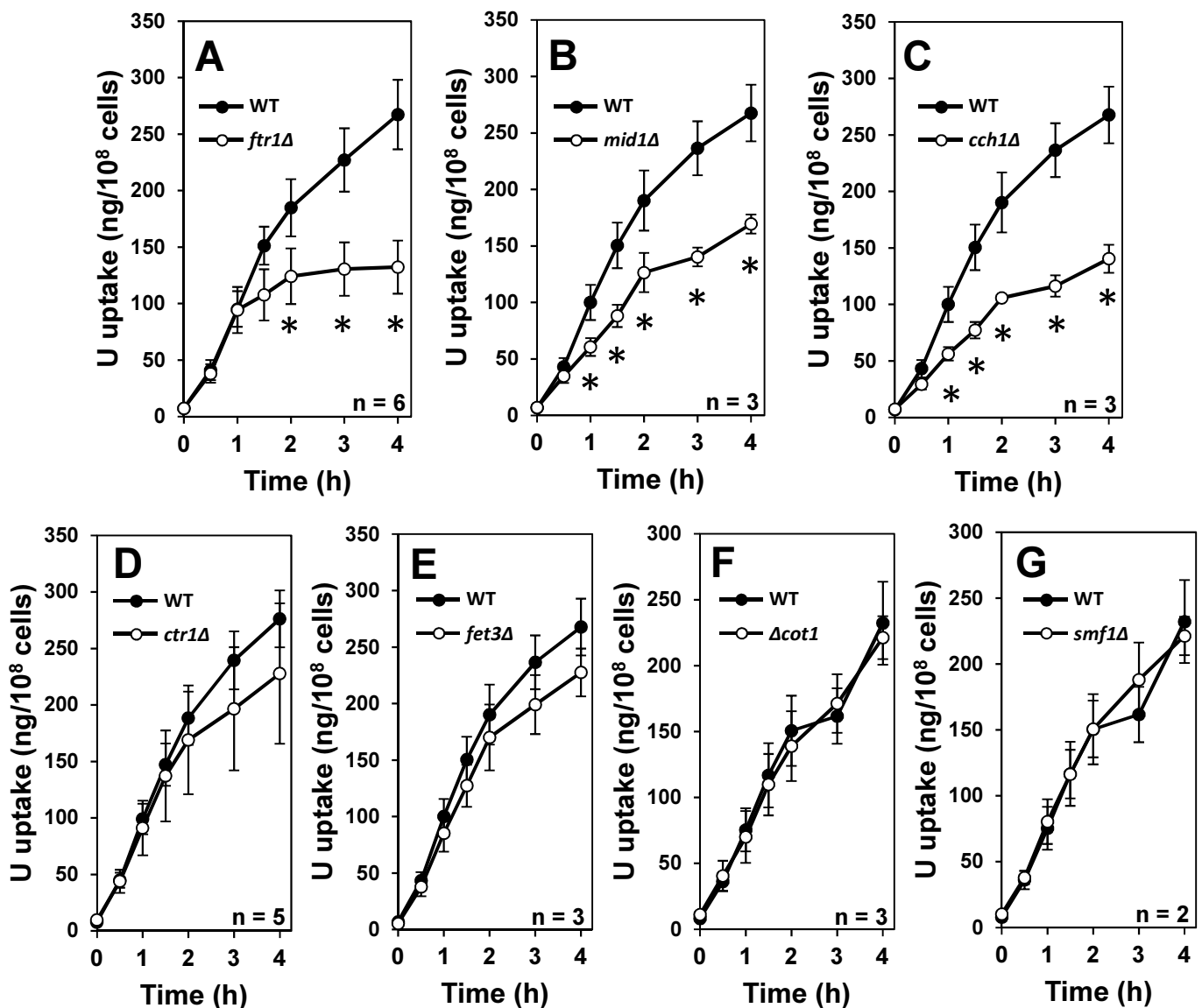


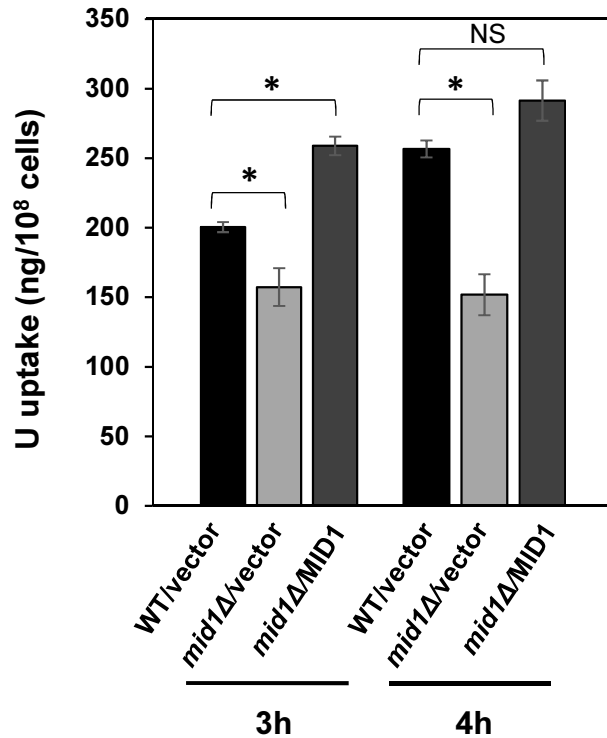
Figure 5 (see legend on the next page)

**Figure 5:** Kinetics of U uptake in *S. cerevisiae* BY4742 strain in the presence of potential essential metal competitors. Cells grown at late-log phase in YD medium were suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer containing 20 mM glucose and various concentrations (0 to 1000  $\mu$ M as indicated) of  $\text{CuSO}_4$  (A),  $\text{FeCl}_3$  (B),  $\text{CaCl}_2$  (C),  $\text{FeSO}_4$  (D),  $\text{MgSO}_4$  (E),  $\text{MnSO}_4$  (F),  $\text{ZnSO}_4$  (G),  $\text{Na}_2\text{MoO}_4$  (H) or  $\text{CoCl}_2$  (I). Cell suspensions were then preincubated for 15 min at 30°C before addition of 10  $\mu$ M uranyl nitrate and further incubation at the same temperature. At the time indicated, 0.5 ml aliquots of the cell suspensions were taken and processed as detailed in the Materials and Methods section. U present in cell pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. Bars = SD when these values exceed the dimensions of the symbols; n = 2 to 4 independent experiments, as indicated.



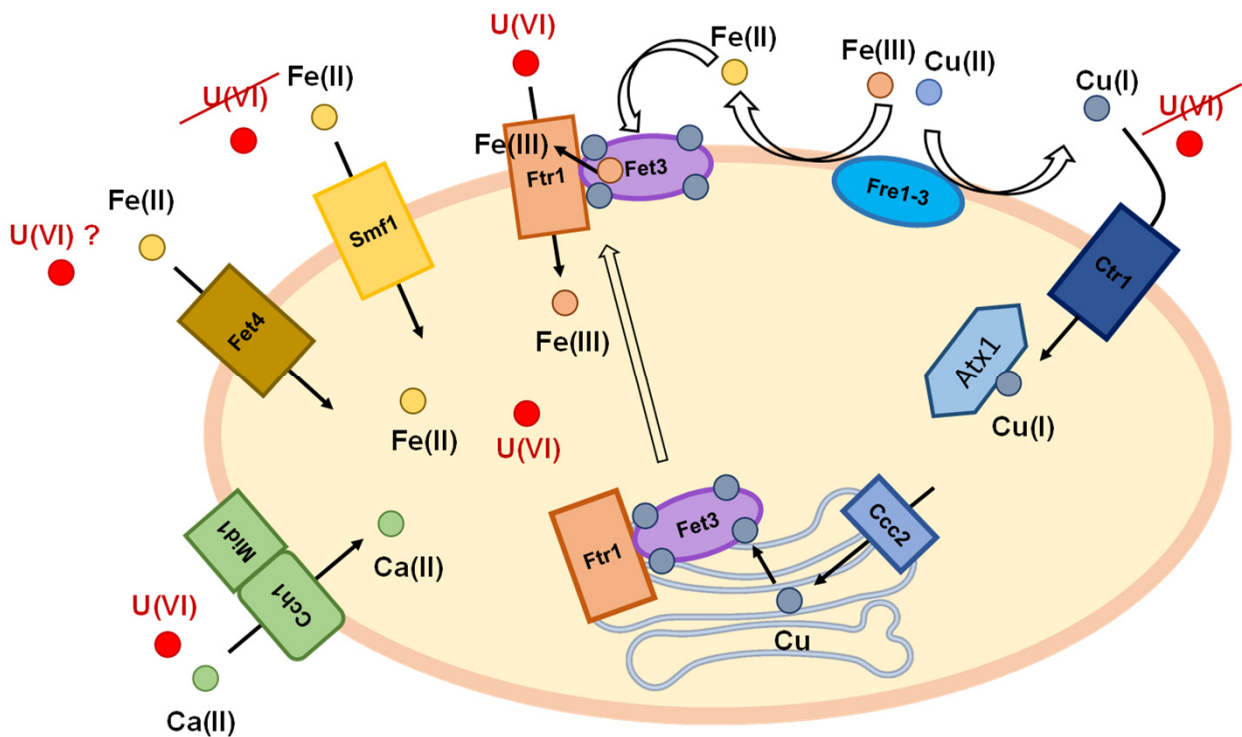
**Figure 6:** Kinetics of U uptake in *S. cerevisiae* mutant strains deleted in essential metal transporters. Yeast strains, *ftr1Δ* (A), *mid1Δ* (B), *cch1Δ* (C), *ctr1Δ* (D), *fet3Δ* (E), *cot1Δ* (F), *smf1Δ* (G) and their wild type counterpart (BY4742 strain) were grown at late-log phase in YD medium and then suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer D containing 20 mM glucose. After a 15 min preincubation at 30°C, 10  $\mu$ M uranyl nitrate was added and cells were further incubated at the same temperature. At the time indicated, 1 ml aliquots of the cell suspensions were taken and processed as detailed in the Materials and Methods section. U present in cell pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. Bars = SD; n = 2 to 6 independent experiments, as indicated. Statistical significance (mutant versus wild-type) was determined using a non-parametric Dunnett's test with  $P < 0.001$  (\*).

**Figure 6**



**Figure 7:** Contribution of the calcium channel Cch1/Mid1 to U entry in *S. cerevisiae* BY4742 strain. The yeast *mid1*Δ mutant as well as the wild-type parental BY4742 strain (WT) were transformed with either the empty low copy number plasmid pRS316 (vector; WT/vector and *mid1*Δ/vector) or YCpMID1 low copy number plasmid (MID1; *mid1*Δ/MID1) encoding the yeast *MID1* gene under the control of its own promoter. Freshly transformed cells were grown at late-log phase in SD/Ca100, plus amino acids and adenine, minus URA medium and then suspended at a concentration of  $2 \times 10^8$  cells/ml in 10 mM MES, pH 5.5 buffer containing 20 mM glucose. After a 15 min preincubation at 30°C, 10 μM uranyl nitrate was added and cells were further incubated at the same temperature. At the time indicated, 1 ml aliquots of the cell suspensions were taken and processed as detailed in the Materials and Methods section. U present in cell pellets washed twice with 10 mM sodium carbonate was quantified by ICP-MS. Error bars = SD; n = 3 independent transformants. Statistical significance was determined using a non-parametric Dunnett's test with  $P < 0.001$  (\*). NS = not significant.

**Figure 7**



**Figure 8:** Recapitulative scheme of U entry routes in *S. cerevisiae*. Our data support the assertion that the high affinity Mid1/Cch1 calcium channel and the high affinity Ftr1/Fet3 iron transport complex promote U uptake in *S. cerevisiae*. Mid1/Cch1: the high affinity calcium channel; Fre1-3: Fre1, Fre2 and Fre3 reductases reduce the Cu(II) to Cu(I) and Fe(III) to Fe(II); Ctr1: the high affinity Cu(I) transporter; Atx1 chaperonin allows copper supply to the Golgi device in concert with Ccc2 transporter. In the path of secretion, copper is incorporated to various metalloproteins including multi-copper ferrous oxidase Fet3. Once copper incorporated in Fet3, the latter is secreted on the cell surface together with the permease Ftr1 with which Fet3 is physically associated, promoting the transport of iron inside the cell (Ftr1/Fet3, the high affinity iron transport complex). Fet4: low affinity and low specificity Fe(II) ion transporter; Smf1: broad-spectrum low affinity and low saturable divalent cation transporter.

**Figure 8**



## Viability of *S. cerevisiae* strains exposed to U

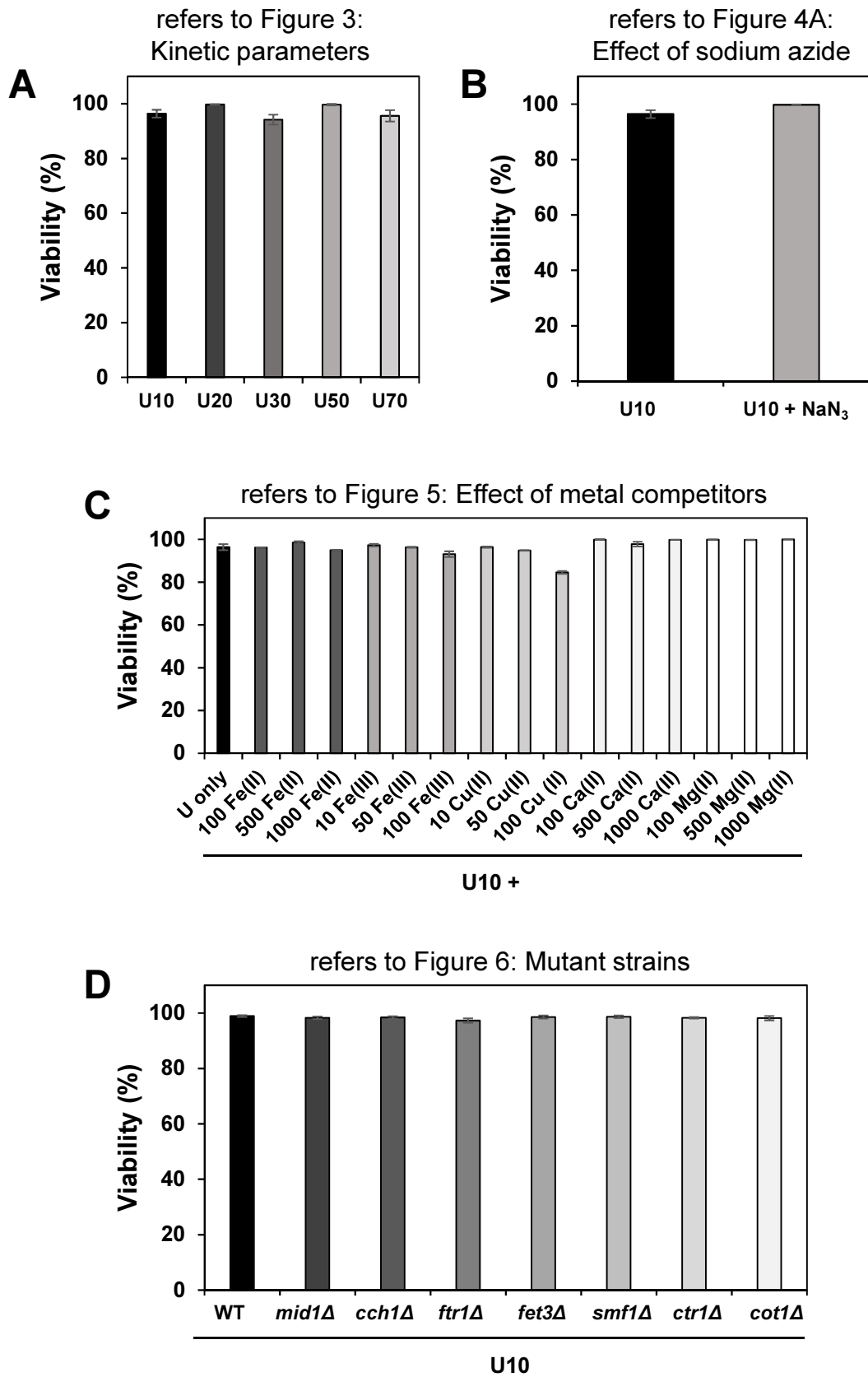


Figure S1 (see legend on the next page)

**Figure S1:** Viability of *S. cerevisiae* strains in the course of the experiments performed in this study. Viability of yeast cell suspensions (Parental BY4742 wild-type strain and the related isogenic mutant strains) was determined by the fluorescein diacetate/propidium iodide staining method using Yeast viability kit and the automated fluorescence cell counter LUNA-FL (Logos Biosystems) after 4h incubation of cells at 30°C in the presence of 20 mM glucose, uranyl nitrate at the indicated concentrations (in  $\mu\text{M}$ ), and other molecules (as indicated) in 10 mM MES pH 5.5 buffer. The details of growth and assay conditions are given in the Materials and Methods section and in the legend of the figures of the corresponding experiments. (A) Viability of cells from experiments in Figure 3 (Kinetics parameters determination); (B) Viability of cells from experiments in Figure 4A (Effect of sodium azide); (C) Viability of cells from experiments in Figure 5 (Effect of metal competitors). Concentrations of competitors are given in  $\mu\text{M}$  from 10 to 1000; (D) Viability of cells from experiments in Figure 6 (Mutant strains deleted in essential metal transporters). The results from viable cell counts are shown as relative values (%) as compared to total counted cells. Error bars = SD; n = 2 to 6 independent experiments, as indicated in the legend of the referred figures.