Supplemental Materials

Molecular Biology of the Cell

Gournas et al.

Supplementary material.

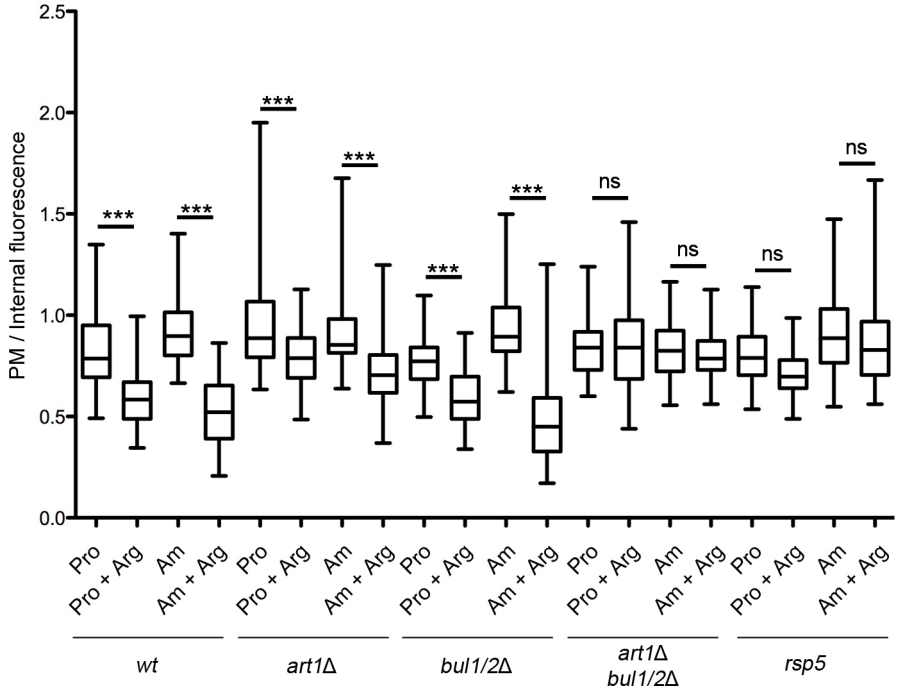


Figure S1. Related to Figure 1.

Quantification of the PM-to-intracellular GFP fluorescence intensity ratios (n, =90 cells) in cells of the Figure 1H experiment, performed as in Figure 1B.

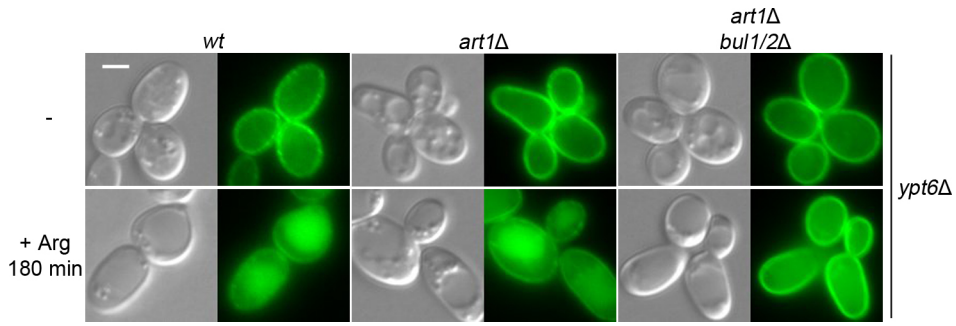
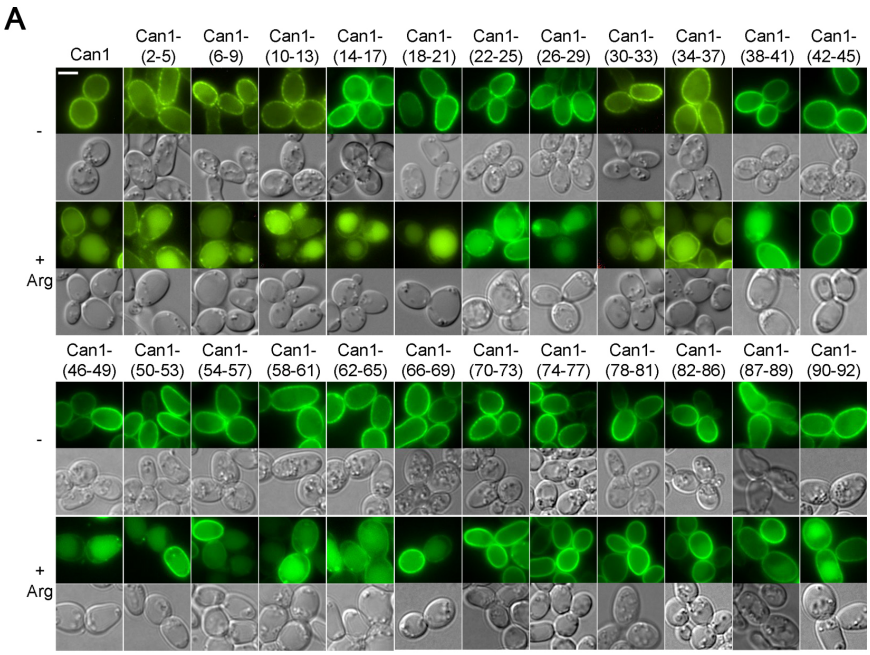
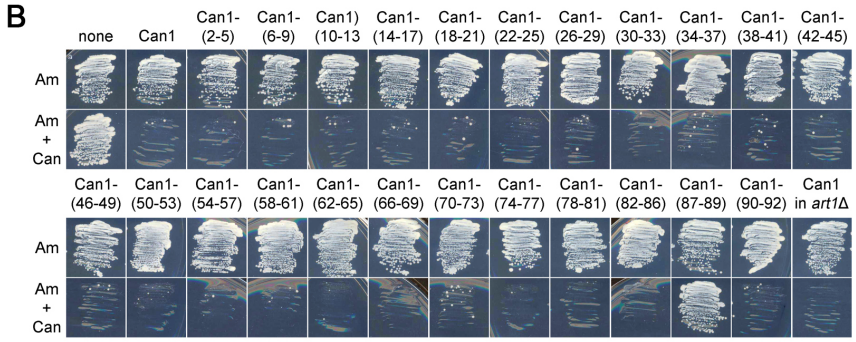
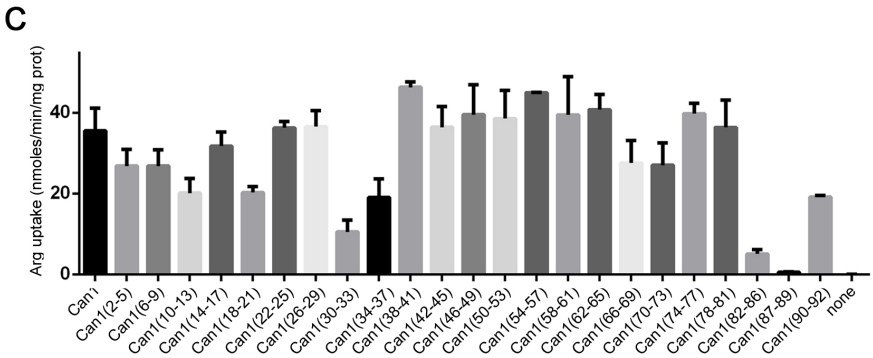


Figure S2. Related to Figure 2.

(A) Strains (all with *ypt6Δ gap1Δ can1Δ* mutations) expressing Can1-GFP from its endogenous promoter were grown in Glu Pro medium. Arg was added for 3 h before imaging by epifluorescence microscopy. The experiment shows that the involvement of Bul1/2 in Can1 downregulation illustrated in Figure 2 is not due to expression of Can1-GFP behind the strong *GAL* promoter.


A
Can1
Can1-(2-5)
Can1-(6-9)
Can1-(10-13)
Can1-(14-17)
Can1-(18-21)
Can1-(22-25)
Can1-(26-29)
Can1-(30-33)
Can1-(34-37)
Can1-(38-41)
Can1-(42-45)
-
+ Arg
Can1-(46-49)
Can1-(50-53)
Can1-(54-57)
Can1-(58-61)
Can1-(62-65)
Can1-(66-69)
Can1-(70-73)
Can1-(74-77)
Can1-(78-81)
Can1-(82-86)
Can1-(87-89)
Can1-(90-92)


B
none
Can1
Can1-(2-5)
Can1-(6-9)
Can1)(10-13
Can1-(14-17)
Can1-(18-21)
Can1-(22-25)
Can1-(26-29)
Can1-(30-33)
Can1-(34-37)
Can1-(38-41)
Can1-(42-45)
Am
Am + Can
Can1-(46-49)
Can1-(50-53)
Can1-(54-57)
Can1-(58-61)
Can1-(62-65)
Can1-(66-69)
Can1-(70-73)
Can1-(74-77)
Can1-(78-81)
Can1-(82-86)
Can1-(87-89)
Can1-(90-92)
Can1 in art1Δ


C
Arg uptake (nmoles/min/mg prot)
40
20
0
Can1
Can1(2-5)
Can1(6-9)
Can1(10-13)
Can1(14-17)
Can1(18-21)
Can1(22-25)
Can1(26-29)
Can1(30-33)
Can1(34-37)
Can1(38-41)
Can1(42-45)
Can1(46-49)
Can1(50-53)
Can1(54-57)
Can1(58-61)
Can1(62-65)
Can1(66-69)
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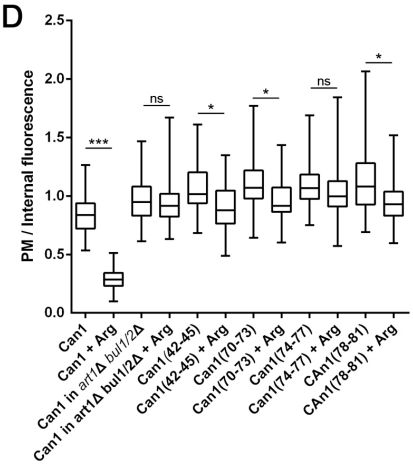
Can1(82-86)
Can1(87-89)
Can1(90-92)
none


D
PM / Internal fluorescence
2.5
2.0
1.5
1.0
0.5
0.0
***
ns
*
*
ns
*
Can1
Can1 + Arg
Can1 in art1Δ bul1/2Δ
Can1 in art1Δ bul1/2Δ + Arg
Can1(42-45)
Can1(42-45) + Arg
Can1(70-73)
Can1(70-73) + Arg
Can1(74-77)
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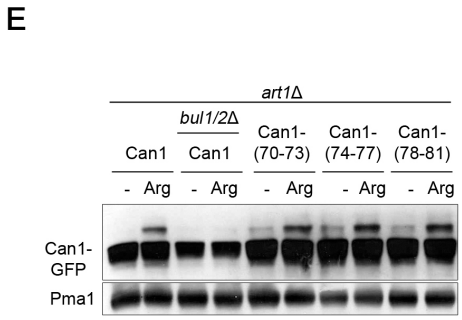
CAn1(78-81)
CAn1(78-81) + Arg


E
art1Δ
bul1/2Δ
Can1
Can1
Can1-(70-73)
Can1-(74-77)
Can1-(78-81)
- Arg
Can1-GFP
Pma1

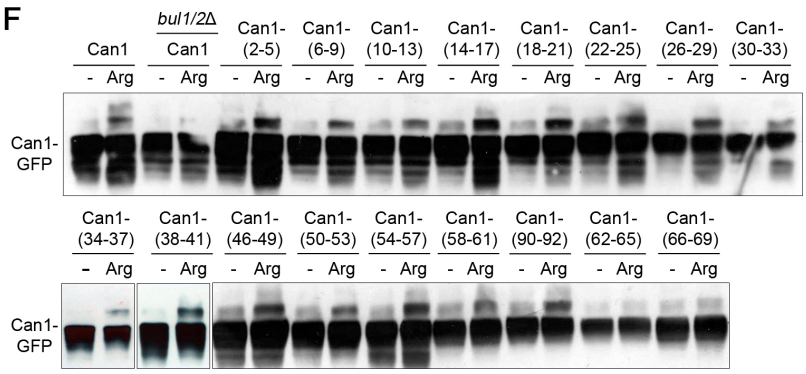

F
bul1/2Δ
Can1
Can1
Can1-(2-5)
Can1-(6-9)
Can1-(10-13)
Can1-(14-17)
Can1-(18-21)
Can1-(22-25)
Can1-(26-29)
Can1-(30-33)
Can1-(34-37)
Can1-(38-41)
Can1-(46-49)
Can1-(50-53)
Can1-(54-57)
Can1-(58-61)
Can1-(90-92)
Can1-(62-65)
Can1-(66-69)
- Arg
Can1-GFP

Figure S3. Mutational analysis of Can1's N-tail and impact on the permease localization, Arg-induced endocytosis, activity, and Bul-dependent ubiquitylation. Related to Figures 3 and 4.

(A) Cells of the *gap1*Δ *can1*Δ strain expessing Can1-GFP or the Can1-GFP mutants carrying Ala substitutions of the indicated residues were grown in Gal Pro. Glu was added for 1.5 h and then Arg for 3h, before analysis by epifluorescence microscopy.

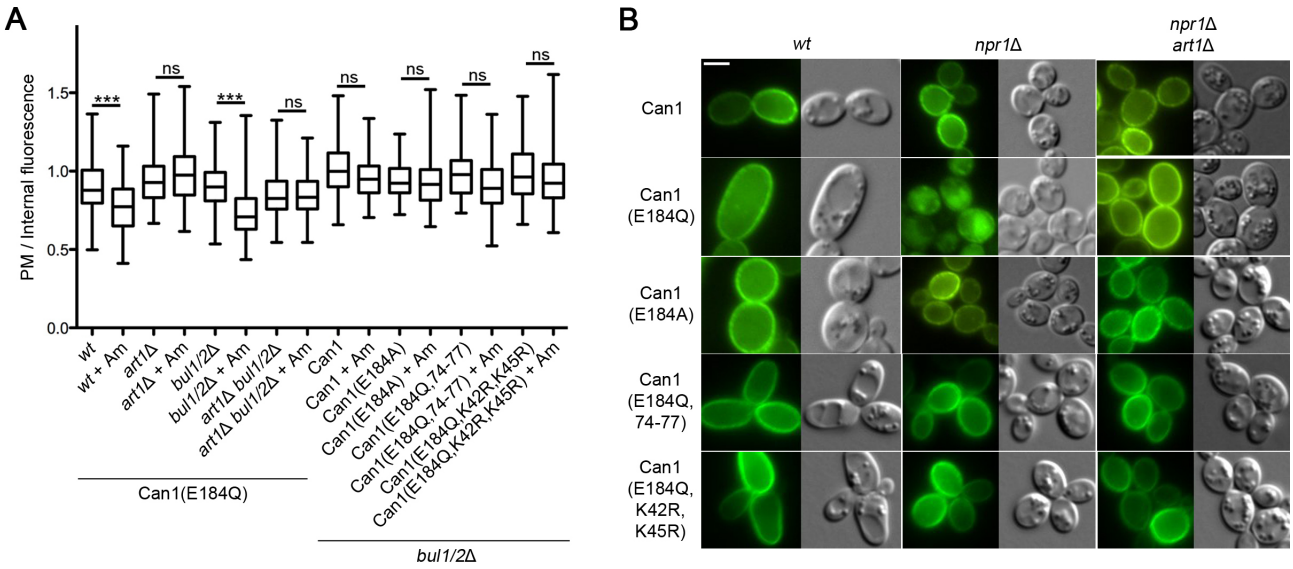
(B) Cells used in A, and cells expressing none Can1-GFP (pURA3, empty vector), were grown for 3 days on solid Glu Am medium with or without canavanine.

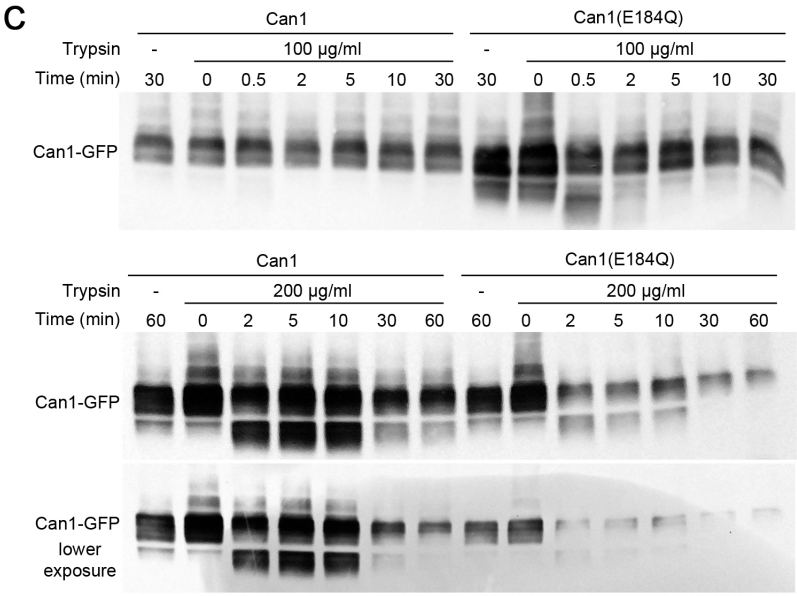
(C) Initial uptake of ^{14}C-Arg was measured in cells used in A/B grown on Gal Pro medium.

(D). Quantifications of the microscopy images of Figure 3B, as in 1B. ***, $P<0.001$; *, P<0,05; ns, nonsignificant, $P>0.05$.

(F, G) Strains (all with *gap1*Δ *can1*Δ mutations) expressing Can1-GFP and the indicated Ala-substituted Can1-GFP mutants were grown on Gal Am medium. Glu was added for 0.5 h and then Arg for 15 min. Total protein extracts from culture samples were then probed with antibodies against GFP.

Comment regarding Figure S3. Can1-GFP mutants bearing Ala substitutions of 3-4 consecutive residues of the N-tail (residues 2-92) have been examined by epifluorescence microscopy (Figure S3A). All were properly targeted to the plasma membrane and most were targeted to the vacuole after Arg addition (Figure S3A). Five active mutants showed impaired Arg-induced vacuolar sorting. One of them is altered in the region 42-KDEK-45 further shown to contain the main Ub-acceptor Lys residues of Can1 (Figures 3C, D, E). The four others cluster in the region between residues 70 and 86 (Figures 3A, S3A). As resistance to Arg-induced endocytosis can be due to a loss of Arg transport activity (Ghaddar *et al.*, 2014b), we tested the functionality of the mutants by growh tests and direct measurement of ^{14}C-Arg uptake. For growth tests, senstivity to canavanine, a toxic arginine analogue transported via Can1, was tested (Figure S3B). The results show that all Can1-GFP mutants are functional, except the one with Ala substitutions of the 87-ELK-89 residues. They also show that the mutants altered in the 70-81 region and resistant to Arg-induced endocytosis are competent for Arg transport. The uptake measurements (Figure S3C) also showed that the mutant bearing Ala substitutions of 82-EVQN-86 residues retained very low Arg uptake capacity, despite confering sensitivity to canavanine (Figure S3B). This result is likely explainable by the very high toxicity of canavanine and illustrates that direct measurements of Can1 activities are needed for accurately determining the functionality of transporter mutants. Thus, the mutant in residues 82-EVQN-86 could be resistant to Arg-induced endocytosis either because it is part of the cluster of residues 70-81, or because of its low Arg transport. As this question remain open, we decided not to include it as part of the putative Art1-binding region (Figure 3A).
We also sought to identify the N-tail region of Can1 involved in Bul-dependent ubiquitylation. For this, we first tested whether the region required for Art1-mediated Can1 endocytosis is also required for the Bul1/2-dependent ubiquitylation. We thus expressed the corresponding mutants in an *art1*Δ strain and tested for Arg-induced ubiquitylation. Interestingly, the mutants altered in the 70-81 region were still ubiquitylated in *art1*Δ cells after Arg addition (Figure S3E) suggesting that the region necessary for the Bul1/2-dependent ubiquitylation of Can1 is different from the one for Art1. We therefore examined Arg-induced ubiquitylation of the remaining Ala-substituted Can1 mutants in an *art1*Δ strain (Figure S3F). Interestingly, two fully functional mutants bearing substitutions of residues 62-69 showed resistance to Bul1/2-dependent ubiquitylation. This region is thus proposed to correspond to the binding site of Bul1/2 (Figure 3A).


A
PM / Internal fluorescence
1.5
1.0
0.5
0.0
***
ns
***
ns
ns
ns
ns
ns
wt
wt + Am
art1Δ
art1Δ + Am
bul1/2Δ
bul1/2Δ + Am
art1Δ bul1/2Δ
art1Δ bul1/2Δ + Am
Can1
Can1 + Am
Can1(E184A)
Can1(E184A) + Am
Can1(E184Q,74-77)
Can1(E184Q,74-77) + Am
Can1(E184Q,K42R,K45R)
Can1(E184Q,K42R,K45R) + Am
Can1(E184Q)
bul1/2Δ
B
wt
npr1Δ
npr1Δ art1Δ
Can1
Can1 (E184Q)
Can1 (E184A)
Can1 (E184Q, 74-77)
Can1 (E184Q, K42R, K45R)


C
Can1
Can1(E184Q)
Trypsin
-
100 µg/ml
Time (min)
30 0 0.5 2 5 10 30
Can1-GFP
200 µg/ml
60 0 2 5 10 30 60
Can1-GFP lower exposure

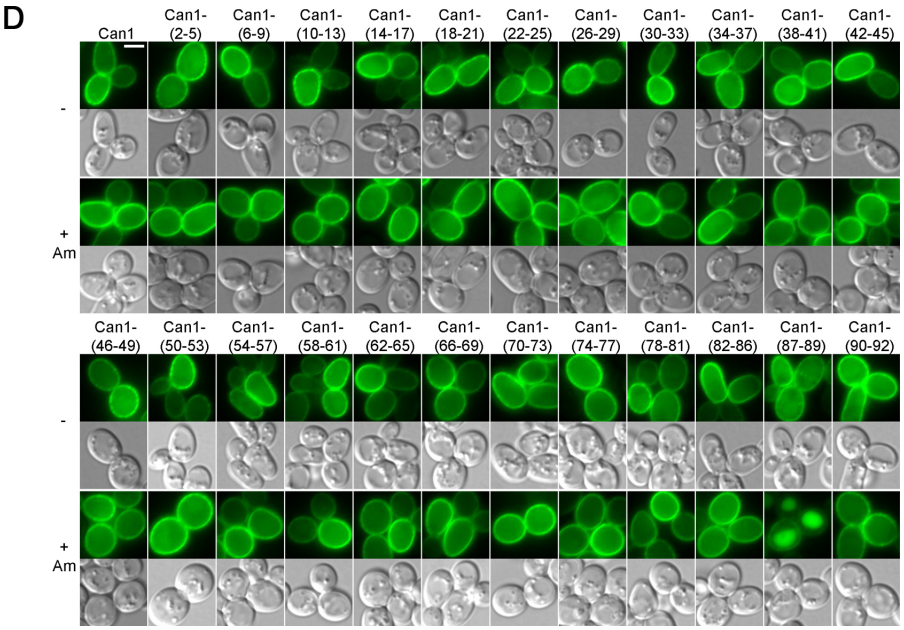

D
Can1
Can1-(2-5)
Can1-(6-9)
Can1-(10-13)
Can1-(14-17)
Can1-(18-21)
Can1-(22-25)
Can1-(26-29)
Can1-(30-33)
Can1-(34-37)
Can1-(38-41)
Can1-(42-45)
Can1-(46-49)
Can1-(50-53)
Can1-(54-57)
Can1-(58-61)
Can1-(62-65)
Can1-(66-69)
Can1-(70-73)
Can1-(74-77)
Can1-(78-81)
Can1-(82-86)
Can1-(87-89)
Can1-(90-92)
-
+ Am

Figure S4. Analysis of Can1 mutants downregulated upon TORC1 activation. Related to Figure 6.

(A) Quantification of the fluorescence signals of Figure 6B, as in Figure 1B. The PM to intracellular GFP fluorescence intensity ratios are plotted (n, =90 cells).

(B) Epifluorescence microscopy analysis of Can1-GFP and the indicated Can1-GFP mutants in wild-type and mutant cells grown on Gal Pro medium. Glu was added for 1.5 h prior to observation.

(C) Immunoblot analysis of membrane enriched protein extracts from cells grown in Gal Pro medium and expressing either Can1-GFP or Can1(184Q)-GFP , incubated or not (-) with 100 (upper) or 200 (lower) µg/ml of trypsin for the indicated times. For the latter condition, two different exposures of the film are shown.

(D) Cells of the *gap1*Δ *can1*Δ strain expressing Can1-GFP or the indicated Ala-substituted N-tail Can1-GFP mutants were grown on Gal Pro medium. Glu was added for 1.5 h, and subsequently Am for 3 h, prior to observation by epifluorescence microscopy.

Comment regarding Fig. S4C. To determine whether Can1(E184Q) adopts a different conformation (see main text), we compared the sensitivity of Can1-GFP and Can1(E184Q)-GFP to limited trypsinolysis. Detergent-free membrane-enriched protein extracts were first isolated from Pro grown cells. In these cell extracts, membrane proteins including Can1 should remain embedded in membranes. A Can1 mutant adopting another conformation could differentially expose the N- and C-tails and/or the extra- and intracellular loops, resulting in altered sensitivity to trypsin. For instance, a similar approach revealed that a Gap1 permease newly synthesized under conditions of sphingolipid depletion is not only inactive but also misfolded (Lauwers et al., 2007). The result shows that Can1(E184Q), in two different experiments using different concentrations of trypsin and different incubation times, is more sensitive to trypsin compared to wt Can1 (Figure S4C). This suggests that the E184Q substitution favors a conformation that is different from the native protein.

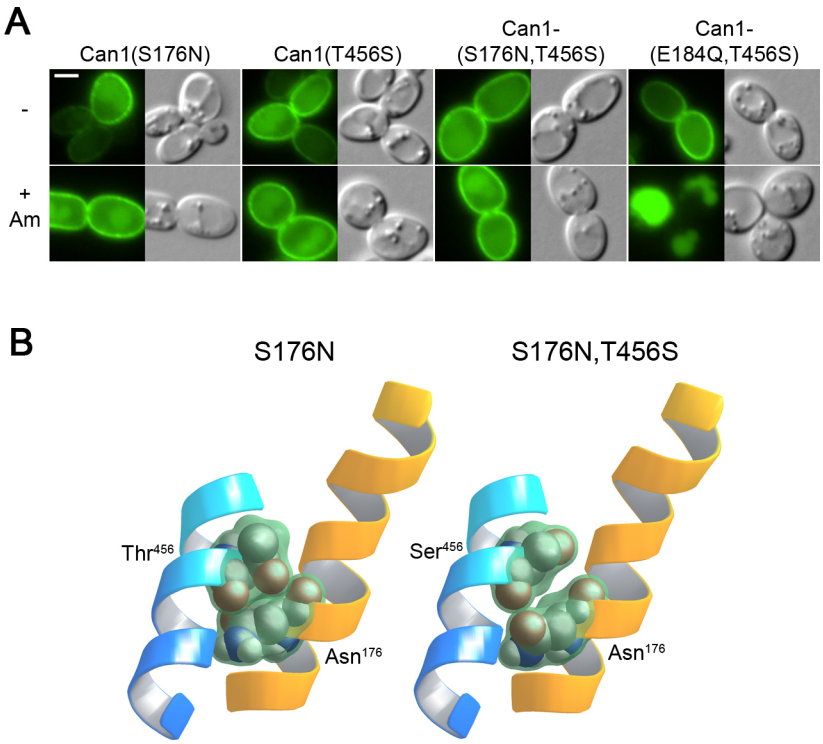


Figure S5. Related to Figure 7.

(A) Epifluorescence microscopy analysis of a *gap1*Δ *can1*Δ *bul1/2*Δ strain expressing Can1-GFP or the indicated Can1-GFP mutants, grown on Gal Pro. Glu was added for 1.5 h and then Am for 3 h.

(B) Representative close view of the region encompassing Asn176 in Can1(S176N,T456S) and in Can1(S176N) showing the structural proximity between the 176 and 456 residues. Residues at positions 176 and 456 are depicted as balls and by their molecule surface. Empty spaces are observed in most of the Can1(S176N/456S) 3D models in contrast to those of Can1(S176N).

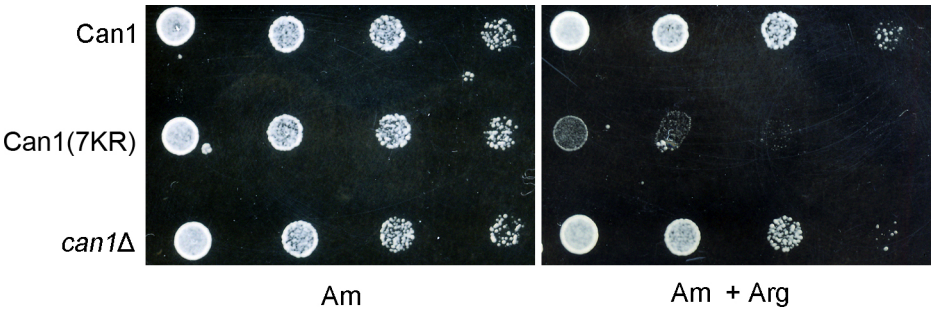


Figure S6. Deficiency in Can1 endocytosis causes Arg toxicity in yeast cells.
Growth tests in Am or Am + Arg media of *gap1*Δ *can1*Δ strains containing plasmids expressing Can1-GFP, Can1(7KR)-GFP, or none Can1-GFP (*can1*Δ).

Supplementary Tables

Table S1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
23344c	*ura3*	Laboratory collection
EK008	*gap1Δ ura3*	Laboratory collection
27038a	*npi1-1(rsp5) ura3*	(Hein *et al.*, 1995)
35283a	*can1Δ gap1Δ npi1-1(rsp5) ura3*	This study
JA937	*art1Δ gap1Δ ura3*	(Ghaddar *et al.*, 2014b)
JA939	*art1Δ bul1Δ bul2Δ gap1Δ ura3*	This study
JA493	*bul1Δ bul2Δ gap1Δ ura3*	(Ghaddar *et al.*, 2014b)
ES029	*can1Δ gap1Δ ura3*	This study
ES031	*art1Δ can1Δ gap1Δ ura3*	This study
ES032	*can1Δ ura3*	This study
CG010	*can1Δ lyp1Δ gap1Δ ura3*	This study
CG052	*art1Δ bul1Δ bul2Δ can1Δ gap1Δ ura3*	This study
CG054	*bul1Δ bul2Δ can1Δ gap1Δ ura3*	This study
CG058	*can1Δ gap1Δ ura3 leu2*	This study
CG059	*art1Δ can1Δ gap1Δ ura3 leu2*	This study
CG063	*art1Δ bul1Δ bul2Δ can1Δ gap1Δ ura3 leu2*	This study
CG064	*ypt6Δ can1Δ gap1Δ ura3*	This study
CG074	*art1Δ ypt6Δ can1Δ gap1Δ ura3*	This study
CG076	*ypt6Δ bul1Δ bul2Δ can1Δ gap1Δ ura3*	This study
CG078	*ypt6Δ art1Δ bul1Δ bul2Δ can1Δ gap1Δ ura3*	This study
JA482	*npr1Δ ura3*	This study
JA410	*bul1Δ bul2Δ npr1Δ ura3*	(Soetens *et al.*, 2001)
CG022	*art1Δ npr1Δ ura3*	This study
CG024	*art1Δ bul1Δ bul2Δ npr1Δ ura3*	This study
Mup1-GFP	*MUP1-GFP his3Δ leu2Δ met15Δ ura3Δ*	Wedlich-Söldner laboratory
MN041	*ssy1Δ mup1Δ mup3Δ gap1Δ ura3*	This study

Table S2. Plasmids used in this study

Plasmid	Description	Reference or source
pFL038	CEN-ARS (URA3)	(Bonneaud *et al.*, 1991)
pJOD010	CEN-ARS GAL1-GAP1-GFP (URA3)	(Nikko *et al.*, 2003)
pCJ313	CEN-ARS LEU2 HIS3 MET25	Lab collection
pAS103	YEp-HA-NPR1 (URA3)	(Schmidt *et al.*, 1998)
pKG036	CEN-ARS CAN1-GFP (URA3)	(Ghaddar *et al.*, 2014a)
pNAM001	CEN-ARS LYP1-GFP (URA3)	(Ghaddar *et al.*, 2014a)
pCJ563	CEN-ARS GAL1-CAN1-GFP (URA3)	(Ghaddar *et al.*, 2014a)
pCJ560	CEN-ARS GAL1-CAN1(E184Q)-GFP (URA3)	This study
pCJ565	CEN-ARS GAL1-CAN1(T180R)-GFP (URA3)	This study
pCJ569	CEN-ARS GAL1-CAN1(T456S)-GFP (URA3)	This study
pCJ574	CEN-ARS GAL1-CAN1(E184A)-GFP (URA3)	This study
pCG002	CEN-ARS GAL1-CAN1(TNSK5-AAAA)-GFP (URA3)	This study
pCG003	CEN-ARS GAL1-CAN1((IEEK13-AAAA)-GFP (URA3)	This study
pCG004	CEN-ARS GAL1-CAN1((EDAD9-AAAA)-GFP (URA3)	This study
pCG005	CEN-ARS GAL1-CAN1((HMYN17-AAAA)-GFP (URA3)	This study
pCG006	CEN-ARS GAL1-CAN1((EPVT21-AAAA)-GFP (URA3)	This study
pCG008	CEN-ARS GAL1-CAN1((SQTH33-AAAA)-GFP (URA3)	This study
pCG010	CEN-ARS GAL1-CAN1((HRRG37-AAAA)-GFP (URA3)	This study
pCG011	CEN-ARS GAL1-CAN1(TLFH25-AAAA)-GFP (URA3)	This study
pCG012	CEN-ARS GAL1-CAN1(DVE28-AAAA)-GFP (URA3)	This study
pCG014	CEN-ARS GAL1-CAN1(SIPL41-AAAA)-GFP (URA3)	This study
pCG015	CEN-ARS GAL1-CAN1(SKEL49-AAAA)-GFP (URA3)	This study
pCG016	CEN-ARS GAL1-CAN1(YPLR53-AAAA)-GFP (URA3)	This study
pCG017	CEN-ARS GAL1-CAN1(SFPT57-AAAA)-GFP (URA3)	This study
pCG018	CEN-ARS GAL1-CAN1(RVNG61-AAAA)-GFP (URA3)	This study
pCG019	CEN-ARS GAL1-CAN1(KDEK45-AAAA)-GFP (URA3)	This study
pCG020	CEN-ARS GAL1-CAN1(EDTF65-AAAA)-GFP (URA3)	This study
pCG022	CEN-ARS GAL1-CAN1(EDEG77-AAAA)-GFP (URA3)	This study
pCG024	CEN-ARS GAL1-CAN1(EVQN81-AAAA)-GFP (URA3)	This study
pCG025	CEN-ARS GAL1-CAN1(ELK89-AAAA)-GFP (URA3)	This study
pCG026	CEN-ARS GAL1-CAN1(QRH92-AAAA)-GFP (URA3)	This study
pCG028	CEN-ARS GAL1-CAN1((S176N-E184Q-T456S)-GFP (URA3)	This study
pCG029	CEN-ARS GAL1-CAN1(E184Q-T456S)-GFP (URA3)	This study
pCG030	CEN-ARS GAL1-CAN1(S176N-T456S)-GFP (URA3)	This study
pCG031	CEN-ARS GAL1-CAN1(S176N-E184Q)-GFP (URA3)	This study
pCG032	CEN-ARS GAL1-CAN1(S176N)-GFP (URA3)	This study
pCG033	CEN-ARS GAL1-CAN1(SMED69-AAAA)-GFP (URA3)	This study
pCG035	CEN-ARS GAL1-CAN1(GIGD73-AAAA)-GFP (URA3)	This study
pCG037	CEN-ARS GAL1-CAN1(EVKR86-AAAA)-GFP (URA3)	This study
pCG054	CEN-ARS GAL1-CAN1(K42R/K45R)-GFP (URA3)	This study
pCG056	CEN-ARS GAL1-CAN1(K42R)-GFP (URA3)	This study
pCG058	CEN-ARS GAL1-CAN1(K45R)-GFP (URA3)	This study
pCG064	CEN-ARS GAL1-CAN1((EDEG77-AAAA/E184Q)-GFP (URA3)	This study
pCG065	CEN-ARS GAL1-CAN1(T180R/E184Q)-GFP (URA3)	This study
pCG069	CEN-ARS GAL1-CAN1(SMED69-AAAA/EDEG77-AAAA)-GFP (URA3)	This study
pCG077	CEN-ARS GAL1-CAN1(K42R/K45R/E184Q)-GFP (URA3)	This study

pCG080	CEN-ARS GAL1-CAN1(K5R/K13R/K42R/K45R/K47R/K85R/K89R)-GFP (URA3)	This study
pCG108	CEN-ARS GAL1-CAN1(K5R/K13R/K42R/K45R/K47R/K85R/K89R)-mCherry (LEU2)	This study
pBOA010	CEN-ARS Sec7-mCherry (LEU2)	(Llinares *et al.*, 2015)

Supplementary References

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