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Quality Control and Substrate-Dependent Downregulation of the Nutrient Transporter Fur4

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Abstract

Upon exposure to stress conditions, unfolded cell-surface nutrient transporters are rapidly internalized and degraded via the multivesicular body (MVB) pathway. Similarly, high concentrations of nutrients result in the downregulation of the corresponding transporters. Our studies using the yeast transporter Fur4 revealed that substrate-induced downregulation and quality control utilize a common mechanism. This mechanism is based on a conformation-sensing domain, termed LID (Loop Interaction Domain), that regulates site-specific ubiquitination (also known as degron). Conformational alterations in the transporter induced by unfolding or substrate binding are transmitted to the LID, rendering the degron accessible for ubiquitination by Rsp5. As a consequence, the transporter is rapidly degraded. We propose that the LID-degron system is a conserved, chaperone-independent mechanism responsible for conformation-induced downregulation of many cell-surface transporters under physiological and pathological conditions.

Keywords

Endocytosis; MVB pathway; ESCRT; ubiquitin; degron; protein degradation; plasma membrane protein; protein trafficking

Introduction

The levels of nutrient transporters at the plasma membrane are regulated by several mechanisms, including regulation at the level of protein synthesis and degradation. These regulatory systems ensure a balance between the uptake of nutrients from the environment and the requirement for these nutrients by the metabolism of the cell. The substrate-dependent regulation of transporters has been studied in detail, utilizing the yeast high-affinity uracil importer Fur4 (reviewed in (1). High uracil concentrations in the growth medium not only suppress the transcription of the *FUR4* gene but also result in the degradation of both its mRNA and protein (2). Maintaining artificially high Fur4 levels under these conditions has been shown to cause cellular accumulation of toxic levels of uracil, demonstrating the importance of Fur4 downregulation in the presence of high-substrate concentrations (2, 3). The substrate-induced degradation of Fur4 involves phosphorylation of a PEST-like sequence in the N-terminal region of the protein and the ubiquitination of two neighboring lysine residues by the ubiquitin-ligase Rsp5 (Figure 1A; (4–6). Although phosphorylation of the PEST region increases the efficiency of Fur4 downregulation, it is not essential for ubiquitination and degradation of the transporter.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ubiquitination of Fur4 causes its rapid internalization and subsequent delivery, via the MVB pathway, to the vacuole for degradation.

Although the general scheme of Fur4 downregulation has been elucidated, the precise mechanism that triggers substrate-dependent Fur4 degradation is not known. Studies of Fur4 and other related transporters indicated that the interaction of the substrate with the substrate-binding site of the transporter is responsible for rapid downregulation of the protein, suggesting that the transporter itself serves as a sensor for the nutrient concentration present. However, conflicting models have been proposed with regard to the mechanism of sensing. Some studies supported the notion that active transport is necessary to induce ubiquitination of transporters (7) whereas other data indicated that the concentration of cytoplasmic substrate is key for the downregulation (3, 8). In both models, conformational changes of the transporter itself are proposed to trigger the degradation of the protein.

Fur4 belongs to the Nucleobase:Cation Symporter-1 (NCS1) family of transporters and imports uracil by using the proton gradient across the yeast plasma membrane. Crystal structure analysis of the bacterial homologue Mhp1 gave detailed insights into the mechanism of substrate import by this group of transporters (9, 10). Fur4 is composed of 12 transmembrane domains that facilitate substrate import described by an alternative-access model. The ground state of the transporter is the outward-facing open conformation that is able to bind extracellular substrate. Upon binding of the substrate, the transporter changes to an outward-facing occluded and then to an inward-facing occluded state. Finally, the transporter releases its substrate into the cytoplasm, resulting in an inward-facing open conformation. Any of these conformational changes might be key to trigger substrate-induced downregulation of the transporter.

Because nutrient transporters are gateways between the extracellular and intracellular environment, the fidelity and specificity of transport activity is of upmost importance for cell survival. Therefore, quality control that ensures the proper function of cell-surface nutrient transporters has to be highly sensitive and efficient in recognizing folding problems. It is well documented that environmental stresses that cause protein unfolding, such as heat shock or exposure to harmful chemicals, result in the rapid downregulation of cell-surface proteins, including Fur4, by a ubiquitin-dependent mechanism (11).

The data presented in this study indicate that both substrate-dependent downregulation and quality control of nutrient transporters are mediated by the same intrinsic, conformation-sensing mechanisms. This mechanism is able to recognize deviations from the ground state of a transporter and trigger its ubiquitination and subsequent degradation.

Results

N-terminal domain is required for Fur4 quality control

Previous studies identified phosphorylation and ubiquitination sites in the N-terminus of Fur4 required for the rapid substrate-dependent degradation of the transporter (PEST and Ub in Figure 1A) (5). Deletion of the first 60 amino acids of Fur4 (Fur4^{N60}), which removes these N-terminal modification sites, results in stabilization of the transporter on the plasma membrane, even in the presence of high uracil concentrations (Figure 2A, lane #2). Surprisingly, the same deletion also inhibited rapid downregulation of Fur4-GFP at high temperature or in the presence of peroxide, conditions that are thought to induce conformational changes, resulting in a non-ground state or unfolded state of the protein. Whereas heat-shock (1h at 37°C) or peroxide treatment (0.005%, 30 min) of yeast cells resulted in the efficient delivery of wild-type Fur4-GFP to the vacuole for degradation, the mutant protein Fur4^{N60}-GFP remained at the plasma membrane (Figure 2A, lane #2).

Uracil-uptake assays demonstrated that peroxide treatment inhibited the uracil import of cells expressing Fur4^{N60}-GFP, supporting the idea that peroxide renders Fur4 non-functional (Figure 2B). However, shifting Fur4^{N60}-GFP expressing cells to 37°C did not inhibit uracil transport, suggesting that these stress conditions are not severe enough to cause irreversible unfolding of Fur4 (data not shown). Together, these observations suggested that the quality control system, which is responsible for the rapid degradation of unfolded or partially unfolded Fur4, is dependent on the same N-terminal modifications that trigger the substrate-dependent downregulation. In particular, ubiquitination at the sites K38 and K41 was found to be essential for Fur4 quality control, since mutating these sites completely abolished stress-induced Fur4-GFP downregulation (Fur4^{K38,41R}-GFP, Figure 2A, lane #3). The ubiquitin ligase Rsp5 has been shown to be responsible for Fur4 ubiquitination at high substrate concentrations or at high temperature (6). Consistent with these previous reports, we observed that yeast strains expressing the mutant allele *rsp5-1* show no uracil- or stress-induced downregulation of Fur4-GFP (Figure 2A, lane #6). This result further supported the notion that Fur4 quality control is mediated by the same mechanism as substrate-dependent downregulation. However, in contrast to uracil-dependent downregulation, stress-induced degradation of Fur4 is not affected by the K272A mutation, a mutation that has been shown to block binding to uracil (Figure 2A, lane #4) (12). This result indicated that Fur4 quality control is independent of substrate binding.

To test if degradation of unfolded Fur4 requires the yeast cytoplasmic quality control system we deleted two key ubiquitin ligases, Ubr1 and San1, that have been shown to play an important role in the degradation of unfolded cytoplasmic proteins (13). In this mutant strain, the trafficking of Fur4-GFP was monitored after heat shock or peroxide treatment. Both stress treatments caused rapid downregulation of Fur4-GFP in the *ubr1 san1* mutant cells indicating that the ubiquitin ligases Ubr1 and San1 are not required for stress-induced degradation of Fur4 (Figure 2A, lane #8).

Screen for Fur4 mutants that confer temperature-sensitive growth

Quality control of multi-spanning transmembrane proteins at the plasma membrane is predicted to play an essential role in maintaining the integrity of the cell. Unfolding of channels or transporters at the cell surface might cause an ion leak that could threaten the survival of the cell. To test this hypothesis, we took advantage of the mutant transporter Fur4^{N60}-GFP that is not downregulated under stress conditions and thus is predicted to remain in the plasma membrane even when unfolded. Low-fidelity PCR was used to randomly mutagenize *fur4^{N60}-GFP*. The resulting mutant constructs were transformed into wild-type yeast and grown on plates at 25°C. The grown yeast colonies were then replica-plated and incubated at 37°C. After two days yeast colonies were identified that lacked growth at 37°C. After re-testing the temperature-sensitive growth of the identified strains, one mutant strain was chosen for further analysis. The mutated *fur4^{N60}-GFP* gene was isolated and DNA sequence analysis identified a single base pair exchange at codon 115, causing an asparagine to histidine exchange in the first transmembrane domain of Fur4 (N115H, Figure 1B).

Growth tests showed that the expression of Fur4^{N60,N115H}-GFP not only impaired single colony growth at 37°C on plates (Figure 2C) but also inhibited growth in liquid medium at 30°C (Figure 2D). Osmotic support in the form of 1M sorbitol suppressed the growth defect in liquid medium (Figure 2D). Mutations in the plasma membrane proton pump Pma1 are known to result in osmosensitive growth (14), suggesting that the observed growth phenotypes caused by the mutant Fur4 protein could be due to a proton-leak across the plasma membrane. To test this idea, lysine 272 of Fur4^{N60,N115H} was mutated to alanine. Lysine 272 is likely the proton carrier in Fur4, a prediction that is based on sequence comparison with the well-studied transporter Mhp1 and based on the observation that lysine

272 is the only charged amino acid within a transmembrane domain required for Fur4 activity (12). Cells expressing Fur4^{N60,N115H,K272A} did exhibit only a weak osmosensitive growth phenotype, supporting the idea that a proton leak is the likely cause for the deleterious affects of Fur4^{N60,N115H} (Figure 2D).

Both Fur4^{N60} and Fur4^{N60,N115H} are functional transporters at 25°C, since expression of each of these Fur4 mutants causes sensitivity to 5-fluoro-uracil, a toxic uracil homologue that is imported by Fur4 (Figure S1A). Consistent with this result, fluorescence microscopy showed normal plasma membrane localization of Fur4^{N60,N115H} (Figure 2A, lane #5). Together, the data suggested that at low temperature Fur4^{N60,N115H} is a functional transporter that when shifted to 37°C unfolds, causing the dramatic growth defect.

If the N-terminal region of Fur4 indeed functions in the quality control of the protein, we would predict that the N115H mutation in the context of the full-length Fur4 protein should cause rapid Fur4 degradation at high temperature. To test this prediction, wild-type Fur4-GFP and the N115H mutant form were transformed into yeast and the resulting strains were grown at 25°C. At exponential growth phase cells were shifted to 37°C for 10min and cells before and after temperature shift were analyzed by microscopy. In contrast to wild-type Fur4-GFP, which to a large extent remained at the plasma membrane, Fur4^{N115H}-GFP was rapidly internalized at 37°C, and a majority of the signal was found in endosomes (Figure 2E). Furthermore, cells expressing Fur4^{N115H} did not exhibit growth defects at high temperature or in liquid media (Figures 2C and 2D), indicating that the Fur4 quality control system was able to detect the temperature induced folding problems in Fur4^{N115H}, trigger its rapid degradation and thus protect the cell from a potentially lethal ion leak. Similar to wild-type Fur4-GFP, rapid degradation of Fur4^{N115H}-GFP required Rsp5 (*rsp5-1*; Figure 2A, lane #7) but was independent of Ubr1 and San1, ubiquitin ligases involved in the cytoplasmic quality control (*san1 ubr1* ; Figure 2A, lane #9).

Substrate-dependent downregulation

Our data suggested that quality control of Fur4 requires the same ubiquitination event that has been shown to trigger downregulation in the presence of high uracil concentrations. Substrate-dependent downregulation is common among many cell-surface transporters. However, the precise mechanism of this induced degradation remains elusive. Based on the observation that the uracil-binding site in Fur4 is involved in sensing high-substrate concentrations, it has been proposed that conformational changes that occur during pumping of the substrate might trigger ubiquitination. Alternatively, a model has been proposed in which a high cytoplasmic uracil concentration is the signal for Fur4 degradation (3). To test these models, we performed a systematic analysis of substrate-dependent Fur4 downregulation.

Extracellular substrate causes Fur4 downregulation

Because Fur4 efficiently imports uracil, adding it to the growth medium increases both the extracellular and cytoplasmic concentrations, making it impossible to differentiate between Fur4 downregulation triggered by intracellular or extracellular substrate. Therefore, we used the K272A mutation in Fur4, which has been shown to inhibit both binding and transport of uracil (12). Consistent with previous studies, we observed no uracil-induced downregulation of Fur4(K272A)-GFP. In contrast, upon addition of 20mg/l uracil, wild-type Fur4-GFP was rapidly endocytosed and delivered to the vacuole for degradation (Figure 3A).

The immunosuppressant drug leflunomide is transported to the cytosol by Fur4 where it inhibits growth, possibly by blocking pyrimidine synthesis (15). This toxic effect of leflunomide is not observed when cells express Fur4(K272A)-GFP in a *FUR4* deletion

strain, indicating that this mutant form of Fur4 is not only impaired in uracil import but also is unable to transport leflunomide into the cell (Figure 3B). Interestingly, we observed leflunomide-induced downregulation of both wild-type Fur4 and Fur4(K272A), suggesting that, unlike uracil, leflunomide is able to efficiently bind to the K272A mutant of Fur4 (Figure 3A). Similar treatment of yeast expressing the methionine transporter Mup1-GFP showed no downregulation of this permease, demonstrating that leflunomide did not cause a general increase of endocytosis but specifically induced downregulation of Fur4 (Figure S1D). Fur4's lysine residues K38 and K41 are targeted for ubiquitination in the presence of high uracil concentrations, a modification that is essential for uracil-dependent downregulation (5). Mutating these two lysine residues to arginine stabilized Fur4 not only in the presence of high uracil but also in the presence of leflunomide (Figure 3A), indicating that uracil and leflunomide trigger the same downregulation mechanism in Fur4.

Together, the data suggested that even in the absence of pump activity leflunomide is able to bind to Fur4 and induce its rapid downregulation. This observation further suggested that the switch of Fur4 from the outward-open or ground state to the outward-occluded conformation is sufficient to trigger its ubiquitination and degradation.

Cytoplasmic uracil causes Fur4 downregulation

Previous studies have observed that high uracil concentrations can redirect the trafficking of newly synthesized Fur4 at the trans-Golgi, resulting in the rapid degradation of the transporter in the vacuole (3). This result suggested that Fur4 downregulation is not induced by uracil transport but by the binding of cytoplasmic uracil to the transporter. To test if this model is correct for plasma membrane localized Fur4, we constructed two strains that would allow us to increase or decrease cytosolic uracil without adding uracil to the growth medium. The first strain constructed was deleted for the cytidine-deaminase gene *CDD1* (*cdd1*⁻), and the cytosine-deaminase gene was overexpressed with the help of a high-copy plasmid (*2μFCY1*). These genetic modifications were expected to allow for efficient conversion of cytosine to uracil, and *vice versa* (Figure 3C). The second strain overexpressed uracil-phosphoribosyltransferase (*2μFUR1*), which was expected to cause rapid conversion of uracil to UMP, thereby lowering cytosolic uracil concentration (Figure 3C). To observe the trafficking of Fur4, wild-type and the two modified yeast strains were transformed with a plasmid expressing Fur4-GFP. Because some of the effects on Fur4 localization were less dramatic than observed in other experiments, for each condition 50 cells were analyzed and the ratio of internal signal (total signal minus plasma membrane signal) versus total signal was determined. The histogram in Figure 3E shows the distribution of these ratios for the three different yeast strains. Because of the cytoplasmic background, the intracellular/total ratios are larger than expected based on the microscopy pictures. For example, the wild-type control shown in Figure 3D has a ratio of 0.42, whereas the uracil-treated sample of the same strain in Figure 3D has a ratio of 0.86. Analysis of these data sets using the Kolmogorov-Smirnov test showed that all discussed differences are statistically relevant.

For the experiments, the yeast strains were grown to exponential phase in minimal-synthetic medium (MS medium) lacking uracil and cytosine. Fluorescence microscopy demonstrated the expected localization of the majority of Fur4-GFP to the plasma membrane in all three strains (Figure 3D). However, the quantitative analysis revealed a shift to a lower intracellular/total ratio for the *FUR1* overexpressing strain (*2μFUR1*, Figure 3E), suggesting that the rapid conversion of uracil to UMP in this strain caused a stabilization of Fur4 on the plasma membrane, more so than the absence of extracellular uracil alone.

As expected, the addition of 5mg/l uracil to the three different strains resulted in the rapid endocytosis and delivery of Fur4-GFP to the vacuole for degradation (Figure 3D). However,

the degree of Fur4 downregulation was reduced in both the *cdd1* Δ *2 μ FCY1* and the *2 μ FUR1* strain (Figure 3E). This result suggested that the conversion of cytosolic uracil either to cytosine or to UMP, respectively, stabilized Fur4 on the plasma membrane.

Yeast cells import cytosine via the Fcy2 transporter (Figure 3C). Previous studies have demonstrated that very high concentrations of cytosine in the growth medium induce Fur4 internalization, possibly caused by cytoplasmic uracil that was converted from imported cytosine (2). However, because of the high cytosine concentration used (40–60mg/l), the study was not able to exclude the possibility that the converted uracil was exported from cells and re-imported, thereby causing the downregulation of Fur4. Therefore, for our experiments, we used low cytosine concentrations (5mg/l) and quantified the effects on Fur4-GFP trafficking in our modified strains. In the wild-type strain, the addition of cytosine resulted in partial Fur4-GFP downregulation, an effect that was suppressed by the overexpression of *FUR1* (Figures 3D and 3E). In contrast, the presence of cytosine caused efficient Fur4-GFP downregulation in the *cdd1* Δ *2 μ FCY1* strain (Figures 3D and 3E). This result suggested that in the *cdd1* Δ *2 μ FCY1* strain the imported cytosine was efficiently converted into uracil, thereby triggering the degradation of Fur4. To ensure that the observed Fur4-GFP downregulation was not caused by extracellular uracil that was produced from cytosine and then exported from cells, a control experiment was performed in which uracil production and the Fur4-GFP reporter were separated into two strains. Wild-type cells expressing Fur4-GFP were mixed with *cdd1* Δ *2 μ FCY1* cells and the effect of cytosine-addition was observed. In contrast to the previous experiment where Fur4-GFP was present in the *cdd1* Δ *2 μ FCY1* cells, the addition of cytosine to the cell mixture did not cause Fur4-GFP downregulation (Figure S1E). Together, the results strongly supported a model in which Fur4 downregulation is caused by high uracil concentrations in the cytoplasm, indicating that uracil import activity of Fur4 is not required to trigger endocytosis of the transporter.

We observed that the K272A mutation impaired substrate-dependent downregulation of Fur4, even in experiments where uracil was intracellularly produced by conversion from cytosine (Figure 3D). This observation not only supported the idea that Fur4 itself is acting as a uracil sensor, but also indicated that uracil sensing was mediated by the Fur4 substrate-binding site. Similar observations were obtained in previous studies, which demonstrated the importance of the K272 site for substrate-dependent redirection of newly synthesized Fur4 at the trans-Golgi (3).

In summary, the analyses of substrate-dependent downregulation suggested that any substrate-bound state of Fur4 induces degradation of the transporter. Downregulation of Fur4 is independent of transporter activity and can be triggered by binding of extracellular as well as intracellular substrate.

The Fur4 Loop Interaction Domain (LID) acts as a conformation sensor

Our substrate-dependent downregulation studies suggested that not a particular conformation but any substrate-bound state is able to trigger Fur4 degradation. This mechanism would explain how stress-induced unfolding of Fur4 causes downregulation by triggering the same ubiquitination as observed in the presence of high substrate concentrations: Any Fur4 conformation that differs from the ground state of the transporter is targeted for degradation. If this model is correct, we would expect to find a domain in Fur4 that senses conformational changes and relays this information to the ubiquitination sites. To identify such a conformation-sensing domain we studied the crystal structure of Mhp1, a bacterial homologue of Fur4 (Figure 1C). The Mhp1 structure showed that the ~20 amino acid region just prior to the first transmembrane domain is in an extended conformation and runs parallel to the membrane along a groove between the cytoplasmic

loops (9). We call this N-terminal region LID, for Loop Interaction Domain (Figures 1A, 1B and 1C). In the outward-facing or ground state of Mhp1, the LID is kept in position by a series of hydrogen-bonding interactions with each of the cytoplasmic loops and the C-terminus (Table 1, Figure S2). Interestingly, about half of these interactions are lost when the transporter switches conformation to the inward-facing state (Table 1, Figure S3).

The structural information suggested that the LID of Mhp1 might stabilize the ground state of the transporter. Furthermore, the LID might function as the predicted conformation sensor that could relay information about the functional state of the transporter to other cellular factors. We envisioned that such a mechanism could be responsible for inducing downregulation of Fur4, a homologue of Mhp1. This model was particularly attractive since the phosphorylation and ubiquitination sites necessary to trigger Fur4 degradation are located adjacent to the LID region of Fur4 (Figure 1A).

The amino acid sequence alignment of Fur4 with other NCS1-type transporters from yeast identified the predicted LID as a region with relative high sequence conservation. In particular, a glutamine and proline residue corresponding to the Fur4 positions 98 and 103, respectively, were identical in all sequences, including Mhp1 (Figure 1B).

Point mutagenesis was used to test if the predicted Fur4 LID and its loop interactions are involved in the downregulation of the transporter. The Mhp1 structure indicated that about half of the LID-loop hydrogen bonds were formed between protein backbone carbonyl- and amino-groups and are therefore not disrupted by changing the amino acid side-chain (Table 1). However, the highly conserved glutamine at position 14 and the arginines at position 21, 25 and 332 of Mhp1 formed hydrogen bonds mediated by their side-chains. Thus, the corresponding positions in Fur4 were changed to alanines (red-labeled amino acids in Figure 1B). Three of these mutations were in the predicted LID region (N98A, E105A, R109A) and one mutation localized to loop 8–9 (K435A). In addition, the conserved proline residue of the Fur4 LID was mutated (P103A). The high conservation of this amino acid suggested that it might play an important structural role for the LID. As a control, two amino acids of the LID that based on the Mhp1 structure were predicted not to be involved in LID-loop interactions were also mutated (E107A, R108A).

The *fur4-GFP* mutant genes were expressed in wild-type cells and microscopy demonstrated that all mutant proteins localized properly to the plasma membrane. Growth tests in the presence of the toxic uracil analogue 5-fluoro-uracil demonstrated that the mutant Fur4 proteins were functional transporters (Figures S1B and S1C). Furthermore, addition of uracil to the growth medium resulted in rapid downregulation of the mutant Fur4 proteins (Figure 4A). Together, the initial analysis of the Fur4 mutants suggested that these transporters function very similar to the wild-type protein. However, in fluorescence microscopy, Fur4(P103A)-GFP and Fur4(R109A)-GFP showed GFP signal surrounding the nucleus, which is reminiscent for ER localized proteins (Figure 4A). This observation suggested that the P103A and R109A mutations affected folding of newly synthesized Fur4, resulting in an inefficient export from the ER. Therefore, as predicted from the Mhp1 structures the LID-loop interactions seem to play an important role in stabilizing the basic fold of the transporter. Consistent with this idea, we observed that N-terminal deletions that removed the Fur4 LID or regions close to the LID caused ER retention and degradation of the mutant Fur4 protein. GFP-tagged versions of these N-terminally deleted Fur4 proteins were barely detectable by fluorescence microscopy and the majority of the remaining signal localized to the ER (Fur4^{N110}-GFP, Fur4^{N90}-GFP, Figure 4B).

Our model predicts that the LID functions as a sensor, which is able to detect substrate- or stress-induced changes in the conformation of Fur4 and trigger the degradation of the

transporter. If this model were correct, we would expect to observe in the presence of low substrate or mild stress conditions increased downregulation of the mutant Fur4 proteins. Therefore, cells expressing either wild-type or mutant Fur4-GFP were treated either with leflunomide or shifted to 37°C for 10min. Leflunomide and not uracil was used for these experiments because this substrate is not metabolically converted and shows weaker affinity to Fur4, which increases the chance to observe differences in the sensitivity of different Fur4 mutants to the presence of substrate. Although both treatments resulted in downregulation of wild-type as well as mutant Fur4-GFP, the extent to which Fur4-GFP was endocytosed was much more pronounced in all mutant forms of Fur4-GFP that were predicted to have impaired LID-loop interactions (N98A, P103A, E105A, R109A, K435A; Table 2). Quantification of cells treated with leflunomide demonstrated that, dependent on the mutation, 18–100% of the mutant Fur4-GFP constructs showed no detectable plasma membrane signal whereas almost all cells expressing wild-type Fur4-GFP or expressing the control mutant forms (E107A and R108A) retained some of the transporter at the cell surface (Figures 4A and 4C). Similarly, the 10min heat shock resulted only in minor endocytosis of wild-type Fur4-GFP and the control mutants (E107A and R108A). In contrast, the same heat treatment caused the majority of the Fur4-GFP mutants predicted to carry destabilizing amino acid exchanges to localize to endosomal structures (Figure 4A, Table 2).

Based on our model, stabilizing the LID-loop interactions should decrease degradation of the transporter. To test this prediction, we used an amber-suppression system to change the methionine at position 96 of Fur4 to BPA (L-2-amino-3-(p-benzoylphenyl)propionic acid), an artificial, photo-crosslinkable amino acid (16). This mutant was expressed in a yeast strain containing an amber suppressor t-RNA and its cognate aminoacyl-tRNA synthetase specific for BPA. The resulting Fur4(M96BPA)-GFP protein properly localized to the plasma membrane where it functioned in uracil import (Figures 4D and S1F). Upon UV exposure, BPA chemically crosslinks with other nearby molecules. Based on the Mhp1 structure, we expected that in UV exposed Fur4(M96BPA)-GFP the BPA side chain would form covalent bonds to amino acids of the nearby cytoplasmic loop 10–11; however, crosslinking of BPA with lipids is also possible. As expected, we observed increased stability of Fur4(M96BPA)-GFP after UV treatment both in the presence of substrate (leflunomide) and stress conditions (heat shock; Figures 4D and 4E). The same UV treatment did not affect downregulation of wild-type Fur4-GFP, indicating that the UV-induced stabilization of the mutant transporter was dependent on the presence of BPA. The fact that UV treatment did not result in a complete block of Fur4(M96BPA)-GFP degradation might be explained by partial crosslinking of BPA to loop 10–11 and/or crosslinking to other molecules that do not restrict LID movements.

In summary, the phenotypes observed with the Fur4 mutants strongly supported the model that the LID functions in sensing conformational changes. Mutating loop-LID interactions mimics the loss of loop-LID interactions that normally occur as a result of substrate binding or unfolding of the transporter and thus the mutations decrease the stability of Fur4. In contrast, stabilizing the loop-LID interaction by chemical crosslinking caused increased stability of Fur4. Furthermore, the wild-type behavior of the control mutants E107A and R108A validated our approach to use the Mhp1 structure in designing the Fur4 mutants and demonstrated the high degree of structural conservation between these two transporters.

Fur4 ubiquitination is regulated by lysine 38,41 accessibility

The data presented above suggested that loss of LID-loop interactions causes ubiquitination of the lysines at position 38 and 41. The key question is: how does the LID regulate the degron? To gain insight into this regulation, we constructed a Fur4 mutant deleted for the first 41 amino acids, which removes the lysines targeted for ubiquitination. As expected,

Fur4(N41)-GFP localized to the plasma membrane even in the presence of substrate or stress conditions (Figure 4F). We then fused the ubiquitination site of Cps1, referred to as “US” (amino acid sequence PVEKAPRS), to the N-terminus of Fur4(N41)-GFP. Cps1 is a transmembrane protein that is constitutively ubiquitinated by Rsp5 and traffics via the MVB pathway to the lumen of the vacuole (17). When expressed in yeast, Fur4(US- N41)-GFP localized to the plasma membrane and, similar to the wild-type transporter, was internalized upon exposure to substrate or heat (Figure 4F). This result showed that the non-regulated Cps1 ubiquitination site was able to substitute for the deleted degron and restore regulated degradation, supporting the idea that regulation of ubiquitination is mediated by the LID.

The ubiquitination site US was also added to the N-terminus of Fur4(60)-GFP, a deletion construct that remains on the plasma membrane even under stress conditions (Figure 2A). In contrast to Fur4(US- N41)-GFP, Fur4(US- N60)-GFP was not internalized upon heat shock or exposure to substrate (Figure 4F), suggesting that the amino acids between position 41 and 60 play an important role in the ubiquitination of Fur4. To test if Fur4 ubiquitination depends on a particular amino acid sequence of the 41–60 region, we inserted a double HA-tag (YPYDVPDYAYPYDVPDYA) downstream of the US sequence in Fur4(US- N60)-GFP, thereby restoring the proper distance between the ubiquitination site and the LID. When expressed in yeast, the resulting construct Fur4(US-2HA- N60)-GFP demonstrated substrate and heat-shock induced downregulation of the transporter (Figure 4F).

Together, our observations suggested that substrate- or stress-dependent ubiquitination of Fur4 is independent of a particular amino acid sequence of the ubiquitination site or the neighboring regions but requires a certain distance between the LID and the lysines recognized by Rsp5. Therefore, we propose a model in which the LID regulates Rsp5’s access to the ubiquitination sites. In the ground state of Fur4, the degron is ‘tucked-in’ and not accessible for Rsp5. However, the loss of loop-LID interactions that occur as a consequence of substrate binding or unfolding results in increased flexibility of the N-terminal region, which in turn allows Rsp5 to ubiquitinate the degron.

Mup1 quality control does not require Art1

Previous studies suggested that a group of proteins, called the arrestin-related trafficking adaptors (ARTs), is responsible for quality-control of cell surface transporters (18). The ART proteins have been shown to bind to transporters and recruit Rsp5. No particular ART protein has been identified necessary for the downregulation of Fur4 (19). However, the methionine transporter Mup1, a member of the APC superfamily of transporters, has been shown to require Art1 for degradation (18).

We tested if quality control of Mup1 depends on the mechanism that is responsible for substrate-induced downregulation. As previously reported, high concentrations of methionine in the growth medium caused rapid Rsp5-dependent internalization of the transporter and its subsequent delivery to the vacuole for degradation (Figure 5, (20)). Similarly, we observed that heat shock or peroxide-treatment induced efficient downregulation of Mup1 in an Rsp5-dependent manner (Figure 5). Furthermore, stress-induced degradation of Mup1 was independent of Ubr1 and San1, the ubiquitin ligases involved in the quality control of cytoplasmic proteins (Figure 5). Together, these data were consistent with the Fur4 results and suggested that Mup1 quality control and methionine-induced downregulation were likely mediated by the same mechanism. However, in a *ART1* deleted strain our fluorescence microscopy analysis showed a delay but not a block in the downregulation of Mup1-GFP triggered either by high substrate or stress conditions (*art1* , Figure 5). This delay in the delivery of Mup1-GFP to the vacuole was more severe in a strain deleted for nine Art proteins (*art1-9* , Figure 5), suggesting some redundancy among the Art proteins in the degradation of Mup1. Together, the data demonstrated that in contrast

to Rsp5, Art1 is not essential for substrate-dependent downregulation or quality control of Mup1 but rather seems to increase efficiency of Rsp5-dependent ubiquitination.

Discussion

Rapid degradation of cell-surface nutrient transporters is initiated either by cellular regulatory systems, such as the starvation response pathway (21), or by protein-specific events, including high-substrate concentration or protein unfolding. Based on our studies of the yeast uracil transporter Fur4 and structural information from homologous bacterial transporters, we propose a model for the mechanism of protein-specific downregulation (Figure 6). The key element in this model is a cytoplasmic region of the transporter, called LID (Loop Interaction Domain) that interacts with intermembrane loop regions, thereby stabilizing the outward-facing or ground-state of the transporter. Conformational changes in the transporter disrupt LID-loop interactions. The resulting increase in flexibility of the LID allows access to the degradation-initiation site in the transporter, referred to as 'degron,' that consequently is targeted for ubiquitination by plasma membrane localized Rsp5. The term degron is used to describe degradation signals that initiate the degradation of a protein in a controlled fashion (reviewed in (22)). The ubiquitinated transporter is efficiently endocytosed and delivered via the MVB pathway to the lysosome/vacuole for degradation. In brief, this degradation model is composed of an intrinsic conformation sensor, the LID, that regulates a ubiquitin site, the degron. The LID-degron system is highly versatile in that various deviations from the conformational ground state can trigger the degradation of the transporter, explaining how one mechanism can mediate both substrate-dependent downregulation and quality control of the protein.

Quality control of plasma membrane proteins is of vital importance for the cell since unfolded multispansing transmembrane proteins have the potential to form pores that compromise cell integrity. Therefore, an efficient system has to be in place that recognizes these unfolded proteins and initiates their rapid endocytosis and degradation. In the past, several studies have attempted to identify quality control factors that are essential for the rapid degradation of unfolded plasma membrane proteins (reviewed in (23)). These studies found that mutations blocking endocytosis or the MVB pathway cause stabilization of the unfolded proteins. However no specific quality control factors were identified. Two recent studies in mammalian cells identified a chaperon-mediated ubiquitination system that is responsible for the rapid turnover of unfolded plasma membrane proteins (24, 25). This system is similar to cytoplasmic protein quality control, in that chaperones recognize the unfolded state of a protein and recruit the ubiquitin ligase CHIP, which then marks the protein for degradation. However, both of these studies were based on membrane proteins containing large unfolded cytoplasmic domains. In these cases, a chaperon-based quality control system similar to that found in the cytoplasm is sensible. However, the question remained how chaperones would be able recognize unfolded transmembrane regions, the type of folding problems that could lead to cell integrity problems.

The LID-degron system proposed by our study is able to explain how unfolding of transmembrane regions or extracellular domains triggers degradation of the transporter without the need of chaperones. This mechanism also explains why different protein unfolding conditions lead to the targeting of the same two lysines in the Fur4 degron, even though 15 other lysines are present within the cytoplasmic regions of the Fur4 protein. Deletion of the degron resulted in a block of Fur4 quality control at the plasma membrane. This lack of quality control has the potential to cause severe damage to the cell, as demonstrated by the toxicity of a degron-deleted Fur4 containing a mutation in the first transmembrane domain (Figure 2). The expression of this mutant form of Fur4 caused

severe growth defects, indicating that neither ER-localized nor cytoplasmic quality control were able to compensate for the lack of the LID-degron system.

Deletion of the LID caused ER retention and rapid degradation of Fur4 (Figure 4B) suggesting that the LID functions not only as a conformation sensor but also plays an important role for proper folding of Fur4. The LID-loop interactions might help arrange the transmembrane domains, thereby stabilizing the ground state of the transporter. This stabilizing role of the LID would explain why the LID is conserved even in the transporters of bacteria, organisms that do not possess ubiquitin-dependent degradation systems. The observation that LID deletions cause ER retention indicated that the ER quality control is independent of the LID-degron system and is able to recognize folding problems in the absence of the N-terminal region. Therefore, the LID-degron system seems to function in the Fur4 quality control past the ER, at the plasma membrane and possibly at Golgi and endosomal compartments.

Substrate-dependent degradation of nutrient transporters is an adaptation mechanism that is part of a regulatory system ensuring that the proper number of transporters are present at the cell surface depending on the nutrient availability and cellular need. High substrate concentrations increase the turnover rate of transporters whereas low substrate availability results in stabilization of the transporters. Previous studies found that the substrate-binding site in Fur4 is required for uracil-induced downregulation, suggesting that Fur4 itself is sensing the presence of uracil, thereby regulating its own turnover rate (2). Furthermore, we found that the presence of both extracellular as well as intracellular substrate is able to induce internalization and degradation of Fur4 (Figure 3), suggesting that any substrate-bound state is able to trigger Fur4 ubiquitination. The LID-degron model fits well with these observations, which predicts that any major conformational change from the ground state of the transporter is sensed by the LID and can cause ubiquitination of the degron. However, our model does not predict that ubiquitination is an obligate step in the transport cycle of Fur4, rather that substrate-bound Fur4 has an increased chance of becoming ubiquitinated. Therefore, the critical parameter for ubiquitination efficiency is the time period of the substrate-bound state, which in turn depends on the uracil concentration. For example, at low cytoplasmic uracil concentrations the substrate-bound conformations are short-lived because uracil is efficiently imported and released by Fur4; and thus, the transporter remains mainly in the ground state. In contrast, high concentrations of cytoplasmic uracil will stabilize the inward-facing substrate-bound state, increasing the chance that the Fur4 degron is targeted by the ubiquitin-ligase Rsp5 (Figure 6). This type of regulation implies a co-evolution of uracil binding affinities of both Fur4 and the enzymes involved in the metabolism of uracil.

We predict that the LID-degron system is not unique to Fur4 but is conserved in a large number of transporters. Consistent with this prediction we found that Mup1, a member of the APC transporter superfamily, showed Rsp5-dependent downregulation both under stress conditions and in presence of high substrate concentrations. In contrast, the ART proteins, Rsp5 adaptors that have been previously suggested to function as Mup1 quality control factors (18), are not essential for stress or substrate-dependent downregulation of Mup1 (Figure 5).

Members of APC superfamily include not only nutrient importers (e.g. Fur4 and Mup1) but also transporters of neurotransmitters, such as the serotonin transporter SERT, that play important roles in modulating neurotransmission in the brain. Structural studies of a bacterial homologue of SERT, called LeuT, demonstrated the presence of several interactions between the N-terminus and cytoplasmic loops. These interactions are only observed in the ground state of LeuT but are lost as a consequence of substrate import (26).

Therefore, SERT is likely to contain a LID-degron system similar to that of Fur4. The idea of an evolutionarily conserved LID-degron system is also supported by published studies of the amino acid transporter Gap1, which showed destabilization in mutants along the N-terminal region before the first transmembrane domain (27).

Materials and Methods

Yeast Strains and Plasmids

S. cerevisiae strains and plasmids used in this work are described in Table 3. Genomic deletions of *FUR4* and *CDD1* were constructed by homologous recombination as previously described (28). All deletion strains were confirmed by PCR. Yeast strains were grown either in standard yeast extract-peptone-dextrose (YPD) or, to maintain plasmids, in synthetic medium supplemented with essential amino acids as required (YNB) (29).

All *FUR4* clonings are based off of the plasmid pFL38-FUR4-GFP (30). For growth assays *FUR4-GFP* was expressed using the constitutive *SNF7* promoter. For microscopy *FUR4-GFP* was expressed using the *CUP1* promoter that was induced with the addition of 0.1mM cupric sulfate. Point mutations in *FUR4* were generated by site directed mutagenesis with the Stratagene Quick Change kit (Agilent Technologies, La Jolla, CA). DNA sequencing was used to confirm the mutations.

Fluorescence microscopy

Cells were grown to midlog phase and analyzed by fluorescence microscopy using a deconvolution microscope (DeltaVision, Applied Precision, Issaquah, WA). For experiments involving Mup1-GFP, cells were grown in minimal media lacking methionine. Quantification of the microscopy pictures was performed utilizing Photoshop software. Images of 50 random cells were deconvolved and saved as a projection in Photoshop format. Individual cells were selected and the boundary of any given cell was determined in the DAPI channel image with the wand selection tool. Total intensity of the whole cell as well as the intracellular region (cell outline contracted by 6 pixels) was recorded. For hydrogen peroxide treatment cells were exposed to 0.005% H₂O₂ for 30min, washed twice with PBS (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na₂HPO₄, 0.24 g/l KH₂PO₄, pH7.2) and resuspended in YNB media. Cells were allowed to recover for 30min before microscopy was performed.

Uracil uptake assay

Cells were grown in minimal medium lacking uracil to mid-log phase. Uracil (5mg/l) was added and after 10min the cells were harvested, washed twice with ice-cold water and lysed in methanol at 50°C (5min). The lysate was cleared twice by centrifugation (10min, 20,000×g) and the resulting supernatant was separated by HPLC chromatography using a Luna-NH2 column (Phenomenex) in presence of a 100-80% acetonitrile/water gradient. Uracil was detected at 260nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MVB	Multivesicular Body
ESCRT	Endosomal Sorting Complex Required for Transport
LID	Loop Interaction Domain

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Figure 1.

Structure of the transporters Fur4 and Mhp1. (A) Schematic representation of Fur4 which contains 12 transmembrane domains. The N-terminally localized degron of Fur4 is composed of the ubiquitination site (Ub) and the PEST-like sequence, which is followed by the LID (Loop Interaction Domain). Deletion of the first 60 amino acids removes the degron (ΔN60). (B) Amino acid sequence alignment of the N-terminal regions of Fur4, Mhp1 and other homologous yeast transporters. Amino acids in Fur4 that have been mutated to alanine are labeled in red. The asparagine 115 of Fur4 is labeled in purple. (C) Side- and bottom-view of the Mhp1 structure (based on crystal structure 2JLN). The LID is labeled in red and the cytoplasmic loops are indicated (L2-3, L6-7, L8-9, L10-11).

Figure 2. Stress-induced downregulation of Fur4 is dependent on the N-terminal degen. (A) Fluorescence microscopy of yeast expressing wild-type and different mutant forms of Fur4-GFP, before and after treatment with uracil, peroxide or heat-shock. (B) Intracellular uracil concentration in cells expressing Fur4^{N60}-GFP. Cells were either not treated or treated with peroxide for 20min and uracil concentration was determined before and after addition of 5μg/ml uracil to the medium. (C) Growth at 37°C of *fur4* strains containing plasmids that express either wild-type or mutant forms of *FUR4-GFP*. (D) Growth of *fur4* strains expressing wild-type or different mutant forms of Fur4-GFP in liquid medium (YNB) at 30°C in the presence or absence of 1M sorbitol. The graph represents the average growth of

three cultures. (E) Downregulation of wild-type and N115H mutant of Fur4-GFP after a 10min heat-shock.

Figure 3.

Extracellular and intracellular substrate initiates downregulation of Fur4. (A) Downregulation of wild-type (WT) and mutant (K272A; K38,41R) Fur4-GFP in the presence of uracil or leflunomide. The fluorescence microscopy pictures are inverted and thus black indicates the localization of GFP. Dashed lines outline cells with no discernable plasma membrane signal. (B) Optical density (OD 600nm) of yeast cultures grown overnight in the presence or absence of leflunomide. Yeast used for the experiment were deleted for Fur4 and transformed either with empty vector (-) or plasmids expressing either wild-type or the K272A mutant of Fur4-GFP. The results show the average growth of three cultures. (C) Schematic of the uracil and cytosine metabolism of yeast. (D) Uracil- and

cytosine-induced downregulation of wild-type and K272A mutant of Fur4-GFP expressed in either wild-type, *cdd1-2 μ FCY1* or *2 μ FUR1* strains. (E) Quantification of the fluorescence microscopy shown in D (50 cells were quantified for each experiment). The graph shows the percentile of cells with a particular range of internal-to-total GFP signal (0.0–0.2, 0.2–0.4, etc.).

Figure 4.

The LID regulates Fur4 degradation. (A) Downregulation of wild-type and LID mutants of Fur4-GFP after treatment with uracil, high temperature or leflunomide. (B) Deletion of the N-terminal 90 or 110 amino acids of Fur4-GFP resulted in ER retention and degradation of the protein. (C) Quantification of the leflunomide treatment shown in A. Approximately 30 cells were analyzed for the presence or absence of plasma membrane localized Fur4-GFP. (D) Heat-shock and substrate-induced downregulation of wild-type Fur4-GFP and M96BPA mutant, before and after UV treatment. (E) Quantification of the analysis shown in D (N=50). (F) Localization of different N-terminal mutants of Fur4-GFP before and after heat-shock or leflunomide exposure.

Figure 5.

Quality control of Mup1 depends on Rsp5 but does not require Art1. Fluorescence microscopy of different Mup1-GFP expressing yeast strains before and after treatment with methionine, peroxide or heat-shock.

Figure 6. Model of substrate- and stress-induced Fur4 downregulation mediated by the LID-degron system.

Table 1

Hydrogen bonds present between LID and the cytoplasmic loops in the ground state of Mhp1 (crystal structure 2JLN; hydrogen bonds missing in the structure 2X79 of the inward-facing occluded state of Mhp1 are marked).

LID		Loop		Distance		Not in 2X79
Position	Atom	Position	Loop	Atom	[Å]	
Arg 10	bb-O	Thr 397	10-11	bb-NH	3.2	X
Ser 11	bb-O	Arg 332	8-9	sc-NH	2.9	
Leu 12	bb-NH	Tyr 395	10-11	bb-O	2.9	X
Leu 13	bb-O	Arg 332	8-9	sc-NH	2.4	X
Asn 14	sc-NH ₂	Pro 331	8-9	bb-O	2.6	X
Asn 17	bb-O	Tyr 395	10-11	sc-OH	2.9	
Thr 20	bb-NH	Gly 87	2-3	bb-O	3.2	
Arg 21	sc-NH	Arg 467	C-term.	bb-O	2.6	
Arg 21	sc-NH	Asp 464	C-term.	bb-O	3.0	X
Tyr 22	sc-OH	Arg 85	2-3	sc-NH	2.7	
Tyr 22	sc-OH	Glu 463	C-term.	sc-O	3.1	
Arg 25	sc-NH	Cys 234	6-7	bb-O	3.2	
Arg 25	sc-NH	Ile 84	2-3	bb-O	2.6	
Arg 25	sc-NH	Gly 87	2-3	bb-NH	3.2	X
Ser 26	bb-NH	Glu 233	6-7	bb-O	3.2	
Val 27	bb-O	Lys 235	6-7	bb-NH	3.1	X

Table 2

Analyzed Fur4 mutants and their phenotype.

Fur4 Mutation	Corresponding Position in Mhp1	<u>Fur4 Stability (relative to WT)</u>	
		Heat shock	Leflunomide
N98 to A	N14	↓	↓
P103 to A	P19	↓	↓
E105 to A	R21	↓	↓
E107 to A	A23	-	-
R108 to A	E24	-	-
R109 to A	R25	↓	↓
K435 to A	R332	↓	↓
M96BPA(+UV)	L12	↑	↑

Table 3

Strains and plasmids used in this study

Strain or plasmid	Descriptive name	Genotype or description	Reference or source
Strains			
SEY6210	WT	MAT <i>leu2-3,112 ura3-52 his3- 200 trp1- 901 lys2-801 suc2- 9</i>	(31)
JKY5	URA3	SEY6210, <i>URA3</i>	This study
JKY6	<i>fur4 URA3</i>	SEY6210, <i>fur4::HIS5, URA3</i>	This study
JKY7	<i>cdd1</i>	SEY62010, <i>cdd1::KanMX</i>	This study
JKY11	<i>mup1</i>	SEY62010, <i>mup1::KanMX</i>	This study
RHY7450	<i>san1 ubr1</i>	BY4741 <i>san1::NatMX ubr1::KanMX</i>	(13)
JPY88	<i>rsp5-1</i>	SEY6210 <i>rsp5::HIS3 + pDsRED415-rsp5(G753I)</i>	(32)
MYY808	<i>rsp5-1</i>	MYY808 MATa, <i>MDM1, smm1, his3, leu2, ura3</i>	(33)
EN60	<i>art1-9</i>	<i>ecm21::G418 csr2::G418 bsd2 rog3::natMX rod1 ygr068c aly2 aly1 ldb19 ylr392c::HIS</i>	(19)
JKY8	<i>art1</i>	SEY6210, <i>art1::HIS5</i>	This study
Plasmids			
pPL4146	P(<i>CUP1</i>)- <i>MUP1-GFP</i>	<i>LEU2</i> (pRS315) P(<i>CUP1</i>)- <i>MUP1-GFP</i>	(34)
pJK19	P(<i>CUP1</i>)- <i>FUR4-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>FUR4-GFP</i>	This study
pJK30	P(<i>CUP1</i>)- <i>fur4(Δ60)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(Δ60)-GFP</i>	This study
pJK28	P(<i>CUP1</i>)- <i>fur4(K272A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(K272A)-GFP</i>	This study
pJK31	P(<i>CUP1</i>)- <i>fur4(Δ60,N115H)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(Δ60,N115H)-GFP</i>	This study
pJK38	P(<i>CUP1</i>)- <i>fur4(N115H)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(N115H)-GFP</i>	This study
pJK25	P(<i>CUP1</i>)- <i>fur4(N98A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(N98A)-GFP</i>	This study
pJK26	P(<i>CUP1</i>)- <i>fur4(P103A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(P103A)-GFP</i>	This study
pJK27	P(<i>CUP1</i>)- <i>fur4(R109A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(R109A)-GFP</i>	This study
pJK29	P(<i>CUP1</i>)- <i>fur4(K435A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(K435A)-GFP</i>	This study
pJK12	P(<i>SNF7</i>)- <i>FUR4-GFP</i>	<i>TRP1</i> (pRS414) P(<i>SNF7</i>)- <i>FUR4-GFP</i>	This study
pJK20	P(<i>SNF7</i>)- <i>fur4(N98A)-GFP</i>	<i>TRP1</i> (pRS414) P(<i>SNF7</i>)- <i>fur4(N98A)-GFP</i>	This study
pJK21	P(<i>SNF7</i>)- <i>fur4(P103A)-GFP</i>	<i>TRP1</i> (pRS414) P(<i>SNF7</i>)- <i>fur4(P103A)-GFP</i>	This study
pJK22	P(<i>SNF7</i>)- <i>fur4(R109A)-GFP</i>	<i>TRP1</i> (pRS414) P(<i>SNF7</i>)- <i>fur4(R109A)-GFP</i>	This study
pJK24	P(<i>SNF7</i>)- <i>fur4(K435A)-GFP</i>	<i>TRP1</i> (pRS414) P(<i>SNF7</i>)- <i>fur4(K435A)-GFP</i>	This study
pJK32	P(<i>SNF7</i>)- <i>FUR4-GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>FUR4-GFP</i>	This study
pJK34	P(<i>SNF7</i>)- <i>fur4(Δ60)-GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4(Δ60)-GFP</i>	This study
pJK35	P(<i>SNF7</i>)- <i>fur4(Δ60,N115H)-GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4(Δ60,N115H)-GFP</i>	This study
pJK33	P(<i>SNF7</i>)- <i>fur4(N115H)-GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4(N115H)-GFP</i>	This study
pJK36	P(<i>SNF7</i>)- <i>fur4(K272A)-GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4(K272A)-GFP</i>	This study
pJK39	P(<i>CUP1</i>)- <i>fur4(K38,41R)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(K38,41R)-GFP</i>	This study
pJK43	P(<i>CUP1</i>)- <i>fur4(E105A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(E105A)-GFP</i>	This study
pJK45	P(<i>SNF7</i>)- <i>fur4(Δ60,N115H,K272A)-GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4(Δ60,N115H,K272A)-GFP</i>	This study
pJK50	P(<i>CUP1</i>)- <i>fur4(E107A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(E107A)-GFP</i>	This study
pJK47	P(<i>CUP1</i>)- <i>fur4(R108A)-GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4(R108A)-GFP</i>	This study
pJK52	P(<i>SNF7</i>)- <i>fur4(E105A)-GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4(E105A)-GFP</i>	This study

Strain or plasmid	Descriptive name	Genotype or description	Reference or source
pJK51	P(<i>SNF7</i>)- <i>fur4</i> (<i>E107A</i>)- <i>GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4</i> (<i>E107A</i>)- <i>GFP</i>	This study
pJK48	P(<i>SNF7</i>)- <i>fur4</i> (<i>R108A</i>)- <i>GFP</i>	<i>LEU2</i> (pRS415) P(<i>SNF7</i>)- <i>fur4</i> (<i>R108A</i>)- <i>GFP</i>	This study
pJK37	P(<i>FCY1</i>)- <i>FCY1</i>	<i>LEU2</i> (pRS425) P(<i>FCY1</i>)- <i>FCY1</i>	This study
pMB449	P(<i>FUR1</i>)- <i>FUR1</i>	<i>LEU2</i> (pRS425) P(<i>FUR1</i>)- <i>FUR1</i>	This study
pMB434	P(<i>SNF7</i>)- <i>fur4</i> (Δ <i>N110</i>)- <i>GFP</i>	<i>URA3</i> (pRS416) P(<i>SNF7</i>)- <i>fur4</i> (Δ <i>111</i>)- <i>GFP</i>	This study
pMB440	P(<i>SNF7</i>)- <i>fur4</i> (Δ <i>N90</i>)- <i>GFP</i>	<i>URA3</i> (pRS416) P(<i>SNF7</i>)- <i>fur4</i> (Δ <i>90</i>)- <i>GFP</i>	This study
pRS415	empty vector		(35)
pRS414	empty vector		(35)
pJK53	P(<i>CUP1</i>)- <i>fur4</i> (<i>M96BPA</i>)- <i>GFP</i>	<i>LEU2</i> (pRS415) P(<i>CUP1</i>)- <i>fur4</i> (<i>M96BPA</i>)- <i>GFP</i>	This study
pJK54	P(<i>CUP1</i>)- <i>fur4</i> (<i>US-ΔN60</i>)- <i>GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4</i> (<i>US-ΔN60</i>)- <i>GFP</i>	This study
pJK55	P(<i>CUP1</i>)- <i>fur4</i> (<i>US-2HA-ΔN60</i>)- <i>GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4</i> (<i>US-2HA-ΔN60</i>)- <i>GFP</i>	This study
pJK56	P(<i>CUP1</i>)- <i>fur4</i> (<i>US-ΔN41</i>)- <i>GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4</i> (<i>US-ΔN41</i>)- <i>GFP</i>	This study
pJK57	P(<i>CUP1</i>)- <i>fur4</i> (Δ <i>N41</i>)- <i>GFP</i>	<i>URA3</i> (pRS416) P(<i>CUP1</i>)- <i>fur4</i> (Δ <i>N41</i>)- <i>GFP</i>	This study