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Amino Acids Induce Peptide Uptake Via Accelerated Degradation of CUP9, the Transcriptional Repressor of the PTR2 Peptide Transporter

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Multiple pathways link expression of PTR2, the transporter of di- and tripeptides in the yeast *Saccharomyces cerevisiae*, to the availability and quality of nitrogen sources. Previous work has shown that induction of *PTR2* by extracellular amino acids requires, in particular, *SSY1* and *PTR3*. SSY1 is structurally similar to amino acid transporters but functions as a sensor of amino acids. PTR3 acts downstream of SSY1. Expression of the PTR2 peptide transporter is induced not only by amino acids but also by dipeptides with destabilizing N-terminal residues. These dipeptides bind to UBR1, the ubiquitin ligase of the N-end rule pathway, and allosterically accelerate the UBR1-dependent degradation of CUP9, a transcriptional repressor of *PTR2*. UBR1 targets CUP9 through its internal degron. Here we demonstrate that the repression of *PTR2* by CUP9 requires TUP1 and SSN6, the corepressor proteins that form a complex with CUP9. We also show that the induction of *PTR2* by amino acids is mediated by the UBR1-dependent acceleration of CUP9 degradation that requires both SSY1 and PTR3. The acceleration of CUP9 degradation is shown to be attained without increasing the activity of the N-end rule pathway toward substrates with destabilizing N-terminal residues. We also found that GAP1, a general amino acid transporter, strongly contributes to the induction of

***PTR2* by Trp. While several aspects of this complex circuit remain to be understood, our findings establish new functional links between the amino acids-sensing SPS system, the CUP9-TUP1-SSN6 repressor complex, the PTR2 peptide transporter and the UBR1-dependent N-end rule pathway.**

Biological processes addressed by this study include the mechanisms and regulation of peptide import. Peptides can serve as a source of amino acids and nitrogen in all organisms. The import of di- and tripeptides (di/tripeptides) in the yeast *Saccharomyces cerevisiae* has been shown to be regulated by the N-end rule pathway, one proteolytic pathway of the ubiquitin (Ub)-proteasome system (1-4). The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (reviewed in refs. (5-7)). Although prokaryotes lack the Ub system, they still contain the N-end rule pathway, albeit Ub-independent versions of it (6,8). In eukaryotes, this pathway recognizes several kinds of degradation signals (degrons), including a distinct set called N-degrons (5-7). Specific N-degrons that are active (recognized) in a cell give rise to that cell's N-end rule. The determinants of an N-degron in a substrate protein are a destabilizing N-terminal residue that bears the unmodified N-terminal amino group, a substrate's internal Lys residue (the site of formation of a poly-Ub chain), and a conformationally mobile region in a vicinity of this residue (9-11).

The N-end rule has a hierarchic structure. In eukaryotes, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu (12). Destabilizing activity of N-terminal Asp and Glu requires their conjugation to Arg, one of the primary destabilizing residues, by the *ATE1*-encoded Arg-tRNA-protein transferase (arginyl-transferase or R-transferase) (13-17). In mammals and other eukaryotes that produce nitric oxide (NO), the set of arginylated residues contains not only Asp and Glu but also N-terminal Cys, which is arginylated after its oxidation to Cys-sulfinate or Cys-sulfonate (13). The *in vivo* oxidation of N-terminal Cys requires NO, as well as oxygen (O_2) or its derivatives (14,17). The N-end rule pathway is thus a sensor of NO, through the ability of this pathway to destroy proteins with N-terminal Cys, at rates controlled by NO, O_2 and their derivatives.

E3 Ub ligases of the N-end rule pathway are called N-recognins (7,18-20). They recognize (bind to) primary destabilizing N-terminal residues. (The term "Ub ligase" denotes either an E2-E3 holoenzyme or its E3 component.) At least four N-recognins, including UBR1, mediate the N-end rule pathway in mammals and other multicellular eukaryotes (7). The known N-recognins share a ~70-residue motif called the UBR box. Mouse UBR1 and UBR2 are the sequelogous (similar in sequence) 200 kDa RING-type E3 Ub ligases that are 47% identical. Several other mammalian N-recognins, either confirmed or putative ones, are HECT-type or SCF-type Ub ligases that share the UBR motif with the RING-type UBR1 and UBR2 but are largely nonsequelogous to them otherwise (21,22). (*A note on terminology*: "sequelog" and "spalog" denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a three-dimensional structure that is similar, to a specified extent, to another three-dimensional structure (23). Besides their usefulness as separate terms for sequence and spatial similarities, the rigor-conferring advantage of "sequelog" and "spalog" is their *evolutionary neutrality*, in contrast to interpretation-laden terms such as "homolog", "ortholog" and "paralog". The latter terms are compatible with the sequelog/spalog terminology and can be used to convey understanding about functions and common descent, if this (additional) information is available.)

The functions of the N-end rule pathway include: (i) The sensing of heme, owing to inhibition, in both yeast and mammals, of the ATE1 R-transferase by hemin (Fe^{3+}-heme), which also inhibits N-recognins, the latter at least in yeast. (ii) The sensing of NO and O_2, and the resulting control of signaling by transmembrane receptors, through the conditional, NO/O_2-mediated degradation of G-protein regulators RGS4, RGS5 and RGS16.

(iii) Control of the import of short peptides, through degradation (modulated by peptides) of CUP9, the repressor of import. (iv) Fidelity of chromosome segregation, through degradation of a separase-produced cohesin fragment. (v) Regulation of apoptosis, through degradation of a caspase-processed inhibitor of apoptosis. (vi) A multitude of processes mediated by the transcription factor c-FOS, a conditional substrate of the N-end rule pathway. (vii) Regulation of the human immunodeficiency virus (HIV) replication cycle, through degradation of HIV integrase. (viii) Regulation of meiosis, spermatogenesis, neurogenesis and cardiovascular development in mammals, and leaf senescence in plants (refs. (2-4,13,14,16-19,24-26) and refs. therein). Mutations in human UBR1, one of E3s of the N-end rule pathway, are the cause of Johansson-Blizzard Syndrome (JBS), which comprises mental retardation, physical malformations, and severe pancreatitis (27).

The N-end rule pathway of *S. cerevisiae* is mediated by a single N-recognin, UBR1, a 225 kDa sequelog of mammalian UBR1 and UBR2 (2,28). *S. cerevisiae* UBR1 contains at least three substrate-binding sites. The type-1 site is specific for basic N-terminal residues of polypeptides (Arg, Lys, His). The type-2 site is specific for bulky hydrophobic N-terminal residues (Trp, Phe, Tyr, Leu, Ile). The third binding site of UBR1 recognizes an internal (non-N-terminal) degron in target proteins. The third binding site of UBR1 is autoinhibited but can be allosterically activated through a conformational change that is caused by the binding of short peptides, such as dipeptides, to the other, type-1 and type-2, binding sites of UBR1. The known substrate of the third binding site of UBR1 is CUP9 (1-3), a homeodomain protein and transcriptional regulator, largely a repressor, of more than 30 genes in *S. cerevisiae*[1]. The regulon of CUP9 includes *PTR2*, a gene encoding the main transporter of di/tripeptides (29,30). The reversal of UBR1 autoinhibition by imported di/tripeptides with destabilizing N-terminal residues accelerates the UBR1-dependent ubiquitylation of CUP9, leads to its faster degradation, and thereby causes a derepression of *PTR2*. The resulting positive-feedback circuit allows *S. cerevisiae* to detect the presence of extracellular peptides and to react by increasing their uptake (2,3).

The evolution of peptide-import circuits is under conflicting selective pressures, as the ability of a cell to import peptides confers both a benefit (utilization of peptides as food) and a vulnerability to toxins that resemble short peptides. While PTR2 is the major transporter of di/tripeptides in *S. cerevisiae*, some di/tripeptides can also be imported (with a low efficacy) by DAL5, whose major function is the import of other nitrogen sources, such as allantoate and ureidosuccinate (4). Another set of *S. cerevisiae* peptide transporters comprises OPT1 (HGT1) and OPT2, which have partially overlapping functions, do not import di/tripeptides but can import peptides of 4-5 residues. In addition, OPT1 (HGT1) is a high-affinity importer of glutathione, a "noncanonical" tripeptide (ref. (31) and refs. therein). Similarly to the PTR2 transporter of di/tripeptides, the expression of OTP2 is downregulated by CUP9, whereas the expression of OPT1 (HGT1) is independent of CUP9 (31). In addition to PTR2 and OPT2, the N-end rule pathway also controls the expression of DAL5, but in a manner opposite to that of the other two transporters: whereas CUP9 is a transcriptional repressor of *PTR2* and *OPT2*, CUP9 apparently upregulates the expression of *DAL5* (29,31). It is unknown whether CUP9 downregulates a repressor of *DAL5* or whether CUP9 acts, in the context of *DAL5*, as a transcriptional activator.

The induction of the PTR2 peptide transporter by di/tripeptides, a process controlled by the UBR1-CUP9 circuit (3), is just one of regulatory inputs that couple PTR2 expression to the availability and quality of nutrients. For example, PTR2 expression is downregulated by certain nitrogen sources, including ammonia, but not by other nitrogen sources, such as urea and allantoin (32). The underlying systems, including the N-end rule pathway, ensure that a cell does not waste resources synthesizing large amounts of the PTR2 transporter in the absence of extracellular peptides, or when a more efficacious nitrogen source, such as ammonia, is present. PTR2 is also induced by extracellular amino acids, particularly leucine (Leu) or

tryptophan (Trp) (33). This response is likely to be adaptive in natural habitats, as the amino acids that *S. cerevisiae* (a scavenging heterotroph) encounters outside laboratory tend to be the breakdown products of peptides and thus signify a likely presence of di/tripeptides.

Extracellular amino acids regulate PTR2 through the SPS (SSY1-PTR3-SSY5) pathway (34-40). SSY1, an integral membrane protein and a sensor of amino acids, is a sequelog of amino acid transporters but does not function as a transporter (36,37,41), a disposition that recurs with other nutrient sensors as well (37,42). Both the inferred design of SSY1 and experimental evidence suggest that it is the concentration ratio of an amino acid across the plasma membrane, rather than the absolute concentration of extracellular amino acid that determines the signaling output by SSY1 (36). Activated SSY1 induces expression of a regulon that includes the PTR2 peptide transporter and amino acid transporters such as AGP1, BAP2, BAP3, TAT1, TAT2 and GNP1 (38). PTR3 and SSY5 are peripheral membrane proteins associated with SSY1 (41). SSY5 is a protease regulated, in particular, by PTR3. SSY5 can cleave, and thereby activate, the latent (conditionally cytosolic) transcriptional activators STP1 and STP2, leading to their import into the nucleus and the induction of genes that encode, in particular, amino acid transporters (34-37,39,40,43).

In the present work, we show that an extracellular amino acid such as Trp acts via the SPS system to induce the PTR2-mediated import of di/tripeptides through the acceleration of degradation of CUP9, the repressor of import, by the UBR1-dependent N-end rule pathway. The bulk of this effect of Trp on the rate of CUP9 degradation requires both SSY1 and PTR3. At the same time, no significant activation of the N-end rule pathway toward substrates with destabilizing N-terminal residues (i.e., toward substrates with N-degrons) was observed under these conditions, suggesting a differential regulation of three substrate-binding sites of the UBR1 Ub ligase. We also show that the repression of *PTR2* by CUP9 requires the global corepressors TUP1 and SSN6, and that GAP1, a general amino acid transporter, strongly contributes to the induction of *PTR2* by Trp. While several aspects of this complex circuit remain to be understood, our findings establish new functional links between the amino acids-sensing SPS system, the CUP9-TUP1-SSN6 repressor complex, the PTR2 peptide transporter and the UBR1-dependent N-end rule pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Genetic Techniques – The *S. cerevisiae* strains used in this study are described in Table 1. AVY24 and AVY25 were constructed in the background of strain RJD347 (*MATα ura3-52*; a gift from Dr. R. Deshaies, California Institute of Technology). A PCR-based deletion strategy (44) was employed, using DNA fragments encoding the myc_3-URA3- myc_3 cassette to precisely delete *SSY1* and *PTR3* open reading frames (ORFs) (myc_3 denotes 3 repeats of a nucleotide sequence encoding the myc epitope, flanked on either side by 50 bp of sequences identical to the sequences of either the *SSY1* or the *PTR3* gene, respectively). This technique was employed to produce deletions of the *SSY1* and *PTR3* ORFs. We then selected for recombination within the *myc_3-URA3-myc_3* module using 5-fluoroorotic acid (FOA), to produce strains AVY27 (*MATα ura3-52 ssy1Δ::myc_3*) and AVY28 (*MATα ura3-52 ptr3Δ::myc_3*), and verified the identity of these strains by Southern hybridization (data not shown). AVY26 (*MATα ura3-52 ubr1Δ::HisG*) was also a derivative of RJD347 (Table 1). It was constructed using the same PCR-based deletion strategy (44), except a *HisG-URA3-HisG* cassette (see below) was used. Strains AVY30 (*MATα leu2-3,112 ubr1Δ::LEU2*), AVY31 (*MATα leu2-3,112 cup9Δ::LEU2*), and AVY32 (*MATα LEU2*) (Table 1) were derivatives of RJD350 (*MATα leu2-3,112*) (a gift from Dr. R. Deshaies).

PCR with *S. cerevisiae* genomic DNA was used to produce a fragment containing *SSN6* flanked by 901 bp and 400 bp of upstream and downstream sequences, respectively. The fragment was ligated to *Not*I/*Eco*RI-cut pRS314 (45). The same procedure was used to construct a fragment containing *TUP1* flanked by 459 bp and 400 bp of upstream and downstream sequences, respectively, followed by ligation to *Spe*I/*Cla*I-cut pRS416 (45). pSsn6Δ, which

carried *ssn6Δ::HisG-URA3-HisG*, was constructed by subcloning the *SSN6*-containing *Not*I-*Eco*RI fragment into *Not*I/*Eco*RI-cut Bluescript (Stratagene), producing pBlueSSN6. Using PCR, a deletion (from 50 bp upstream of the *SSN6* start codon to position 2,585 of its ORF) was introduced, while simultaneously creating an *Ecl*XI site at the upstream breakpoint of the deletion. The *Ecl*XI-*Pst*I fragment of pAS315 (a gift from Dr. A. Sil, University of California, San Francisco) containing *HisG-URA3-HisG*, was then inserted. *In vivo* integration of the *Not*I-*Eco*RI fragment of the resulting plasmid (pSsn6Δ), carrying *ssn6Δ::HisG-URA3-HisG*, produced a deletion of *SSN6* that spanned the region from -50 to 2,585, relative to start codon. pTup1Δ, which carried *tup1Δ::HisG-URA3-HisG*, was constructed similarly, using the *Spe*I-*Cla*I fragment containing *TUP1* (from pTUP1), its subcloning into pBluescript (thus yielding pBlueTUP1), and PCR to produce a deletion allele of *TUP1* (from 7 bp upstream of its start codon to position 2,016 of the *TUP1* ORF) that contained a (PCR-added) *Bam*HI site at the deletion breakpoint. The *Bam*HI-*Eco*RI fragment of pAS135 was then inserted into pBlueTUP1. *In vivo* integration of the *Spe*I-*Xho*I fragment of the resulting plasmid (carrying *tup1Δ::HisG-URA3-HisG*) produced a deletion of *TUP1*, from -7 to +2,016, relative to start codon. To select for recombination/excision within the integrated *HisG-URA3-HisG* cassette and thereby to produce *ssn6Δ::HisG* and *tup1Δ::HisG* alleles, 5-fluoroorotic acid (FOA)-resistant yeast colonies were isolated, and the identities of strains were verified by Southern hybridization (data not shown). This procedure was performed in the JD52 background to construct strains AVY60 and AVY61; in the JD55 (*ubr1Δ*) background (Table 1) (24) to construct AVY62 and AVY63; in the AVY50 background (1) to construct AVY64 and AVY65; and in the AVY51 background (1) to construct AVY66 and AVY67 (Table 1).

SSN6 tagged at the C-terminus with two copies of the myc epitope ($SSN6_{myc2}$) and expressed from its own promoter on a high copy (2μ) plasmid was a gift from Dr. R. Zitomer (State University of New York, Albany). For plasmid-based complementation assays, plasmids encoding SSN6 (pSSN6) and TUP1 (pTUP1) were constructed using standard techniques. ^{f}DHFR-Ub^{K48R}-$CUP9_{NSF}$ was expressed from the P_{MET25} promoter in the plasmid pMET416UPRCUP9$_{NSF}$, based on the low copy (*CEN*) vector p416MET25 (3). The previously described (28) pUb23-X plasmids expressed X-βgal reporter proteins (derived from the corresponding Ub-X-β-galactosidase fusions; X=Met, Ala, His or Tyr) from the P_{GAL1} promoter in a high copy vector.

Coimmunoprecipitation, Pulse-Chase, β-Galactosidase and Northern Hybridization Assays – For CUP9-SSN6 coimmunoprecipitation assays, *S. cerevisiae* carrying the indicated plasmids were grown to A_{600} of ~1 in SD medium containing auxotrophic supplements. Cells from a 10-ml culture were harvested by centrifugation, washed in 1 ml of water, and resuspended in 0.8 ml of ice-cold buffer C (0.25 M NaCl, 1 mM EDTA, 50 mM HEPES, pH 7.5) containing ovalbumin at 1 mg/ml. Cells were disrupted by vortexing with 0.5 ml (packed volume) of 0.5-mm glass beads in the presence of protease inhibitors (Roche). Extracts were prepared and immunoprecipitations were carried out as described (46), with two modifications. First, after incubation of an extract with anti-flag beads (Sigma, St. Louis, MO) for 45 min at 4°C, the resulting immunoprecipitate was washed once with 1 ml of buffer C, and then twice more with 1 ml of buffer C lacking ovalbumin. Second, immunoprecipitates were eluted from anti-flag beads through a 15-min incubation, at room temperature, with the flag peptide at 0.5 mg/ml, in buffer C lacking ovalbumin. An equal volume of 2 x SDS-PAGE sample buffer was added, followed by SDS-13% PAGE and immunoblotting with anti-myc antibody (Covance, Berkeley, CA), followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (BioRad, Hercules, CA) at 1:1,000 dilution, and thereafter a detection of the latter antibody using ECL assay (GE Healthcare, Piscataway, NJ).

For pulse-chase assays, *S. cerevisiae* was grown to A_{600} of ~0.8 in SHM medium (2% glucose, 0.1% allantoin, 0.17% yeast nitrogen base without amino acids and without ammonium sulfate) that either contained or

lacked tryptophan (Trp) (98 μM; 20 μg/ml), an inducing amino acid (33). Cells were harvested, washed in 0.8 ml of SHM ±Trp, resuspended in 0.4 ml of the same medium, and labeled for 5 min at 30°C with 0.16 mCi of ^{35}S-EXPRESS (Perkin-Elmer, Waltham, MA, USA). Thereafter cells were pelleted, resuspended in fresh SHM ± tryptophan containing 4 mM L-methionine and 2 mM L-cysteine, and incubated further at 30°C. Samples of 0.1 ml were taken at indicated time points and transferred to chilled tubes, each containing 0.5 ml of 0.5-mm glass beads, 0.7 ml of ice-cold lysis buffer (1% Triton-X100, 0.15 M NaCl, 5 mM EDTA, 50 mM HEPES, pH 7.5), and a mixture of protease inhibitors (final concentrations: 1 mM freshly prepared phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin). Extracts were prepared and immunoprecipitations carried out as described (46), using anti-flag M2 beads (Sigma). Immunoprecipitated proteins were fractionated by SDS-13% PAGE, followed by autoradiography. Some of pulse-chase assays were carried out in the presence of cycloheximide (at 0.2 mg/ml) during the chase.

For β-galactosidase (βgal) activity assays, *S. cerevisiae* carrying a pUB23-X plasmid (28) and expressing, from the P_{GAL1} promoter, an X-βgal test protein (derived from Ub-X-β-galactosidase, with X=Met, Ala, His or Tyr) (47) were grown in SHM medium overnight. Cultures were diluted to A_{600} of ~0.1 in SHM containing 2% galactose instead of glucose and either containing or lacking 98 μM Trp, followed by growth until A_{600} of ~0.5. Cell extracts were thereafter prepared and processed for assays with o-nitrophenyl o-nitrophenyl-β-D-galactopyranoside (ONPG) as previously described (28). For Northern analyses of *PTR2* induction by amino acids, cells were grown to A_{600} of ~0.6 in SHM containing or lacking 98 μM (20 μg/ml) Trp. Total RNA was prepared as described previously (15), using RNeasy Mini Kit (Qiagen, Valencia, CA). Samples containing 25 μg of RNA were electrophoresed in 1% formaldehyde-agarose gels, followed by blotting for Northern analysis (3). Northern assays of the effects of deletions of specific genes on *PTR2* expression were carried out identically, in the absence of added Trp.

RESULTS AND DISCUSSION

Repression of PTR2 Transcription by CUP9 Involves the CUP9-TUP1-SSN6 Corepressor Complex – UBR1, the E3 Ub ligase of the *S. cerevisiae* N-end rule pathway, regulates the import of peptides by controlling, through degradation of the transcriptional repressor CUP9, the expression of the peptide transporter PTR2 (1). CUP9 was identified using a bypass screen for mutations that restored dipeptide uptake and *PTR2* expression in *ubr1*Δ cells (1). Further analysis, described below, of our collection of bypass mutants identified additional isolates that did not belong to the *CUP9* complementation group. Many of these non-*cup9* mutants (Fig. 1*A*) exhibited flocculation (aggregation during growth in liquid culture) and had a reduced mating ability. These phenotypes are characteristic of a defect in either *TUP1* or *SSN6*, which encode two "global" corepressors that form a complex interfering with transcription initiation (48-50)). The TUP1-SSN6 complex, which does not bind to DNA by itself, is recruited to individual promoters through interactions with specific DNA-binding proteins. One such protein is the mating-type regulator MATα2 (51,52). In the absence of either TUP1 or SSN6, the MATα2-mediated repression of *MAT**a***-specific genes is defective, resulting in mating-defective, pseudodiploid cells (48).

To determine whether the above non-*cup9* isolates (Fig. 1*A*) were either *tup1* or *ssn6* mutants, they were transformed with low-copy plasmids that expressed either *TUP1* or *SSN6* from their natural promoters. These plasmids indeed suppressed the clumpy-growth and peptide-uptake phenotypes of all of the flocculating bypass mutants. The entire collection of our 201 bypass mutants (including *cup9* mutants) was partitioned into complementation groups using a combination of the above plasmid-based complementation tests as well as mating-based complementation assays (the latter could not be comprehensive, owing to mating deficiency of some isolates). The 201 bypass suppressors were found to belong to 3 complementation groups, defined by *CUP9, TUP1* and *SSN6*. Apart from genes required for cell viability, it is likely that our

(nonconditional) screen was an exhaustive one, given the numbers of isolates in each of the 3 complementation groups thus identified: 104, 67, and 30 isolates of *cup9*, *ssn6* and *tup1* mutants, respectively (Fig. 1*A*).

To verify the role of *TUP1* and *SSN6* in regulating peptide uptake, we tested the effects of disrupting either one of these genes on the ability of a *ubr1*Δ strain to import dipeptides. Congenic [*tup1*Δ *ubr1*Δ] and [*ssn6*Δ *ubr1*Δ] double mutants were constructed using homologous recombination in the lysine (Lys)-auxotrophic (*lys2*) genetic background, and the identities of mutants were verified by Southern hybridization (data not shown). The ability of these Lys-requiring double mutants to import dipeptides was then assayed by monitoring their growth on a minimal medium containing the dipeptide Lys-Ala as the sole source of Lys (Fig. 1*B*). A *lys2* strain containing the intact *UBR1* gene was able to import sufficient amounts of Lys-Ala dipeptide to support its growth on this medium, whereas a congenic [*ubr1*Δ *lys2*] strain could not grow under these conditions (it could be rescued by free Lys) (Fig. 1*B*). In contrast to the [*ubr1*Δ *lys2*] strain, both [*tup1*Δ *ubr1*Δ *lys2*] and [*ssn6*Δ *ubr1*Δ *lys2*] triple mutants formed colonies on Lys-Ala plates, as did a [*cup9*Δ *ubr1*Δ *lys2*] strain (Fig. 1*B*). Thus, a deletion of either *CUP9,* or *TUP1,* or *SSN6* is each capable of restoring dipeptide uptake to a *ubr1*Δ strain. The slightly smaller colonies formed by the [*ssn6*Δ *ubr1*Δ *lys2*] strain on Lys-Ala plates could be caused by a growth defect of *ssn6*Δ strains (49), a phenotype that we also observed with the [*ssn6*Δ *ubr1*Δ *lys2*] strain on a control (Lys-supplemented) plate (Fig. 1*B*).

The fact that *cup9, tup1* and *ssn6* mutants all bypassed the block to dipeptide uptake in a *ubr1*Δ strain suggested that CUP9 represses *PTR2* expression by functioning as a part of a TUP1/SSN6-containing complex. To address this model, we compared the levels of *PTR2* mRNA in *ssn6*Δ and *tup1*Δ mutants with those in other strains, in the absence of added *PTR2* inducers such as dipeptides or amino acids. In a strain retaining all 5 of the studied genes, *PTR2*, *UBR1*, *CUP9*, *SSN6* and *TUP1*, and also in a *ubr1*Δ strain, the level of *PTR2* mRNA was low enough to be undetectable at the sensitivity of Northern assays used (Fig. 2*A*, lanes a, b; compare with lanes c-i). With *ubr1*Δ strains, no *PTR2* mRNA could be detected even by higher-sensitivity Northerns (data not shown), whereas wild-type (*UBR1*) strains, in the absence of either added dipeptides or "inducing" amino acids, contained low but detectable levels of *PTR2* mRNA, as shown previously (1,3). In contrast, the levels of *PTR2* mRNA were high in the absence of either *CUP9*, or *SSN6*, or *TUP1*, irrespective of the presence or absence of *UBR1* (Figs. 2*A*, lanes c-i; compare with lanes a, b; see also Fig. 3A, lane c versus lane i). Thus, the tight block to dipeptide uptake in a *ubr1*Δ strain, caused by increased levels of the CUP9 repressor, could be bypassed not only through the removal of CUP9, but also through the removal of either SSN6 or TUP1 (Fig. 2*A*).

High levels of *PTR2* mRNA in the absence of either CUP9, SSN6, or TUP1 (Figs. 2*A* and 3*A*) indicated that each of these proteins was essential for repression of the *PTR2* gene. Both the demonstrated functional dependence of CUP9 on SSN6/TUP1 (Figs. 1 and 2*A*) and the close analogy between the homeodomain repressors CUP9 and MATα2, the latter of which is known to interact with SSN6/TUP1 (see above), suggested that CUP9 also interacted with SSN6/TUP1. To address this experimentally, we carried out a coimmunoprecipitation assay, using epitope-tagged SSN6 and CUP9 (Fig. 2*B*). Since overexpression of wild-type CUP9 inhibits cell growth, most likely owing to its interactions with DNA (data not shown), we employed a previously constructed nontoxic derivative of CUP9 (3). CUP9 interacts with DNA through its homeodomain motif. Based on the previously studied interactions of other homeodomain proteins with DNA, an Asn (N) → Ser (S) substitution at position 265, within the recognition helix of the CUP9 homeodomain, was predicted to strongly reduce the affinity of CUP9 for DNA without causing a significant structural perturbation (51). This nontoxic CUP9 derivative, tagged at the C-terminus with the flag epitope, was termed $CUP9_{NSF}$ (3). Extracts were prepared from *S. cerevisiae* stably expressing both $CUP9_{NSF}$ and SSN6 ($SSN6_{myc2}$) (the latter C-terminally

tagged with a double myc epitope), followed by immunoprecipitation with anti-flag antibody, SDS-PAGE, and immunoblotting with anti-myc antibody. $SSN6_{myc2}$ was coimmunoprecipitated with $CUP9_{NSF}$ in this assay (Fig. 2*B*, lane c). No $SSN6_{myc2}$ was detected in immunoprecipitates of extracts from cells that did not also express $CUP9_{NSF}$ (Fig. 2*B*, lane b), and the expression levels of $CUP9_{NSF}$ and $SSN6_{myc2}$ were similar in cells that coexpressed the two proteins (data not shown). Thus, in agreement with the above expectation, these results (Fig. 2*B*) suggested that untagged SSN6 and untagged CUP9, at their (lower) natural expression levels in *S. cerevisiae*, would also form a complex that is analogous to the SSN6/MATα2 complex (50), a model that accounts for the observed functional interdependence among CUP9, SSN6 and TUP1 (Figs. 1*B*, 2*A* and 3*A*). While we did not examine the immunoprecipitated $CUP9_{NSF}$-containing complexes for the presence of TUP1, extensive previous work has established that SSN6 and TUP1 form a stable complex both *in vivo* and as purified proteins (49), indicating that it is the complex of SSN6 and TUP1 that interacts with CUP9, similarly to the previously characterized interaction between SSN6/TUP1 and MATα2, another homeodomain repressor (50).

Both CUP9 and UBR1 Are Required for Inducibility of PTR2 by Amino Acids – When an amino acid, particularly a bulky hydrophobic one such as Leu or Trp, is added to a culture of *S. cerevisiae* growing on a poor nitrogen source such as, for example, allantoin, cells induce their *PTR2* gene and thus increase their capacity for the import of di/tripeptides (33). Effective concentrations of extracellular Leu or Trp can be as low as 1 μM (33). Previous work has shown that an accelerated degradation of CUP9 by the UBR1-dependent N-end rule pathway in response to imported dipeptides with destabilizing N-terminal residues, strongly enhances *PTR2* expression (3). This result led us to consider whether other signals that up-regulate *PTR2* expression, e.g., amino acids, may also act through acceleration of CUP9 degradation.

To begin addressing this possibility, we examined the effects of Trp, a strongly inducing amino acid, on *PTR2* mRNA levels in several *S. cerevisiae* strains, AVY32, AVY24, AVY25, AVY30 and AVY31, that were constructed to be completely prototrophic, and in particular did not require extracellular amino acids for viability (Table 1). These strains were grown in allantoin-based media (SHM) containing or lacking Trp. Allantoin is a nonrepressing nitrogen source (33), so the expression of *PTR2* was not influenced by the nitrogen catabolite repression under these conditions (53). *PTR2* mRNA levels were extremely low in wild-type [*UBR1 CUP9*] cells grown in medium lacking amino acids, but in the presence of 98 μM (20 μg/ml) Trp the expression of *PTR2* was greatly increased (Fig. 3*A*, lanes a, b). By contrast, in the absence of CUP9 (in a *cup9*Δ strain), *PTR2* was highly expressed in either the presence or absence of Trp (Fig. 3*A*, lanes i, j; compare with lanes a, b). Finally, *PTR2* mRNA was undetectable in a *ubr1*Δ strain grown in either the presence or absence of Trp (Fig. 3*A*, lane c, d). Thus, both *UBR1* and *CUP9* are required for inducibility of *PTR2* expression by an amino acid.

Three genes, *SSY1*, *PTR3*, and *SSY5*, are a part of the SPS amino acid-sensing pathway in *S. cerevisiae* (see Introduction). We examined the influence of added Trp on the levels of *PTR2* mRNA in *ssy1*Δ and *ptr3*Δ strains, comparing the consequences of deleting these genes with the effects of deleting either *CUP9* or *UBR1*. The induction of *PTR2* by Trp was dramatically reduced in both *ssy1*Δ and *ptr3*Δ strains (Fig. 3*A*, lanes e-h). However, a weak Trp-mediated increase in *PTR2* expression was still observed in these strains, in contrast to the complete absence of *PTR2* expression (and of Trp effect) in *ubr1*Δ cells, and in contrast to a high level of *PTR2* mRNA in *cup9*Δ cells (Fig. 3*A*). Thus the bulk (but apparently not all) of the Trp effect on the expression of *PTR2* requires *SSY1* and *PTR3*.

We next examined whether the general amino acid permease GAP1 (37,54) was required for Trp-mediated induction of *PTR2* mRNA. Expression of *PTR2* in wild-type cells and a congenic *gap1*Δ strain was measured by Northern analysis as a function of time after the addition of Trp. Wild-type cells exhibited a

strong induction of *PTR2* by 20 min in the presence of Trp (Fig. 3*D, E*, lanes a-c). Remarkably, the induction of *PTR2* by Trp, while still occurring in the absence of GAP1, was found to be strongly decreased (Fig. 3*D, E*, lanes d-f; compare with lanes a-c). To the best of our knowledge, this is the first indication of a major involvement of GAP1 in the amino acid-mediated induction of the PTR2 peptide transporter. Being an inducer of *PTR2* and other transporter genes via the amino acid sensor SSY1, the extracellular Trp exerts its effect without its import across the plasma membrane (see Introduction). Therefore the absence of GAP1 as an importer of Trp would not be expected, *a priori*, to influence the PTR2-inducing effect of Trp. Moreover, Trp can also be imported by the Trp-specific permease TAP2 (SCM2) (55). In sum, the observed decrease (but not elimination) of *PTR2* induction in the absence of *GAP1* (Fig. 3*D, E*, lanes a-f) stems from a function of GAP1 that is separate from its role as a transporter of amino acids. Indeed, *S. cerevisiae* GAP1 is known to function as an amino acid sensor for activation of protein kinase-A (PKA) targets. The PKA and SCH9 kinases have both overlapping and distinct functions in controlling the adaptation of *S. cerevisiae* to nutrient availability, and GAP1 plays a role at least in the PKA-mediated part of this circuit (54,56). Thus, while the mechanistic understanding of GAP1's involvement in the induction of *PTR2* by Trp remains to be attained, the above finding (Fig. 3*D, E*) revealed a new aspect of *PTR2* control and yet another function of GAP1 as a nutritional regulator.

We also measured, in the *gap1* background, the influence of STP1 and/or STP2 on the Trp-mediated induction of *PTR2* mRNA. STP1 and STP2 are two sequelogous, conditionally active transcriptional activators that mediate the signaling by the SPS pathway (see Introduction) (35,39,40,43). Northern analyses of *PTR2* expression as a function of time after addition of Trp showed that the Trp-mediated induction of *PTR2* was largely retained in a [*gap1*Δ *stp1*Δ] strain (which lacked the STP1 transcriptional activator), in comparison to congenic *gap1*Δ strain, whereas the STP2 transcriptional activator was essential for *PTR2* induction by Trp: both a [*gap1*Δ *stp2*Δ] mutant and a triple mutant [*gap1*Δ *stp1*Δ *stp2*Δ] did not exhibit a significant induction of *PTR2* by Trp, in contrast to parental *gap1*Δ and [*gap1*Δ *stp1*Δ] strains (Fig. 3*D, E*). Thus, as could be expected from the presence of STP1/2-recognized UAS_{AA} nucleotide sequence motifs (57) upstream of the *PTR2* gene (data not shown), the induction of *PTR2* proceeds both through its transcriptional upregulation, in particular by the Trp-activated STP2 (Fig. 3*D, E*), and through a decrease in its repression, owing to the Trp-induced acceleration of degradation of the CUP9 repressor by the N-end rule pathway, as described below (Fig. 4).

The SPS Pathway Induces PTR2 Expression Through Acceleration of CUP9 Degradation – Some of the above results (Fig. 3*A*) suggested that the induction of PTR2 transporter by amino acids might occur through an increase in the rate of degradation of CUP9 repressor. A precedent for this possibility was the previously demonstrated acceleration of CUP9 degradation by dipeptides with destabilizing N-terminal residues (3). In the latter case, the bulk of CUP9 degradation, both before and after the addition of dipeptides to the medium, was carried out by the UBR1-dependent N-end rule pathway (3). To determine whether the rate of CUP9 degradation was altered by a PTR2-inducing amino acid such as Trp, we carried out pulse-chase assays with CUP9. These experiments utilized $CUP9_{NSF}$, the above-described nontoxic, single-residue mutant of CUP9. $CUP9_{NSF}$ was expressed as a ^{f}DHFR-Ub^{K48R}-$CUP9_{NSF}$ fusion, where ^{f}DHFR was the N-terminally flag-tagged mouse dihydrofolate reductase. Deubiquitylating enzymes (DUBs) cotranslationally cleave this fusion at the Ub^{K48R}-$CUP9_{NSF}$ junction, yielding the long-lived reference protein ^{f}DHFR-Ub^{K48R} and the test protein $CUP9_{NSF}$ (3). The reference ^{f}DHFR-Ub^{K48R} served as a "built-in" internal control for variations in expression levels and immunoprecipitation efficiency. This generally applicable method, called the UPR (Ub-protein-reference) technique (refs. (10,47) and refs. therein), increases the accuracy of pulse-chase assays. The *in vivo* degradation of $CUP9_{NSF}$ was indistinguishable from that of wild-type CUP9, and was also not altered significantly by the incorporation of this protein into the above

(cotranslationally processed) UPR-type fusion (3).

We expressed $CUP9_{NSF}$ from a *URA3*-bearing plasmid in *S. cerevisiae* strains that did not require extracellular amino acids for viability (RJD347, AVY26, AVY27 and AVY28; see Table 1). In wild-type (*UBR1*) cells grown in the SHM medium lacking amino acids, $CUP9_{NSF}$ was degraded with $t_{1/2}$ of ~10 min (Fig. 4*A, B, E, F*). Remarkably, the addition of Trp (to 98 μM, or 20 μg/ml) to the medium resulted in a strong acceleration of $CUP9_{NSF}$ degradation, with $t_{1/2}$ of ~2 min between 0 and 5 min of chase, and even less than 2 min between 5 and 10 min of chase (Fig. 4 *A, B, E, F*). This effect of Trp on the rate of $CUP9_{NSF}$ degradation was reproducible in separate and independent pulse-chase assays, including a set of assays in which cycloheximide, a translation inhibitor, was present during chase (Fig. 4*C, G*). (The other assays, in Fig. 4*A, B, E, F*, did not involve cycloheximide.) Although in cycloheximide-based assays (Fig. 4*C, G*) the $CUP9_{NSF}$ protein was degraded more slowly ($t_{1/2}$ of ~20 min and 3-4 min in the absence and presence of Trp, respectively) than in cycloheximide-free assays (Fig. 4*A, B, E, F*), the effect of Trp was comparably strong in both cases (Fig. 4). In addition to increasing the accuracy of pulse-chase assays, the UPR technique, specifically its "built-in", long-lived reference protein, makes it possible to determine the *relative* level of a test protein (measured as the ratio of ^{35}S in a test versus reference protein) at the beginning of chase, i.e., at the end of pulse labeling (10). In Fig. 4*E-G*, 100% was assigned, in each of three panels, to the relative amount of ^{35}S (relative to the reference protein) of $CUP9_{NSF}$ at time "0" (the end of pulse labeling) in the absence of Trp in the medium. Thus, a value below 100% at time "0" reflects the relative extent of $CUP9_{NSF}$ degradation during the 5-min pulse. In all of pulse-chase assays (Fig. 4*E-G*), these initial (time-zero) levels of $CUP9_{NSF}$ were found to correlate, consistently, with Trp-induced changes in the rates of $CUP9_{NSF}$ degradation that were measured during the chase.

The presence of *SSY1* (Fig. 4*A, E*) and also, independently, of *PTR3* (4*B, F*) was found to be strongly but partially required for the Trp-accelerated degradation of $CUP9_{NSF}$. Specifically, the bulk of enhanced degradation of $CUP9_{NSF}$ upon the addition of Trp was absent in *ssy1*Δ and *ptr3*Δ mutants (Fig. 4*A, B, E, F*). Interestingly, however, the degradation of $CUP9_{NSF}$ was still detectably accelerated by Trp in these mutant strains (Fig. 4*A, B, E, F*), in agreement with the weak but still detectable induction, by Trp, of *PTR2* mRNA in *ssy1*Δ and *ptr3*Δ mutants (Fig. 3*A*). As we observed previously as well (3), $CUP9_{NSF}$ was greatly but not completely stabilized in *ubr1*Δ cells (Fig. 4*G* and data not shown). Interestingly, the addition of Trp to *ubr1*Δ cells resulted in a weak but still detectable Trp-induced destabilization of the (now long-lived) $CUP9_{NSF}$, despite the absence of the N-end rule pathway (Fig. 4*G*, open circles versus open triangles). The "residual" (UBR1-independent) instability of $CUP9_{NSF}$ and the "residual" sensitivity of this degradation to Trp suggest that CUP9 may also be targeted, at a much lower rate, by a UBR1-independent proteolytic pathway. Our attempts to identify a relevant E3 Ub ligase, through the testing of non-*UBR1* E3 mutants, have not been successful, thus far (data not shown).

The Levels of UBR1 mRNA Are Not Changed Significantly by a PTR2-Inducing Amino Acid – Previous work has shown that the *in vivo* concentration of UBR1 is rate-limiting for degradation of N-end rule substrates by the *S. cerevisiae* N-end rule pathway (reviewed in ref. (5). To determine whether the Trp-induced acceleration of CUP9 degradation (Fig. 4) was accompanied by an increased level of the UBR1 Ub ligase, we used Northern hybridization to compare the levels of *UBR1* mRNA before and after the addition of Trp. Extracellular Trp did not cause a significant alteration in the levels of *UBR1* mRNA (Fig. 3*B, C*). Together with findings described in the next section (Fig. 5), these results (Fig. 3*B, C*) strongly suggested that the observed acceleration of CUP9 degradation in the presence of extracellular Trp (Fig. 4) was not caused by increased levels of UBR1. In addition, the levels of *UBR1* mRNA remained the same or nearly the same in the absence of *CUP9* (Fig. 3*B, C*), indicating that *UBR1* is not a part of the CUP9 regulon.

Degradation of Reporter Proteins Bearing N-Degrons Is Not Changed

Significantly By a PTR2-Inducing Amino Acid – CUP9 lacks an N-degron, and is recognized by the UBR1 Ub ligase through a C-terminus-proximal degron (see Introduction). To determine whether the acceleration of CUP9 degradation by Trp (Fig. 4) was accompanied by increased activity of the N-end rule pathway toward substrates with N-degrons, we employed X-β-galactosidase (X-βgal) reporters. They were produced through cotranslational deubiquitylation of the corresponding Ub-X-βgal fusions, where X was a variable residue (47,58). Previous work has shown that the enzymatic activity of βgal in extracts from cells that expressed an X-βgal reporter protein was a reliable measure of the reporter's metabolic stability *in vivo* (28). Of the four X-βgal reporters in Fig. 5, Met-βgal and Ala-βgal had stabilizing N-terminal residues, whereas His-βgal and Tyr-βgal had, respectively, a type-1 and type-2 primary destabilizing N-terminal residues. As would be expected from the UBR1-dependent degradation of His-βgal and Tyr-βgal (5), their levels in wild-type (*UBR1*) cells were much lower than the levels of long-lived Met-βgal and Ala-βgal under the same conditions (Fig. 5). We found that the addition of Trp, which led to a strong acceleration of the UBR1-dependent degradation of CUP9 (Fig. 4), did not result in significant alterations of the relative steady-state levels of His-βgal and Tyr-βgal reporters, in comparison to those of Met-βgal and Ala-βgal (Fig. 5). These findings were independently confirmed by carrying out pulse-chase assays with X-βgal reporter proteins (data not shown). Thus the activity of the N-end rule pathway toward substrates with N-degrons did not change significantly upon the addition of Trp.

Concluding Remarks – Extracellular Trp does not increase the activity of the N-end rule pathway toward its N-degron-bearing substrates, while it does accelerates degradation of the CUP9 repressor by the same pathway (Figs. 4 and 5), thereby making possible the induction of the PTR2 peptide transporter. This dichotomy constrains possible interpretations of our findings. Yet another constraint stems from the fact that the bulk of the Trp-induced acceleration of CUP9 degradation by the N-end rule pathway requires the amino acid-sensing SPS pathway (Figs. 3*A* and 6). CUP9 is targeted for degradation via its internal degron, through its interaction with a third substrate-binding site of the UBR1 E3 Ub ligase, which functions as the UBR1-RAD6 holoenzyme (see Introduction). The autoinhibited CUP9-binding site of UBR1 can be activated, allosterically, by dipeptides with destabilizing N-terminal residues that interact with the other two (type-1 and type-2) binding sites of UBR1 (2,3). Conformational equilibria, in the *in vivo* ensembles that contain UBR1, would determine a fraction of UBR1 that is active toward CUP9. We suggest that such equilibria can be shifted toward CUP9-targeting conformations of UBR1 not only in response to UBR1-binding di/tripeptides, as demonstrated previously (2,3), but also through other routes, for example UBR1 phosphorylation. Other studies have shown that signaling by the SSY1 sensor of amino acids that results in the PTR3/SSY5-dependent (proteolytic) activation of the latent transcription factors STP1 and STP2 is mediated, at least in part, by an increased phosphorylation of PTR3, in reactions mediated by the SSY1-associated YCK1/YCK2 kinases (35,40,59). In sum, a parsimonious, testable model that can account for our findings (Fig. 1-5) and is also consistent with other evidence is that the Trp-activated signaling by the SPS system (Fig. 6) may increase the rate of CUP9 degradation by the N-end rule pathway through a phosphorylation of the UBR1 Ub ligase. This phosphorylation may be mediated, at least in part, by the previously described SSY1-associated kinases that mediate the SSY1-induced phosphorylation of PTR3 (35,40,59). (As shown in Figs. 3 and 4, both SSY1 and PTR3 are required for the bulk of Trp-induced acceleration of CUP9 degradation.)

Our recent findings[2] indicate that UBR1 is a multiply phosphorylated protein. They also indicate that UBR1 phosphorylation involves a set of several kinases that include YCK1/YCK2, and that the resulting modifications of UBR1 regulate its functions[2]. Work is under way to dissect UBR1 phosphorylation *in vivo* and to determine whether specific species of phosphorylated UBR1 underlie the Trp-induced acceleration of degradation of the CUP9 repressor (Fig. 4). Work is also under way to identify a putative E3 Ub ligase(s) that appears

to mediate the "residual" degradation of CUP9 in *ubr1*Δ cells, which lack the N-end rule pathway. Yet another aspect of these circuits that remains to be understood is a mechanism that underlies our finding (Fig. 3*D, E*) that the general amino acid permease GAP1 contributes to the induction of *PTR2* by Trp. Given recent studies of GAP1 as an amino acid sensor (54,56), its Trp-mediated effect on *PTR2* is likely to involve a phosphorylation-based pathway. A better understanding of the spatiotemporal aspect of CUP9 regulation would also be important. Because steady-state levels of (short-lived) CUP9 in wild-type cells are very low, and because overexpression of wild-type CUP9 is toxic, this and previous studies (2,3) utilized $CUP9_{NSF}$, a missense mutant that does not bind to cognate DNA sites that CUP9 normally recognizes. The Trp-induced acceleration of $CUP9_{NSF}$ degradation (Fig. 4) implies that *in vivo* interactions of CUP9 with DNA do not play a significant role in the Trp effect. Nevertheless, it would be important to determine whether wild-type CUP9 can be targeted for degradation by the N-end rule pathway directly at sites of its association with DNA *in vivo*, whether a modification of CUP9, e.g., its phosphorylation, might also be involved in the Trp-induced, UBR1-dependent acceleration of CUP9 degradation, and whether this degradation takes place in the context of CUP9-containing complexes such as CUP9-SSN6-TUP1. Some of the other transcriptional repressors, such as MATα2, are metabolically stabilized by their association with SSN6-TUP1 (52).

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FOOTNOTES

[1]C. S. H. and A. V., unpublished data.
[2]C. S. H. and A. V., unpublished data.

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Legends to Table 1 and Figures

TABLE 1. *S. cerevisiae* strains used in this study.

FIGURE 1. Mutations in *SSN6* or *TUP1* rescue the block to dipeptide import in *ubr1*Δ *S. cerevisiae*, which lack the N-end rule pathway. (*A*) A collection of 201 mutants in which the ability of a *ubr1*Δ strain to import dipeptides had been restored (*cup9* members of that collection were examined in the earlier study (1)) were classed into complementation groups that included *SSN6* and *TUP1* using plasmid-based or mating-based complementation tests (1). The number of isolates in each complementation group, *cup9*, *tup1*, and *ssn6,* is shown. (*B*) *ssn6*Δ and *tup1*Δ restore dipeptide import to a *ubr1*Δ strain. Strains JD52, AVY107, AVY51, AVY62 and AVY63 (Table 1), with relevant genotypes indicated on the plate diagram, were tested for their ability to use the dipeptide Lys-Ala as a source of essential nutrient, lysine. All strains were *lys2* and thus auxotrophic for Lys. Strains were tested for growth on SD medium lacking Lys (left), or supplemented with Lys-Ala (center), or supplemented with Lys (right). Only strains capable of importing dipeptides could form colonies on the [-Lys; +[Lys-Ala]] plate. Deletion of either *SSN6*, *TUP1,* or (as shown previously (1)) *CUP9* restored dipeptide import to a *ubr1*Δ strain (center plate).

FIGURE 2. CUP9 represses *PTR2* transcription via SSN6 and TUP1. (*A*) *SSN6*, *TUP1* and *CUP9* are required for repression of the peptide transporter gene *PTR2*. Northern analysis of *PTR2* mRNA in the wild-type strain JD52, and in congenic *ssn6*Δ (AVY60), *tup1*Δ (AVY61), *cup9*Δ (AVY50) and *ubr1*Δ (AVY107) mutants, as well as in various combinations thereof (AVY51, and AVY62-AVY67) (Table 1). The status of each gene (-, deleted; +, wild-type allele) is shown in the upper diagram. *PTR2* and *ACT1* (actin) mRNA are indicated. *S. cerevisiae* strains were grown in SHM without PTR2-inducing amino acids or peptides. (*B*) Coimmunoprecipitation of myc-tagged SSN6 ($SSN6_{myc2}$) with $CUP9_{NSF}$, a flag-tagged CUP9 derivative (see Results and Discussion). Extracts from cells expressing the indicated combinations of $SSN6_{myc2}$ and $CUP9_{NSF}$ were immunoprecipitated with a monoclonal anti-flag antibody, followed by SDS-PAGE and immunoblotting with a

monoclonal anti-myc antibody (see Experimental Procedures).

FIGURE 3. Northern analyses of *PTR2* and *UBR1* under different conditions and in different genetic backgrounds. (*A*) Amino acid-inducible expression of *PTR2* requires *UBR1* and *CUP9*. Congenic strains of the indicated genotype (AVY32, AVY30, AVY24, AVY25, AVY31) (Table 1) were grown in SHM medium containing or lacking 98 μM (20 μg/ml) tryptophan (Trp). Isolated total RNA was analyzed by Northern hybridization with *PTR2* and *ACT1* DNA probes, as indicated. (*B, C*) Levels of *UBR1* expression do not change significantly in the presence versus absence of Trp. Total RNA from the strains AVY30-AVY32 (Table 1) was analyzed by Northern hybridization with *UBR1* (*B*) and *ACT1* (*C*). Lane a, RNA from *ubr1*Δ cells (negative control). Lanes b, c, RNA from wild-type (*UBR1*) cells either in the presence or absence of added Trp (98 μM). Lanes d, e, same as lanes b, c but RNA from *cup9*Δ cells. (*D*) Trp-mediated induction of *PTR2* in wild-type and mutant *S. cerevisiae*. Total RNA was isolated from congenic strains of indicated genotypes either before the addition of 98 μM Trp, or 20 min thereafter, or 60 min thereafter, and the relative levels of *PTR2* mRNA were determined by Northern hybridization. (*E*) Ethidium-stained rRNA (loading controls) in agarose gel before the transfer of RNA to hybridization membrane.

FIGURE 4. The bulk of Trp-accelerated CUP9 degradation requires SSY1, PTR3 and UBR1. Pulse-chase analyses, using the UPR technique and ^{f}DHFR-Ub^{K48R} reference protein, of the CUP9 degradation. *S. cerevisiae* of the indicated genotypes were grown in SHM medium containing or lacking 98 μM Trp (20 μg/ml). ^{35}S-Met/Cys labeling for 5 min, followed by chases for 5 and 10 min (*A, B*) or for 5, 15 and 30 min (*C, D*) , the latter with cycloheximide at 0.2 mg/ml. (*E, F*) PhosphorImager-based quantitation of pulse-chase patterns in *A* and *B*, respectively. Closed circles and triangles, wild-type cells, without and with added Trp, respectively. Open circles, either *ssy1*Δ cells (*A*) or *ptr3*Δ cells (*B*) in the absence of added Trp. Open triangles, same but with added Trp. (*G*) Quantitation of pulse-chase patterns in *C, D*. Closed circles and triangles, wild-type cells, without and with added Trp, respectively. Open circles and triangles, same but with *ubr1*Δ cells. Data in *C, D* and *G* were from experiments independent of those in *A, B, E, F*, and in addition were carried out with cycloheximide. See Experimental Procedures for additional details.

FIGURE 5. Relative metabolic stabilities of X-βgal test proteins in the absence or presence of added tryptophan. *In vivo* degradation of X-βgal proteins (X=Met, Ala, His, Tyr) was assessed by comparing their steady-state levels in wild-type *S. cerevisiae* in the absence or presence of added Trp. In contrast to CUP9, which is targeted for UBR1-dependent degradation through its internal, C-terminus-proximal degron, the short-lived His-βgal and Tyr-βgal (bearing, respectively, a type-1 and type-2 destabilizing N-terminal residue) are targeted by UBR1 via their respective N-degrons.

FIGURE 6. The amino acid-sensing SPS pathway and its connections with the PTR2-CUP9-UBR1 circuit, including the amino acid-mediated acceleration of degradation of CUP9, a transcriptional repressor of the PTR2 peptide transporter. See the main text for details, including the evidence for involvement of a GAP1-mediated pathway.

A

Gene	number of mutant isolates
cup9	104
ssn6	67
tup1	30

B

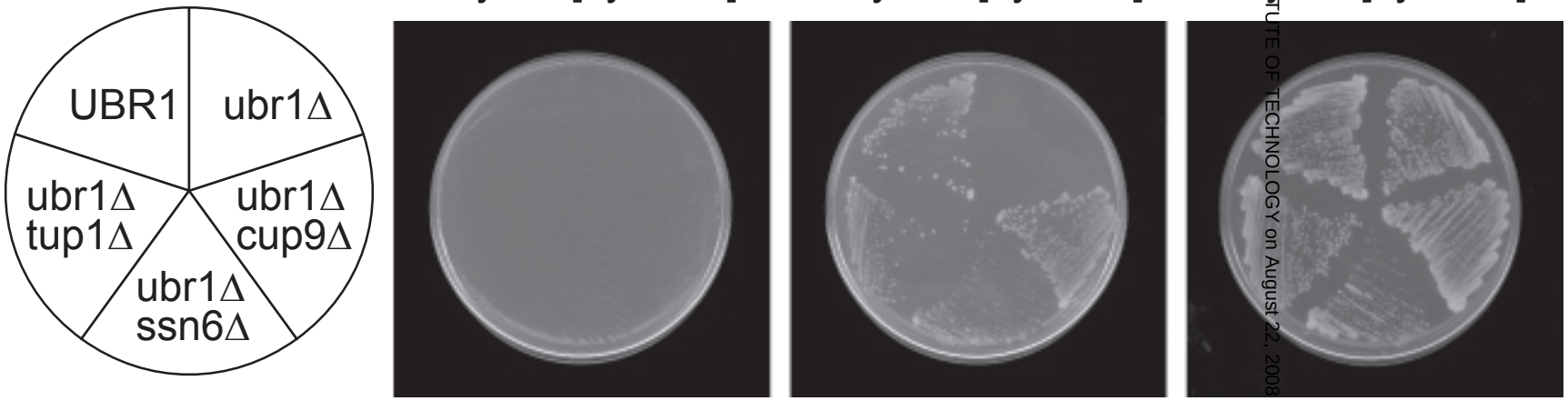


Fig. 1, Xia et al.

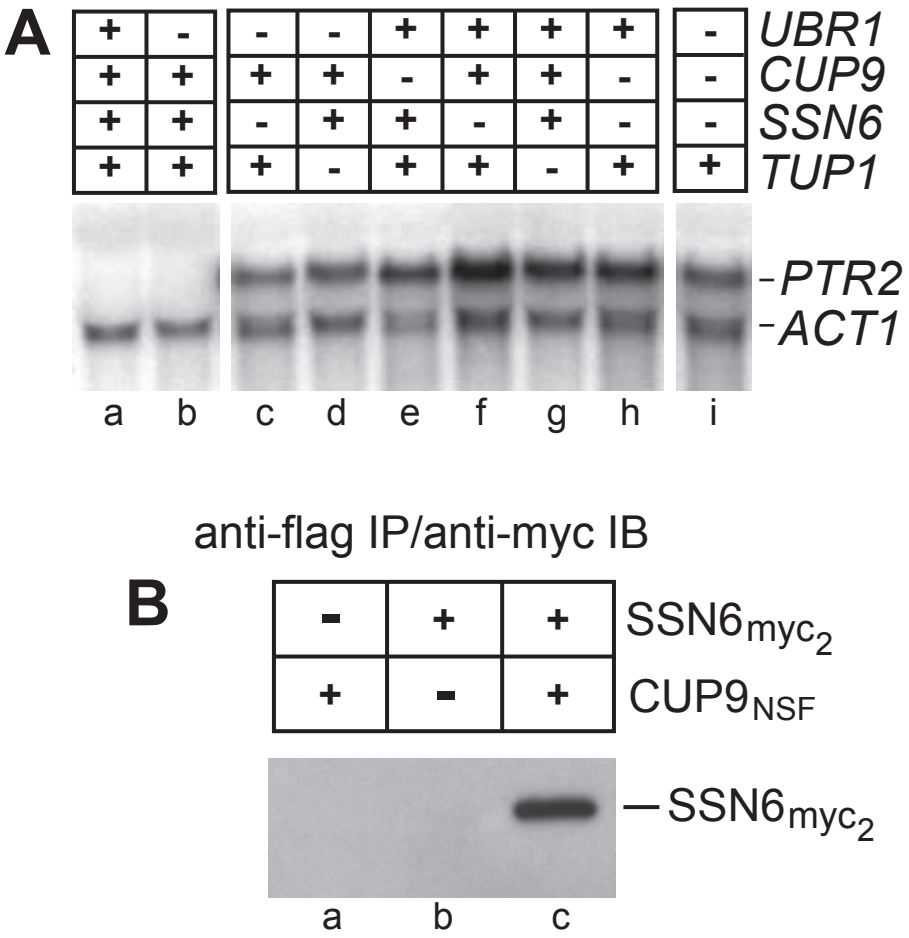


Fig. 2, Xia et al.

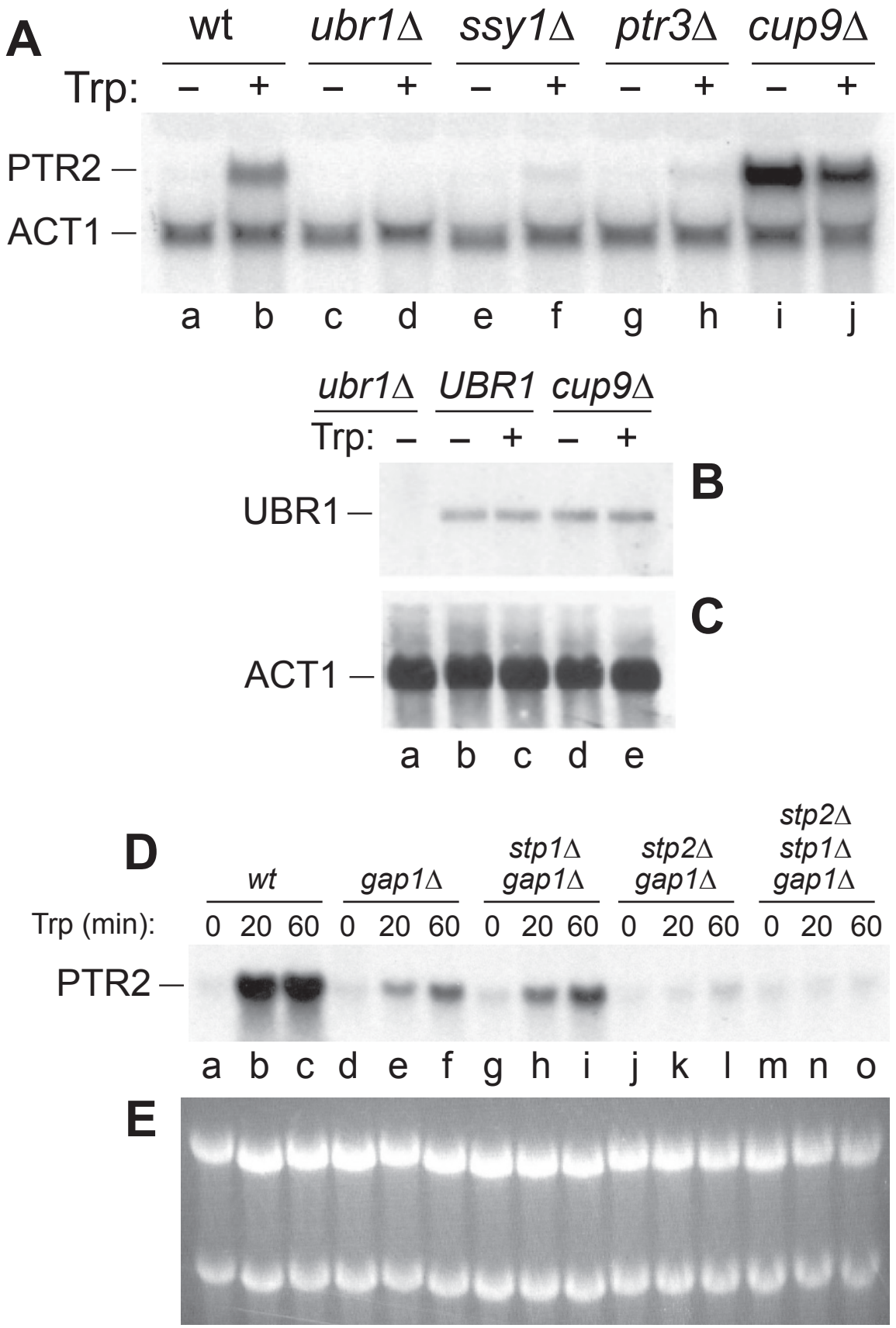
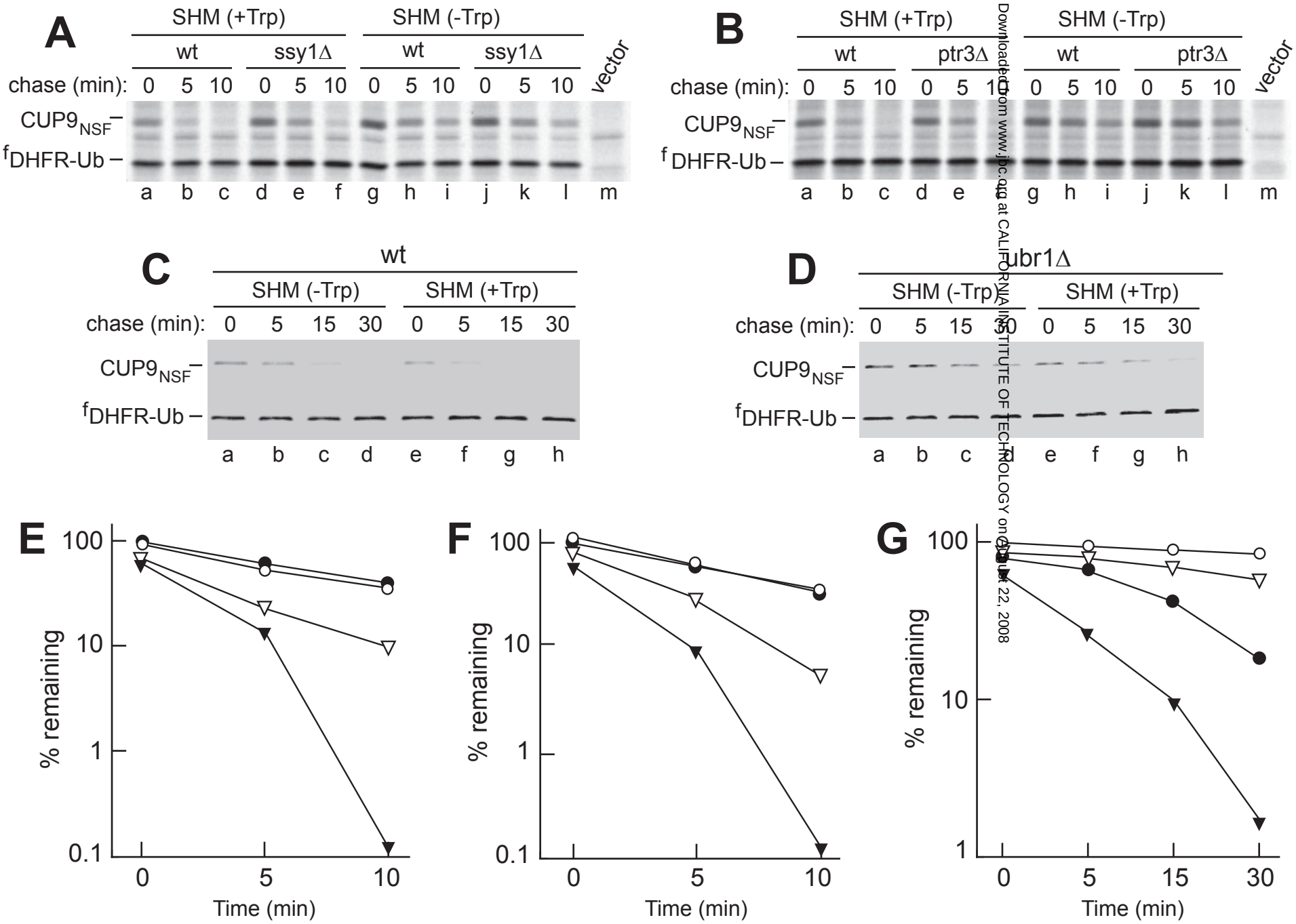


Fig. 3, Xia et al.



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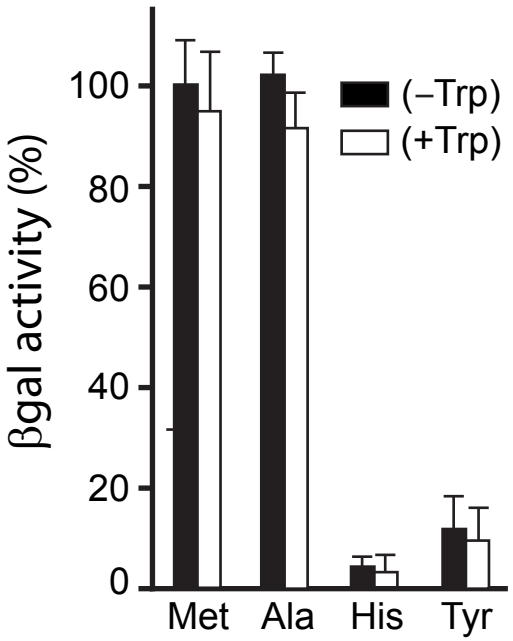

Fig. 5, Xia et al.

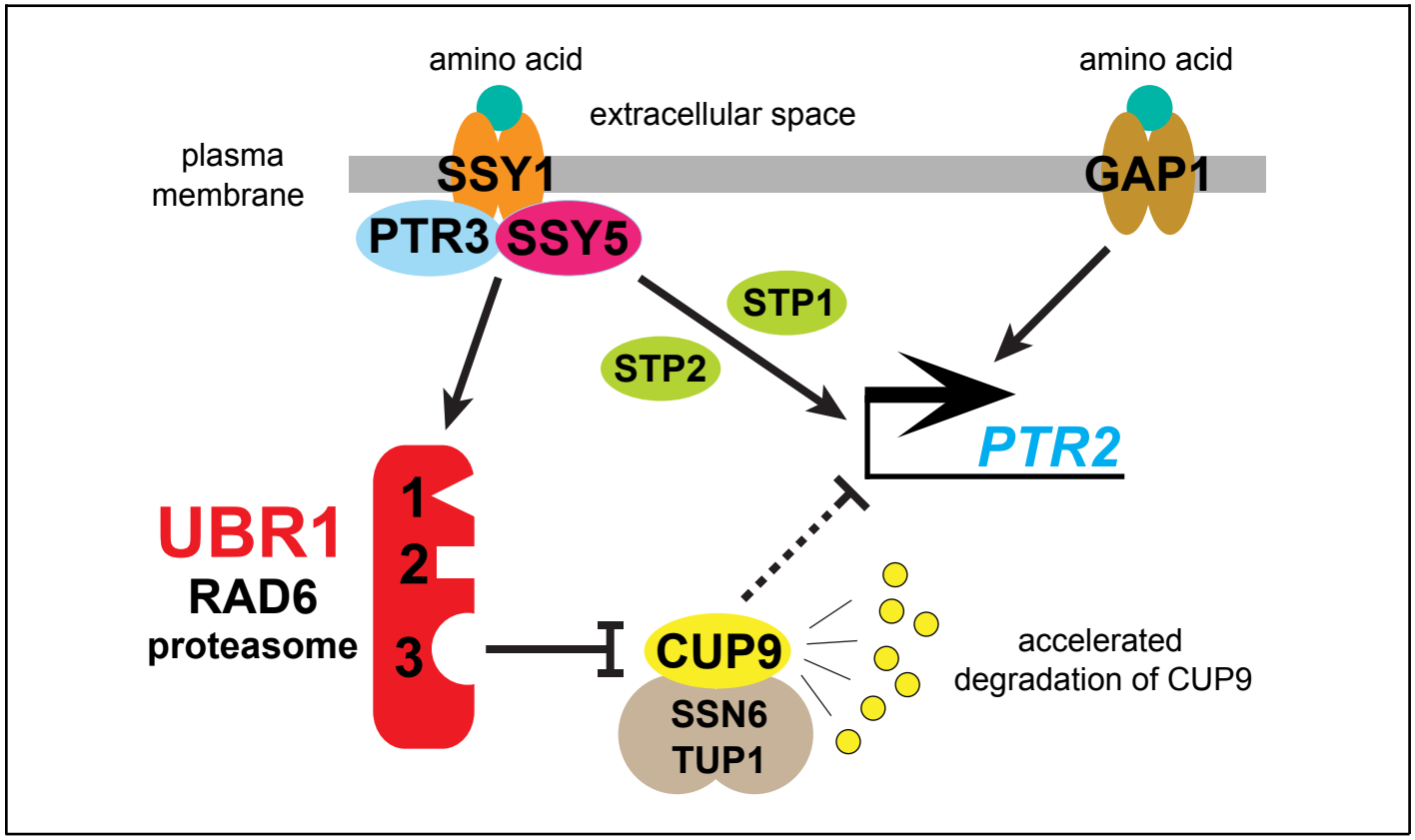

Fig. 6, Xia et al.