# Regulation of peptide import through phosphorylation of Ubr1, the ubiquitin ligase of the N-end rule pathway

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**Substrates of the N-end rule pathway include proteins with destabilizing N-terminal residues. These residues are recognized by E3 ubiquitin ligases called N-recognins. Ubr1 is the N-recognin of the yeast *Saccharomyces cerevisiae*. Extracellular amino acids or short peptides up-regulate the peptide transporter gene *PTR2*, thereby increasing the capacity of a cell to import peptides. Cup9 is a transcriptional repressor that down-regulates *PTR2*. The induction of *PTR2* by peptides or amino acids involves accelerated degradation of Cup9 by the N-end rule pathway. We report here that the Ubr1 N-recognin, which conditionally targets Cup9 for degradation, is phosphorylated in vivo at multiple sites, including Ser$^{300}$ and Tyr$^{277}$. We also show that the type-I casein kinases Yck1 and Yck2 phosphorylate Ubr1 on Ser$^{300}$, and thereby make possible (''prime'') the subsequent (presumably sequential) phosphorylations of Ubr1 on Ser$^{296}$, Ser$^{292}$, Thr$^{288}$, and Tyr$^{277}$ by Mck1, a kinase of the glycogen synthase kinase 3 (Gsk3) family. Phosphorylation of Ubr1 on Tyr$^{277}$ by Mck1 is a previously undescribed example of a cascade-based tyrosine phosphorylation by a Gsk3-type kinase outside of autophosphorylation. We show that the Yck1/Yck2-mediated phosphorylation of Ubr1 on Ser$^{300}$ plays a major role in the control of peptide import by the N-end rule pathway. In contrast to phosphorylation on Ser$^{300}$, the subsequent (primed) phosphorylations, including the one on Tyr$^{277}$, have at most minor effects on the known properties of Ubr1, including regulation of peptide import. Thus, a biological role of the rest of Ubr1 phosphorylation cascade remains to be identified.**

Mck1 | proteolysis | Yck1

The uptake of di- and tripeptides (di/tripeptides) in the yeast *Saccharomyces cerevisiae* is regulated by the N-end rule pathway, one proteolytic pathway of the Ub-proteasome system (Fig. 1*A*) (1–4). The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal residue (5–8). Degradation signals (degrons) that are targeted by the N-end rule pathway include a set called N-degrons (5, 9). The main determinant of an N-degron is a destabilizing N-terminal residue of a substrate protein (5, 9).

The N-end rule has a hierarchic structure that involves the primary, secondary, and tertiary destabilizing N-terminal residues (Fig. 1*A*). Destabilizing activities of these residues differ by their requirements for a preliminary enzymatic modification, a set of reactions that includes N-terminal deamidation and arginylation (8–12). In mammals and other multicellular eukaryotes, 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. The in vivo oxidation of N-terminal Cys requires nitric oxide (NO), oxygen (O2), and/or their derivatives (Fig. 1*A*) (8, 11).

E3 Ub ligases of the N-end rule pathway are called N-recognins (5, 8, 13, 14). They bind to primary destabilizing N-terminal residues. At least 4 N-recognins, including Ubr1, mediate the N-end rule pathway in mammals and other multicellular eukaryotes (8). N-recognins share a ≈70-residue motif called the UBR box. Mouse Ubr1 and Ubr2 are the sequelogous (15) 200-kDa RING-type E3 Ub ligases. 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 (Fig. 1*A*) (2, 13, 14). Ubr1 contains at least 3 substrate-binding sites. The type-1 and type-2 sites are specific for basic (Arg, Lys, His) and bulky hydrophobic (Trp, Phe, Tyr, Leu) N-terminal residues, respectively. The third binding site of Ubr1 recognizes, in particular, an internal (non-N-degron) degradation signal in Cup9, a transcriptional repressor of a regulon that includes *PTR2*, which encodes the transporter of di/tripeptides (16). This site of Ubr1 is autoinhibited but can be activated through the binding of di/tripeptides to the type-1/2 sites of Ubr1 (1, 2, 13). The resulting acceleration of the Ubr1-dependent degradation of Cup9 decreases its levels and thereby induces the Ptr2 transporter. This positive-feedback circuit allows *S. cerevisiae* to detect the presence of extracellular peptides and to react by increasing their uptake (1, 2).

The functions of the N-end rule pathway include the sensing of NO, oxygen, short peptides, and heme (Fig. 1*A*); the maintenance of high fidelity of chromosome segregation, through the degradation of a separase-produced fragment of cohesin subunit; regulation of peptide import, through the degradation of Cup9 (see above); regulation of signaling by transmembrane receptors, through the $NO/O_2$-dependent degradation of specific RGS proteins that down-regulate G proteins; regulation of apoptosis, meiosis, spermatogenesis, neurogenesis, and cardiovascular development in mammals, and regulation of leaf senescence in plants (refs. 1, 2, 4, 8, 10–12, 17 and refs. therein). Mutations in human Ubr1 are the cause of Johansson–Blizzard Syndrome (JBS), which includes mental retardation, physical malformations and severe pancreatitis (18).

In the present study, we show that the Ubr1 Ub ligase is phosphorylated in vivo at multiple sites, including Ser$^{300}$ and Tyr$^{277}$. We found that the type-I casein kinases Yck1 and Yck2 phosphorylate Ubr1 on Ser$^{300}$ and thereby make possible (“prime”) the subsequent phosphorylation of Ubr1 on Ser$^{296}$, Ser$^{292}$, Thr$^{288}$, and Tyr$^{277}$. The latter phosphorylations are mediated by Mck1, a member of the glycogen synthase kinase 3 (Gsk3) family. We also show that phosphorylation of Ubr1 on Ser$^{300}$ plays a major role in the control of peptide import by the N-end rule pathway.

## Results and Discussion

**Identification of in Vivo Ubr1 Phosphorylation Sites Using Mass Spectrometry.** To determine the sites of Ubr1 phosphorylation, we used tryptic digestion of Ubr1, capillary liquid chromatography, and peptide sequencing by mass spectrometry (cLC-MS/MS) [supporting information (SI) *Text* and Fig. S1]. The following Ubr1-derived phosphopeptides were identified: (*i*) **p*Y*$^{277}$**ENDYMFDGTTTAK and YENDYMFDGTT**p*T*$^{288}$**AK, (*ii*) **p*T*$^{291}$**SPSN**p*S*$^{296}$**PEASPLAKorT**p*S*$^{292}$**PSN**p*S*$^{296}$**PEASPLAK, (*iii*) **p*T*$^{291}$**SPSN**p*S*$^{296}$**PEA**p*S*$^{300}$**PLAK or T**p*S*$^{292}$**PSN**p*S*$^{296}$**-PEA**p*S*$^{300}$**PLAK, (*iv*) EHE**p*S*$^{1196}$**EFDEQDNDVDMVGEK, (*v*) NLDEDD**p*S*$^{1938}$**DDNDDDER, and (*vi*) NLDEDD**p*S*$^{1938}$**-

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The authors declare no conflict of interest.

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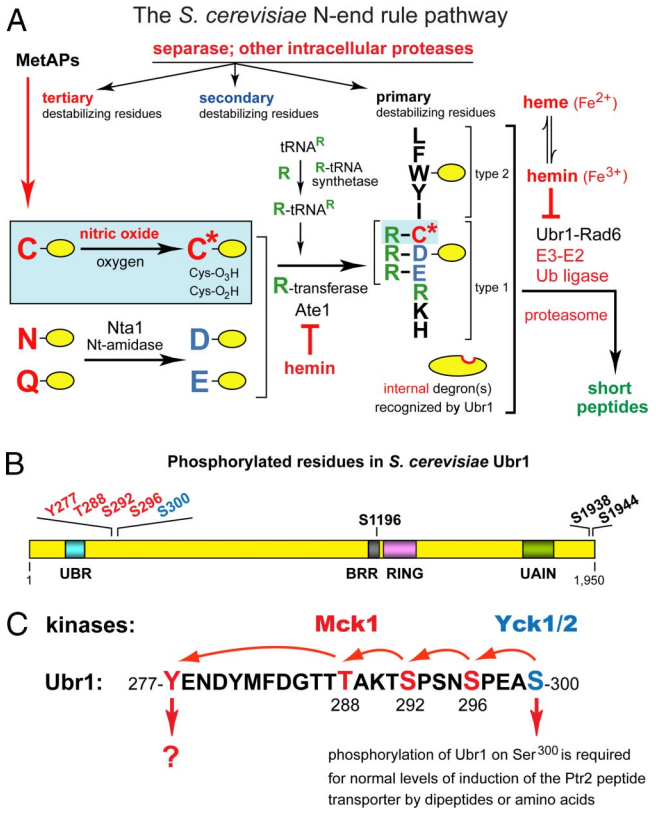


**Fig. 1.** The N-end rule pathway, in vivo phosphorylation sites in *S. cerevisiae* Ubr1, and the primed cascade of Ubr1 phosphorylation. (*A*) The *S. cerevisiae* N-end rule pathway. N-terminal residues are indicated by single-letter abbreviations for amino acids. Yellow ovals denote the rest of a protein substrate. Hemin ($Fe^{3+}$-heme) inhibits arginylation by R-transferase in both *S. cerevisiae* and mammals (12). MetAPs, methionine aminopeptidases. Reactions in the shaded rectangle are a part of the N-end rule pathway that is active in eukaryotes which produce NO (11). These reactions may also be relevant to *S. cerevisiae*, which lacks NO synthase but can produce NO by other routes, and can also be influenced by NO from extracellular sources. C* denotes oxidized Cys, either Cys-sulfinate or Cys-sulfonate (11). Type-1 and -2 destabilizing N-terminal residues of N-end rule substrates are recognized by Ubr1, the sole E3 Ub ligase of *S. cerevisiae*. Through its third substrate-binding site, Ubr1 targets internal (non-N-terminal) degrons in substrates (denoted by a larger oval) such as Cup9, a transcriptional repressor (1, 3, 4). (*B*) Phosphorylated residues of *S. cerevisiae* Ubr1 identified in the present work. Also indicated are the conserved Ubr1 regions such as the UBR box, the BRR (basic residues-rich) domain, the Cys/His-rich RING domain, and the UAIN (UBR autoinhibitory) domain (2). (*C*) The cascade of Ubr1 phosphorylation discovered in the present work. Initial phosphorylation of Ubr1 on $Ser^{300}$ by the CKI-type Yck1/Yck2 kinases makes possible ("primes") the subsequent (apparently sequential) phosphorylations of Ubr1 by Mck1 (a Gsk3-type kinase) on $Ser^{296}$, $Ser^{292}$, $Thr^{288}$, and $Tyr^{277}$. Also indicated is the identified function of $Ser^{300}$ phosphorylation.

DDNDD**p*S*$^{1944}$**DER. Although cLC-MS/MS alone could not distinguish between phosphorylation at $Thr^{291}$ vs. $Ser^{292}$, the latter residue was the more likely one, given the presence of a consensus phosphorylation site for a Gsk3-type kinase (S/T-X-X-X-S/T). We changed $Ser^{292}$ of Ubr1 to Ala and found this mutation (S292A) abolished phosphorylation on $Tyr^{277}$. Together, these analyses identified 8 in vivo phosphorylation sites (Fig. 1*B*). Five of these sites were clustered in a 25-residue region of the 1,950-residue Ubr1, between the previously delineated locations (13) of its type-1 and -2 substrate-binding sites (Fig. 1*B* and Fig. S1).

**In Vivo Phosphorylation of Ubr1 on $Tyr^{277}$.** As a part of this study, we produced an affinity-purified rabbit antibody (termed $Ab1^{ScUbr1(1–1140)}$) to N-terminal half of *S. cerevisiae* Ubr1. To detect the in vivo phosphorylation of $Tyr^{277}$ by a method other than MS, we also produced an affinity-purified rabbit antibody (termed $Ab1^{pY277Ubr1}$) against a peptide that contained phospho-$Tyr^{277}$ and adjacent Ubr1 sequences. $Ab1^{pY277Ubr1}$ recognized both the phosphopeptide and $Tyr^{277}$-phosphorylated Ubr1 ($Ubr1^{pY277}$) and did not recognize the unphosphorylated peptide or $Ubr1^{Y277F}$, which contained a nonphosphorylatable (Phe) residue at position 277 (Fig. 2 *A* and *B*). The epitope(s) recognized by $Ab1^{pY277Ubr1}$ could be removed by treating Ubr1 with lambda protein phosphatase; the epitope was retained in the presence of phosphatase inhibitors (Fig. 2*C*).

The in vivo levels of $Ubr1^{pY277}$ (relative to total Ubr1) were increased upon the addition of glucose, an amino acid such as Trp, or a type-1 dipeptide such as Arg-Ala (Fig. 2 *F–H*). We also expressed Ubr1 in *S. cerevisiae* CHY50, a [*ubr1*Δ *pdr5*Δ] strain that was sensitive to a proteasome inhibitor such as MG132. The levels of $Ubr1^{pY277}$ were not increased by treatment with MG132 (Fig. 2*D*). This test was performed in part because some Ubr1 mutants at position 277, such as $Ubr1^{Y277A}$, and $Ubr1^{Y277E}$, were found to be short-lived in vivo (Fig. S2 *D* and *E*). These results indicated that that the identity of a residue at position 277 is critical for a metabolically stable conformation of Ubr1. The above Ubr1 mutants are described solely in *SI* (Fig. S2 *B–E*), because the levels of $Ubr1^{pY277}$ were not altered by inhibition of the proteasome (Fig. 2*D*), suggesting (but not proving) that the metabolic stability of $Ubr1^{pY277}$ is comparable to that of Ubr1 unphosphorylated on $Tyr^{277}$.

Truncated derivatives of Ubr1 (2, 13) and the $Ab1^{pY277Ubr1}$ antibody were used to determine "large-scale" requirements for the in vivo phosphorylation of Ubr1 on $Tyr^{277}$. Remarkably, no phosphorylation on $Tyr^{277}$ was observed with the $^{f}Ubr1^{1–717}$ fragment, in contrast to "wild-type" levels of $Tyr^{277}$ phosphorylation with $Ubr1^{1–1140f}$, $^{f}Ubr1^{1–1367}$, $^{f}Ubr1^{1–1700}$, and $^{f}Ubr1^{1–1818}$ fragments (Fig. S3*A*). Thus, a region between positions 717 and 1,140 (Fig. 1*B*) was essential for the phosphorylation of Ubr1 on the far-upstream $Tyr^{277}$ residue (Fig. S3*A*), suggesting that this phosphorylation may require an appropriate conformation and/or a preceding phosphorylation at other residues of Ubr1.

**$Tyr^{277}$ of Ubr1 Is Phosphorylated by Mck1, a Member of the Gsk3 Kinase Family.** We used $Ab1^{pY277Ubr1}$ (Fig. 2 *A–C*) and a library of yeast mutants in 27 putative Tyr kinases (see *SI Text*) to screen for the ability of these mutants to phosphorylate Ubr1 on $Tyr^{277}$ in vivo. A mutant lacking the Mck1 kinase was found to be nearly incapable of phosphorylating Ubr1 on $Tyr^{277}$ (Fig. S4*A*, lane 5). This defect could be rescued by wild-type Mck1 but could not be rescued by $Mck1^{D164A}$ (19), a catalytically inactive mutant (Fig. S4*B*). Expression of $Mck1^{Y199F}$, a possibly leaky mutant in the activation loop of this kinase (ref. 19 and refs. therein), weakly but detectably rescued the $Tyr^{277}$-specific phosphorylation of Ubr1 (Fig. S4*B*).

Mck1 is a member of the Gsk3 family of kinases, present in all eukaryotes (19, 20). In addition to Mck1, the *S. cerevisiae* genome encodes 3 other kinases of this family, Rim11, Mrk1, and Ygk3. In contrast to the near absence of $Tyr^{277}$-specific phosphorylation of Ubr1 in *mck1*Δ cells, this phosphorylation remained unperturbed in *rim11*Δ, *mrk1*Δ, or *ygk3*Δ cells (Fig. S4*A*). Moreover, double and triple mutants, the latter lacking all 3 Gsk3-type kinases other than Mck1, did not exhibit a significant decrease in the $Tyr^{277}$-specific Ubr1 phosphorylation (data not shown), indicating a unique role of Mck1 in phosphorylating Ubr1 on $Tyr^{277}$. We also overexpressed $His_6$-tagged derivatives of either wild-type Mck1 or its catalytically inactive $Mck1^{D164A}$ mutant in *S. cerevisiae* and purified them to near homogeneity (Fig. S3*B*). Wild-type Ubr1 and $Ubr1^{Y277F}$ were expressed in the *mck1*Δ strain (Fig. S4*A*, lane 5) and also purified. Ubr1 or $Ubr1^{Y277F}$ were incubated with Mck1 or $Mck1^{D164A}$ and γ[$^{32}$]ATP, followed by SDS/PAGE and autoradiography. Wild-type Mck1 phosphorylated wild-type Ubr1, whereas the amounts of $^{32}P$

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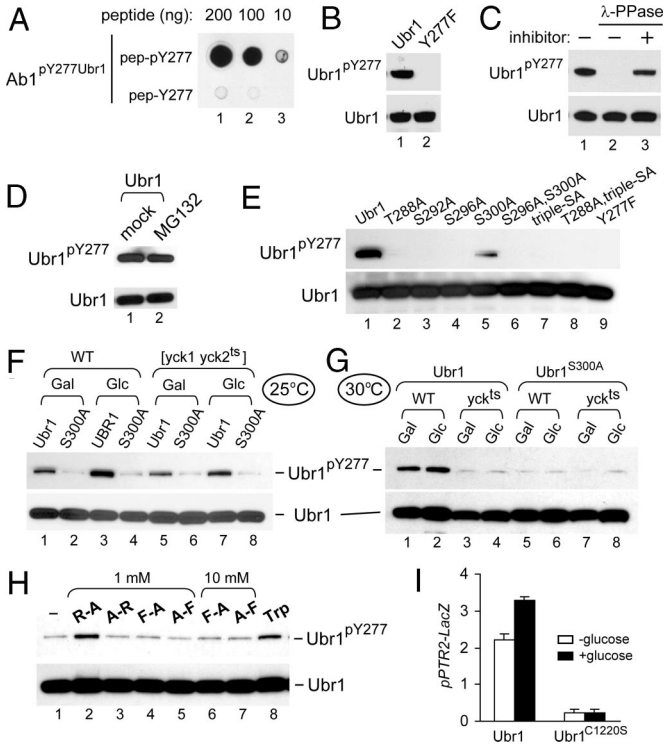


**Fig. 2.** Phosphorylation of Ubr1 on Tyr$^{277}$. (*A*) Characterization of antibody, termed Ab1$^{pY277Ubr1}$, that was raised against a Ubr1 phosphopeptide QFLNDLKp*Y*$^{277}$ENDY. Dot immunoblotting with affinity-purified Ab1$^{pY277Ubr1}$, using indicated amounts of the spotted QFLNDLKp*Y*ENDY phosphopeptide and its unphosphorylated counterpart. (*B*) Specificity of Ab1$^{pY277Ubr1}$. Purified wild-type Ubr1 (which contains Ubr1$^{pY277}$) and Ubr1$^{Y277F}$ were subjected to SDS-NuPAGE (4–12%) and immunoblotted with affinity-purified Ab1$^{pY277Ubr1}$ (1:1,000), and thereafter with Ab1$^{ScUbr1(1–1140)}$. (*C*) Purified wild-type Ubr1 (0.25 $\mu$g) was incubated with 400 units of $\lambda$ phosphatase ($\lambda$-PPase) in the presence or absence of phosphatase inhibitors for 2 h at 30 °C, followed by SDS/PAGE and sequential immunoblotting, as in *B*. (*D*) *S. cerevisiae* CHY50 [*ubr1*Δ *pdr5*Δ] expressing the N-terminally flag-tagged Ubr1 ($^{f}$Ubr1), was grown in SC(-Leu) and treated for 3 h either with MG132 (at 50 $\mu$M) or with an equivalent volume of its stock-solution solvent dimethyl sulfoxide. Cell extracts were subjected to SDS/PAGE and sequential immunoblotting, as in *B*. (*E*) Ubr1 phosphorylation on Tyr$^{277}$ depends on phosphorylation at other sites. JD55 (*ubr1*Δ) cells were transformed with pFlagUBR1SBX-based plasmids that expressed wild-type Ubr1 or its indicated derivatives. Cells were grown to $A_{600}$ of ≈1 in SC(-Leu). Cell extracts were subjected to SDS-NuPAGE (4–12%) and sequential immunoblotting, as in *B*. (*F* and *G*) Wild-type LRB906 [*YCK1 YCK2*] or LRB756 [*yck1*Δ *yck2*$^{ts}$] strains (Table S1) that expressed wild-type $^{f}$Ubr1 or $^{f}$Ubr1$^{S300A}$ (in the *ubr1*Δ background) were grown in a galactose-containing medium, with or without a subsequent addition of glucose, either at 25 °C (permissive temperature for Yck2$^{ts}$) or at 30 °C (semi-permissive temperature for Yck2$^{ts}$). Cell extracts were subjected to SDS/PAGE and sequential immunoblotting with Ab1$^{pY277Ubr1}$ and anti-flag antibodies. (*H*) AVY26 (*ubr1*Δ) *S. cerevisiae* expressing wild-type Ubr1 from pSOB33 (Tables S1 and S2) was grown to $A_{600}$ of ≈0.8 in SHM medium in the presence of indicated dipeptides, at 1 mM or 10 mM, or in the presence of Trp (98 $\mu$M). Cell extracts were subjected to SDS/PAGE and immunoblotting, as in *B*. (*I*) CHY201 (*ubr1*Δ) *S. cerevisiae* (Table S1) carried the plasmid pSS4 ($P_{PTR2}$-*lacZ*) and either pCH100 (wild-type Ubr1) or pCH159 (Ubr1$^{MR1}$), the latter expressing Ubr1$^{C1220S}$, which is inactive as an E3 (14). Cultures were grown at 25 °C in galactose-containing SGal(-Ura,-Leu) medium to $A_{600}$ of ≈0.8, followed by the addition of either galactose (to 4% total) (white bars) or glucose (to 4%) (black bars), further incubation for 2.5 h, and measurements of $\beta$gal activity in cell extracts.

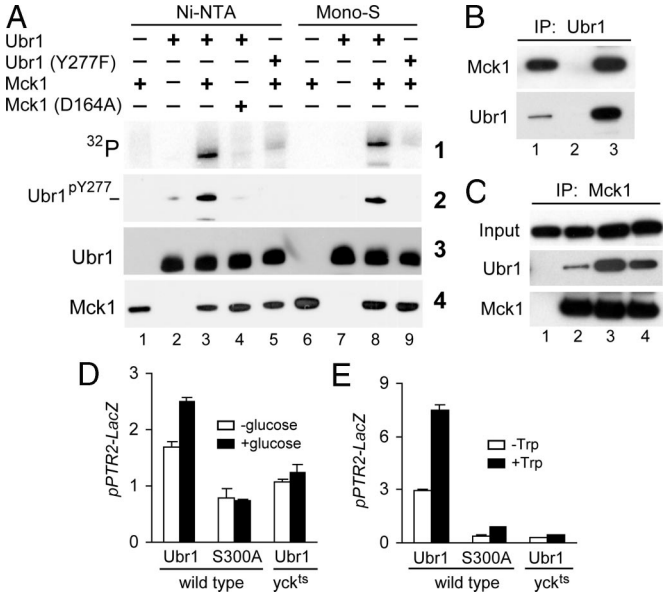


**Fig. 3.** Phosphorylation of Ubr1 by Yck1/Yck2 and Mck1 kinases. (*A*) Purified Mck1 phosphorylates Ubr1 on Tyr$^{277}$. Wild-type $^{f}$Ubr1 and its $^{f}$Ubr1$^{Y277F}$ derivative were purified from *mck1*Δ *S. cerevisiae*; 0.1 $\mu$g of $^{f}$Ubr1 or $^{f}$Ubr1$^{Y277F}$ were incubated with 0.1 $\mu$g of either partially purified ("Ni-NTA") or additionally purified ("Mono-S") ha-tagged Mck1, or with its indicated derivatives in the presence of $\gamma$-[$^{32}$]ATP at 30 °C for 30 min, followed by SDS/PAGE and sequential immunoblotting with Ab1$^{pY277Ubr1}$, anti-flag, and anti-ha antibodies. (*B*) Extracts from JD55 (*ubr1*Δ) cells expressing $^{f}$Ubr1 and Mck1-ha were incubated with beads only (lane 2) or anti-flag beads (lane 3). Bound proteins were eluted and analyzed by SDS/PAGE and immunoblotting with anti-HA and anti-flag antibodies. Lane 1, 0.1% of total extract. (*C*) Extracts from JD55 (*ubr1*Δ) cells expressing $^{f}$Ubr1 or $^{f}$Ubr1$^{Y277F}$ and coexpressing Mck1-ha were processed for immunoprecipitation, SDS/PAGE, and immunoblotting, as in *B*. "Input" samples before immunoprecipitation, with immunoblotting using anti-flag. Lane 1, cells expressed $^{f}$Ubr1 alone (control). Lane 2, same as lane 1 but cells expressed both $^{f}$Ubr1 and Mck1-ha. Lane 3, CHY89 (*mck1*Δ *ubr1*Δ) cells that lacked endogenous Mck1 and expressed both $^{f}$Ubr1 and Mck1$^{D164A}$-ha. Lane 4, same as lane 2 but with cells that expressed $^{f}$Ubr1$^{Y277F}$ and Mck1-ha. (*D*) CHY201 (*ubr1*Δ) and CHY202 (*ubr1*Δ *yck1*Δ *yck2*$^{ts}$) *S. cerevisiae* carried pSS4 ($P_{PTR2}$-*lacZ*) and either pCH100 (wild-type Ubr1) or pCH114 (Ubr1$^{S300A}$) (Tables S1 and S2). Cultures were grown in SGal(-Ura,-Leu) at 25 °C to $A_{600}$ of ≈0.8, and thereafter were shifted to 37 °C for 20 min, followed by the addition of either galactose (to 4% total) (white bars) or glucose (to 4%) (filled bars) and further incubation at 37 °C for 2.5 h, followed by measurements of $\beta$gal activity in cell extracts. (*E*) CHY203 (*ubr1*Δ) and CHY204 (*ubr1*Δ *yck1*Δ *yck2*$^{ts}$) *S. cerevisiae* carried pSS4 ($P_{PTR2}$-*lacZ*) and either pCH100 (wild-type Ubr1) or pCH114 (Ubr1$^{S300A}$) (Tables S1 and S2). Cultures were grown to $A_{600}$ of ≈0.8 in SHM medium (see *SI Text*) at 25 °C and thereafter were shifted to 37 °C for 2.5 h in the same medium either without or with the addition of Trp to 98 $\mu$M, followed by processing as in *D*.

were much lower (but not zero; see below) with Ubr1$^{Y277F}$, which could not be phosphorylated at position 277 (Fig. 3*A*). Immunoblotting of the same reaction products with Ab1$^{pY277Ubr1}$ directly confirmed that Mck1 phosphorylated Ubr1 on Tyr$^{277}$ (Fig. 3*A*). We conclude that Mck1 is by far the major (possibly the sole) kinase that phosphorylates Ubr1 on Tyr$^{277}$.

Reciprocal coimmunoprecipitation tests confirmed that Ubr1 specifically interacted with Mck1 (Fig. 3 *B* and *C*). As would be expected, the apparent affinity of Mck1 for Ubr1 was higher with either unphosphorylated Ubr1 (isolated from *mck1*Δ cells expressing catalytically inactive Mck1$^{D164A}$-ha) or with Ubr1$^{Y277F}$ (which could not be phosphorylated at position 277) than with wild-type Ubr1 from cells containing wild-type Mck1 (Fig. 3*C*). The binding of the Ubr1 Ub ligase to Mck1 did not make the latter short-lived in vivo (data not shown).

**A "Primed" Ubr1 Phosphorylation Cascade That Begins at Ser$^{300}$ Involves 3 Other Serines/Threonines and Concludes with Tyr$^{277}$.** We identified Thr$^{288}$, Ser$^{292}$, Ser$^{296}$, and Ser$^{300}$ of Ubr1 as the sites of its in vivo phosphorylation downstream of Tyr$^{277}$, a residue phosphorylated by Mck1 (Figs. 1*B* and 3*A* and Fig. S1). A NetPhosK program (www.cbs.dtu.dk/services/NetPhosK) pinpointed Thr$^{288}$, Ser$^{292}$, Ser$^{296}$, and Ser$^{300}$ of Ubr1 as putative sites of phosphorylation by

Gsk3-type kinases. To address these issues, we constructed Ubr1 mutants that retained $Tyr^{277}$ but contained single or multiple mutations to Ala at the above Ser/Thr sites. We then used $Ab1^{pY277Ubr1}$, which recognized $Ubr1^{pY277}$, to determine the extent of in vivo phosphorylation of mutant Ubr1 proteins on $Tyr^{277}$. Strikingly, no phosphorylation on $Tyr^{277}$ was observed with $Ubr1^{T288A}$, $Ubr1^{S292A}$, and $Ubr1^{S296A}$, and also with multisite mutants $Ubr1^{S296A,S300A}$, $Ubr1^{S292A,S296A,S300A}$, and $Ubr1^{T288A,S292A,S296A,S300A}$, whereas $Ubr1^{S300A}$ exhibited a detectable but greatly reduced phosphorylation on $Tyr^{277}$ (Fig. 2*E*). Thus, the Mck1-mediated phosphorylation on $Tyr^{277}$ is the last step of a phosphorylation cascade that involves the obligatory preceding ("priming") phosphorylations of Ser/Thr residues that are close to position 277 of Ubr1 (Fig. 1 *B* and *C*). These data, together with a low but significant *in vitro* Mck1-mediated phosphorylation of $Ubr1^{Y277F}$, which could not be phosphorylated at position 277 (Fig. 3*A*), strongly suggested (but did not formally prove) that the observed phosphorylation of Ubr1 on $Thr^{288}$, $Ser^{292}$, and $Ser^{296}$ is also carried out by Mck1 (Fig. 1*C*).

**Yck1 and Yck2 Are "Priming" Kinases That Phosphorylate Ubr1 on $Ser^{300}$ and Initiate the Mck1-Mediated Phosphorylations on $Thr^{288}$, $Ser^{292}$, $Ser^{296}$, and $Tyr^{277}$.** In a cascade that is referred to as "primed" phosphorylation, specific residues of a protein must be phosphorylated sequentially rather than independently of one another, in that a "downstream" phosphorylation event requires one or more of preceding phosphorylations (21). Kinases that phosphorylate at a position required for subsequent phosphorylations are referred to as "priming" kinases (refs. 20–22 and refs. therein). In the known Gsk3 cascades, a frequently encountered preference is for a prior phosphorylation, often by a distinct kinase, of a residue at +4 position relative to a residue to be phosphorylated next. This previously established rule is consistent with the distances between the $Ser^{300}$, $Ser^{296}$ $Ser^{292}$, and $Thr^{288}$ residues of Ubr1 (Fig. 1*C*). A prior phosphorylation by a priming kinase is not always strictly essential, in that in most cases a priming phosphorylation greatly increases the activity of a Gsk3-type kinase at a "dependent" phosphorylation site, typically by 100- to 1,000-fold (21).

According to the NetPhosK program, $Ser^{300}$ of *S. cerevisiae* Ubr1 was a plausible phosphorylation site for either Gsk3-type kinases or Pho85, of the CDK kinase family. We found that null mutations in either *S. cerevisiae* Pho85 or the non-Mck1 kinases of the Gsk3 family (Rim11, Mrk1, and Ygk3) did not affect the Mck1-mediated phosphorylation of Ubr1 on $Tyr^{277}$ in vivo (Fig. S4*A*). Previous work has shown that Yck1/Yck2, 2 sequelogous *S. cerevisiae* kinases of the casein kinase-I (CKI) family (23) are essential components of systems such as glucose-mediated signaling pathways (24), bud-localized mRNA translation (25), and the signaling mediated by extracellular amino acids, particularly the SPS pathway (26–28). In mammals, CKI-type kinases regulate a multitude of processes, including the cell cycle, circadian rhythms, membrane trafficking, chromosome segregation, and apoptosis (23).

*S. cerevisiae* contains 4 sequelogous kinases of the CKI family: Yck1, Yck2, Yck3, and Hrr25. Yck1 and Yck2 are peripheral membrane proteins whose localization at the plasma membrane requires palmitylation of their C-terminal Cys-Cys motif (refs. 27 and 28 and refs. therein). Yck1 and Yck2 complement each other in a number of functions (27). We carried out coimmunoprecipitation with the flag-tagged Ubr1 ($^{f}$Ubr1) and myc-tagged Yck1 (Yck1-myc) that were coexpressed in *ubr1*Δ cells. $^{f}$Ubr1 was coimmunoprecipitated with Yck1-myc (Fig. S4 *C* and *D*). Moreover, $^{f}$Ubr1 could be efficiently phosphorylated, in the presence of $\gamma[^{32}]$ATP, in immunoprecipitates with wild-type (C-terminally tagged) Yck1-myc but not with inactive $Yck1^{K98R}$-myc or with beads alone (Fig. S4*E*). Yck1 assays were also carried with GST-$Ubr1^{227–327}$ and GST-$Ubr1^{227–327,S300A}$. GST-$Ubr1^{227–327}$ was phosphorylated in immunoprecipitates with wild-type Yck1-myc but not with inactive $Yck1^{K98R}$-myc (Fig. S4*F*). Crucially, the otherwise identical GST-$Ubr1^{227–327,S300A}$, which contained Ala at position 300 of Ubr1, was at most weakly phosphorylated by Yck1-myc (Fig. S4*F*). Together, these results indicated that the CKI-type Yck1 and Yck2 kinases (either one of them was sufficient; data not shown) functioned as priming kinases of Ubr1, phosphorylating its $Ser^{300}$ and thereby making possible ("priming") the cascade of Mck1-mediated phosphorylations that converged on $Tyr^{277}$ (Fig. 1*C*).

**Yck1/Yck2-Primed, Mck1-Mediated Phosphorylation of Ubr1 on $Tyr^{277}$ Is Increased by Glucose, Amino Acids, or Type-1 Dipeptides.** The addition of glucose, an amino acid such as Trp, or a type-1 dipeptide such as Arg-Ala to a growth medium significantly increased the in vivo levels of $Ubr1^{pY277}$ (Fig. 2 *F–H*). To determine whether glucose-mediated increases of $Ubr1^{pY277}$ required the priming Yck1/Yck2 kinases that initially phosphorylate Ubr1 on $Ser^{300}$, we used a wild-type [*YCK1 YCK2*] strain and a temperature-sensitive (ts) [*yck1*Δ *yck2$^{ts}$*] strain that lacked Yck1 and contained a ts-Yck2 kinase ($Yck2^{ts}$). These strains (Table S1) were constructed to express either wild-type Ubr1 or $Ubr1^{S300A}$, which could not be phosphorylated at position 300. Exponential cultures in a 2% galactose-containing medium at 25 °C (permissive temperature for $Yck2^{ts}$) or at 30 °C (semipermissive temperature) were left growing with galactose or were modified by the addition of glucose to 4% and further incubation for 2.5 h at either 25 °C or 30 °C. With [*YCK1 YCK2*] cells, phosphorylation of Ubr1 on $Tyr^{277}$ was increased upon the addition of glucose at 25 °C. By contrast, at 30 °C the level of $Ubr1^{pY277}$ was greatly reduced in [*yck1*Δ *yck2$^{ts}$*] cells (Fig. 2 *F* and *G*). In addition, glucose did not increase phosphorylation of Ubr1 on $Tyr^{277}$ in [*yck1*Δ *yck2$^{ts}$*] cells when these cells were cultured further at the nonpermissive temperature of 37 °C for 2.5 h (data not shown). The increased phosphorylation on $Tyr^{277}$ required $Ser^{300}$, because no change in the levels of $Ubr1^{pY277}$ was observed in otherwise identical experiments with $Ubr1^{S300A}$ (Fig. 2 *F* and *G*). In agreement with this result, the level of $Ubr1^{pY277}$ increased upon the addition of glucose at 25 °C but not at 30 °C in [*yck1*Δ *yck2$^{ts}$*] cells (Fig. 2 *F* and *G*).

**Normal Induction of the Ptr2 Peptide Transporter by Amino Acids, Dipeptides, or Glucose Requires Phosphorylation of Ubr1 on $Ser^{300}$ by Yck1/Yck2.** By measuring the expression of $P_{PTR2}$-*lacZ* transcriptional reporter, we found that the addition of glucose to cells growing on galactose led to a modest (≈1.6-fold) but reproducible induction of the transporter-encoding *PTR2* gene in wild-type (*UBR1*) cells but not in cells that expressed the catalytically inactive $Ubr1^{C1220S}$ mutant (Fig. 2*I*). Glucose-mediated increase of *PTR2* expression required the Yck1/Yck2 kinases and phosphorylation of Ubr1 on $Ser^{300}$, because no induction of *PTR2* by glucose occurred at 37 °C either in [*yck1*Δ *yck2$^{ts}$*] cells that expressed wild-type Ubr1 or in [*YCK1 YCK2*] cells that expressed $Ubr1^{S300A}$ (Fig. 3*D*).

Addition of an amino acid such as Trp significantly increased the levels of $Ubr1^{pY277}$ (Fig. 2*H*, lane 8; compare with lane 1 or 3). In vitro kinase assays (data not shown) indicated this effect of Trp was not mediated by an increase in the activity of the Mck1 kinase. The addition of Trp also induced the expression of *PTR2* by >2-fold (Fig. 3*E*). The induction of *PTR2* by Trp was nearly abolished in [*YCK1 YCK2*] cells that expressed $Ubr1^{S300A}$ instead of wild-type Ubr1 or in [*yck1*Δ *yck2$^{ts}$*] cells at 37 °C in the presence of wild-type Ubr1 (Fig. 3*E*). Together, these findings strongly suggested that Trp causes the formation of $Ubr1^{pY277}$ through the demonstrated (ref. 27 and refs. therein) activation of the Yck1/Yck2 kinases by the SPS pathway or through inhibition of a phosphatase(s) that dephosphorylates the phospho-$Ser^{300}$ residue of Ubr1.

$Ubr1^{S300A}$ was indistinguishable from wild-type Ubr1 in mediating the in vivo degradation of a type-1 N-end rule substrate such as Arg-βgal (Fig. 4*A*). However, $Ubr1^{S300A}$ was considerably impaired in mediating the degradation of either a type-2 N-end rule substrate such as Tyr-βgal (Fig. 4*A*) or the Cup9 repressor (Fig. S4*G* and data not shown). Cup9 is targeted by Ubr1 through an

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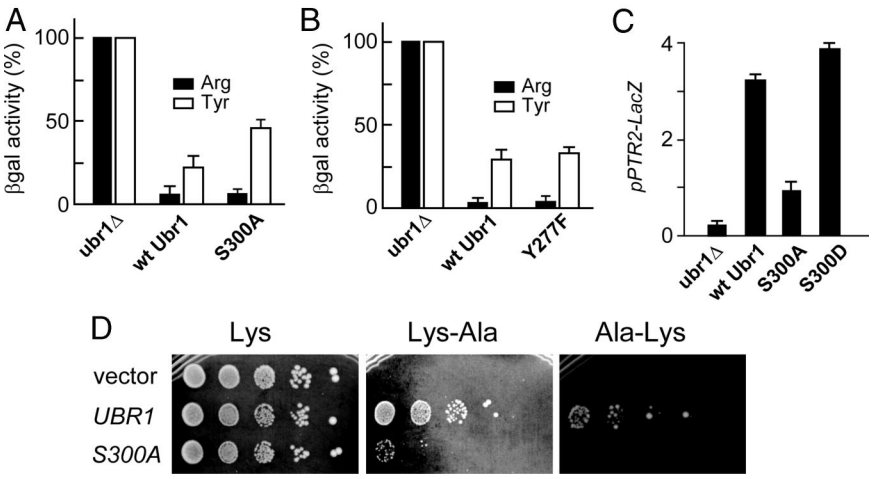


**Fig. 4.** Functional consequences of Ubr1 alterations at position 300. (*A*) Relative levels of Arg-βgal (filled bars) and Tyr-βgal (white bars), a type-1 and a type-2 N-end rule substrate, respectively (Fig. 1*A*), in *ubr1*Δ *S. cerevisiae* or in the same strain that expressed either wild-type Ubr1 or Ubr1$^{S300A}$. (*B*) Same as in *A* but an independent experiment, and Ubr1$^{Y277F}$ instead of Ubr1$^{S300A}$. (*C*) Effects of Ubr1 mutations at position 300 on *PTR2* expression. *S. cerevisiae* JD55 (*ubr1*Δ) carried pSS4 ($P_{PTR2}$-*lacZ*) and either pCH100 (wild-type Ubr1), pCH114 (Ubr1$^{S300A}$) pCH246 (Ubr1$^{S300D}$). Cultures were grown to $A_{600}$ of ≈0.8 at 30 °C in SC containing a mixture of compounds (including amino acids) required by a strain, followed by measurements of βgal activity in cell extracts. (*D*) JD55 (*ubr1*Δ) *S. cerevisiae* carrying either the empty vector (pRS315), or pCH100 (wild-type Ubr1), or pCH114 (Ubr1$^{S300A}$) (Table S2) were serially diluted 5-fold and spotted on SC(-Leu, -Lys) plates containing 110 μM Lys, or 66 μM Lys-Ala, or 66 μM Ala-Lys. The plates were incubated at 30 °C for 3 days.

internal degron (see Introduction). A strongly impaired ability of Ubr1$^{S300A}$ to induce *PTR2* could also be observed in experiments where a mixture of amino acids was present (Fig. 4*C*). The (previously described) Trp-induced acceleration of Cup9 degradation (4) was found to be nearly absent in cells that expressed Ubr1$^{S300A}$ instead of wild-type Ubr1 (Fig. S4*G* and data not shown). This result accounted, at least in part, for the decreased ability of Ubr1$^{S300A}$ to mediate the induction of *PTR2* by amino acids (Figs. 3*E* and 4*C*).

Type-1 dipeptides such as Lys-Ala (which bind to Ubr1 and thereby accelerate Cup9 degradation) can up-regulate peptide import and thus enable survival of cells on media with peptides as sole sources of a required amino acid (1). In agreement with other data (see above) about functional significance of Ubr1 phosphorylation on Ser$^{300}$, Ubr1$^{S300A}$ was strongly impaired in the growth-based peptide import assay, in contrast to wild-type Ubr1 (Fig. 4*D*). A different test involved Ubr1$^{S300D}$, in which Ser$^{300}$ was converted to Asp, a "mimic" of phospho-Ser. This experiment provided yet another, independent evidence for the functional importance of phosphorylation on Ser$^{300}$. Specifically, the Ubr1$^{S300D}$ mutant was more active than wild-type Ubr1 (and much more active than Ubr1$^{S300A}$) in mediating the expression of *PTR2* (Fig. 4*C*). Whereas a type-1 dipeptide such as Arg-Ala (at 1 mM) significantly increased the levels of Ubr1$^{pY277}$, a type-2 dipeptide such as Phe-Ala did not have this effect even at 10 mM (Fig. 2*H*), for reasons that remain to be understood.

## Concluding Remarks

Several E3 Ub ligases of the Ub-proteasome system have been shown to be regulated through phosphorylation (refs. 29–31 and refs. therein). We discovered a "primed" phosphorylation cascade that involves Ser$^{300}$, Ser$^{296}$, Ser$^{292}$, Thr$^{288}$, and Tyr$^{277}$ of Ubr1, the E3 Ub ligase (N-recognin) of the N-end rule pathway in *S. cerevisiae* (Figs. 1 *B* and *C* and 5). This cascade is mediated by Yck1 and Yck2, 2 sequelogous type-I casein kinases that act as "priming" kinases for the Mck1 kinase of the Gsk3 family. Phosphorylation of Ubr1 on Ser$^{300}$ by the functionally overlapping Yck1/Yck2 kinases makes the resulting Ubr1$^{pS300}$ a substrate of Mck1, which phosphorylates 3 Ser/Thr residues of Ubr1 and completes the cascade by phosphorylating Tyr$^{277}$ (Fig. 1 *B* and *C*). The Tyr$^{277}$ residue of Ubr1 is an example of a tyrosine phosphorylation by Mck1, outside of its autophosphorylation activity. We also showed that the initial (priming) phosphorylation of Ubr1 on Ser$^{300}$ (Fig. 1*C*) plays a major role in the control of import of di/tripeptides by the N-end rule pathway (Figs. 1*C* and 5).

Phosphorylation of Ubr1 on Tyr$^{277}$ is up-regulated by an extracellular amino acid such as Trp (Fig. 2*H*). Trp induces the Ptr2 transporter and thereby up-regulates the import of di/tripeptides (32). The mechanism of Ptr2 induction by Trp was recently found to involve acceleration of the Ubr1-dependent degradation of the Cup9 repressor (4). This acceleration requires the amino acid-sensing SPS (*S*sy1-*P*tr3-*S*sy5) pathway (26, 28, 33). Ssy1, the plasma membrane-embedded amino acid sensor, transduces its interaction with an extracellular amino acid such as Trp into a changed phosphorylation state of Ptr3, an Ssy1-associated protein that functions downstream of Ssy1. The Trp-induced phosphorylation of Ptr3 is mediated by the Ssy1-dependent activation of the Yck1/Yck2 kinases (27, 28). Remarkably, the same kinases were found to phosphorylate Ubr1 on Ser$^{300}$, thereby priming the rest of Ubr1 phosphorylation cascade (Figs. 1 *B* and *C* and 5). Thus, the addition of Trp up-regulates the cascade phosphorylation of Ubr1 through the same Ssy1-mediated activation of the Yck1/Yck2 kinases that

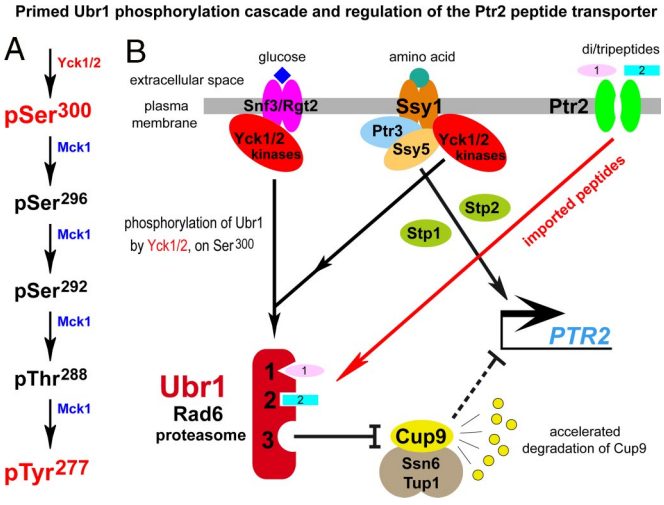


**Fig. 5.** Summary of main findings. (*A*) The cascade of Ubr1 phosphorylation. See Fig. 1*C* for another diagram of this cascade. (*B*) The Yck1/Yck2 kinases mediate the activity of the amino acid-sensing SPS pathway and the glucose-sensing Snf3/Rgt2 pathway. We found that Yck1/Yck2 also function as "priming" kinases in the Ubr1 phosphorylation cascade (*A* and Fig. 1*C*) that involves the Yck1/Yck2 and Mck1 kinases. Stp1 and Stp2 are latent transcription factors, activated through cleavages by Ssy5, that up-regulate, in particular, the expression of the peptide transporter gene *PTR2*, in a process counteracted by the Cup9 repressor (28). Also shown is the characterized positive-feedback circuit (1, 2) that involves the binding of di/tripeptides with type-1 or -2 destabilizing N-terminal residues (Fig. 1*A*) to the types-1/2 binding sites of Ubr1, and the resulting allosteric activation of the third binding site of Ubr1 that targets Cup9 for ubiquitylation. This activation, which accelerates Cup9 degradation and thereby up-regulates *PTR2*, was found to require phosphorylation of Ubr1 on Ser$^{300}$ by the Yck1/Yck2 kinases.

also leads to phosphorylation of Ptr3, a component of the SPS pathway (27, 28, 33).

The impaired ability of Ubr1$^{S300A}$ to increase the rate of Cup9 degradation in response to amino acids or type-1 dipeptides can account, at least in part, for the demonstrated impairment of Ubr1$^{S300A}$ in the induction of *PTR2* by these effectors (Figs. 3*E* and 4*C*; Fig. S4*G*). In summary, the induction of *PTR2* by Trp, a process that involves the SPS pathway and the Ubr1-dependent acceleration of Cup9 degradation (4), requires phosphorylation of Ubr1 on Ser$^{300}$ by the Yck1/Yck2 kinases. These kinases also play a role in the signaling by glucose in yeast (24), in agreement with the Yck1/Yck2-dependent up-regulation of Ubr1 phosphorylation on Tyr$^{277}$ by glucose, an effect that is not observed with Ubr1$^{S300A}$ (Fig. 2 *F* and *G*). In contrast to the Yck1/Yck2-mediated phosphorylation of Ubr1 on Ser$^{300}$ that affects both the activity of Ubr1 and the control of peptide import by the N-end rule pathway, the other (primed) phosphorylations of Ubr1 (Fig. 1*C*) appear to have at most minor effects on the known Ubr1 functions. Thus, a biological role of the post-Ser$^{300}$ part of Ubr1 phosphorylation cascade (Fig. 1*C*) remains to be identified. We also found that mutational replacements of Tyr$^{277}$ by either Ala or Glu (the latter a mimic of phospho-Tyr at this position) converted Ubr1 into a short-lived protein in vivo (Fig. S2 *B–E*). Surprisingly, a proteasome-inhibitor test with Ubr1$^{pY277}$ (wild-type Ubr1 phosphorylated on Tyr$^{277}$) suggested its metabolic stability (Fig. 2*D*), in contrast to a short in vivo half-life of, for example, Ubr1$^{Y277E}$ (Fig. S2 *B–E*). It remains to be determined definitively whether the phosphorylation of Ubr1 on Tyr$^{277}$ can lead to its accelerated degradation in vivo.

Although *S. cerevisiae* Ubr1 is sequelogous to the mammalian Ubr1/Ubr2 N-recognins (8), the latter lack a sequence that resembles the Tyr$^{277}$-containing region of *S. cerevisiae* Ubr1. Nevertheless, a counterpart of the functionally critical Ser$^{300}$ residue of yeast Ubr1 (Fig. 1*C*) may well be present in mammalian Ubr1/Ubr2, which have several potential sites of phosphorylation by a CKI-type kinase. In other words, the main discovery of the present work (Figs. 1*C* and 5) suggests that phosphorylation will be found to regulate N-recognins of multicellular eukaryotes as well.

## Methods Summary

For descriptions of materials and methods, including *S. cerevisiae* strains, plasmids and PCR primers, see *SI Text*, and Tables S1–S3. Standard techniques, including PCR, were used to construct specific strains and plasmids, including plasmids that encoded Ubr1 mutants. Epitope-tagged Mck1, Ubr1 and their mutant derivatives or GST fusions to Ubr1 fragments were overexpressed in *S. cerevisiae* or *Escherichia coli* and purified through steps that included affinity chromatography. Sites of in vivo phosphorylation of Ubr1 in *S. cerevisiae* were mapped using trypsin-produced fragments of isolated Ubr1 and mass spectrometry (MS). Ab1$^{ScUbr1(1–1140)}$, an antibody that recognized all species of *S. cerevisiae* Ubr1 and Ab1$^{pY277Ubr1}$, an antibody to a phosphopeptide that encompassed phospho-Tyr$^{277}$, were produced in rabbits. All procedures, including the making and characterization of antibodies, the immunoblotting and coimmunoprecipitation assays, pulse–chase assays, and in vitro kinase assays are described in *SI Text*.

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