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Regular paper

Hem12, an enzyme of heme biosynthesis pathway, is monoubiquitinated by Rsp5 ubiquitin ligase in yeast cells*

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Heme biosynthesis pathway is conserved in yeast and humans and *hem12* yeast mutants mimic porphyria cutanea tarda (PCT), a hereditary human disease caused by mutations in the *UROD* gene. Even though mutations in other genes also affect UROD activity and predispose to sporadic PCT, the regulation of UROD is unknown. Here, we used yeast as a model to study regulation of Hem12 by ubiquitination and involvement of Rsp5 ubiquitin ligase in this process. We found that Hem12 is monoubiquitinated *in vivo* by Rsp5. Hem12 contains three conserved lysine residues located on the protein surface that can potentially be ubiquitinated and lysine K8 is close to the 36-LPEY-39 (PY) motif which binds WW domains of the Rsp5 ligase. The *hem12-K8A* mutation results in a defect in cell growth on a glycerol medium at 38°C but it does not affect the level of Hem12. The *hem12-L36A,P37A* mutations which destroy the PY motif result in a more profound growth defect on both, glycerol and glucose-containing media. However, after several passages on the glucose medium, the *hem12-L36A,P37A* cells adapt to the growth medium owing to higher expression of *hem12-L36A,P37A* gene and higher stability of the mutant Hem12-L36A,P37A protein. The Hem12 protein is downregulated upon heat stress in a Rsp5-independent way. Thus, Rsp5-dependent Hem12 monoubiquitination is important for its functioning, but not required for its degradation. Since Rsp5 has homologs among the Nedd4 family of ubiquitin ligases in humans, a similar regulation by ubiquitination might be also important for functioning of the human UROD.

Key words: yeast; heme biosynthesis; Hem12; ubiquitination; Rsp5 ligase; protein degradation

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INTRODUCTION

Rsp5 is a unique yeast member of the Nedd4 family of ubiquitin ligases which have a common C2-WW-HECT modular structure with the lipid-binding domain C2, protein-binding domains WW and catalytic HECT domain (Kaliszewski & Zoladek, 2008). The Rsp5 ligase monoubiquitinates and polyubiquitinates substrates with K63-linked ubiquitin chains *in vivo* and *in vitro* (Kee *et al.*, 2006). Some Rsp5 substrates are then transported *via* endocytosis to the vacuole for degradation (Lauwers *et al.*, 2010), others are proteolytically processed (Hoppe *et al.*, 2000) or have reduced activity (Novoselova *et al.*, 2013). Rsp5 ubiquitinates a transcriptional activator, Spt23, thereby promoting its release from the endoplasmic reticulum, also affecting its nuclear transport and transcriptional activation of the *OLE1* gene encoding a desaturase of fatty acids. This is an essential function since *rsp5*Δ strain cannot grow unless cells bear a plasmid encoding a constitutively active variant $Spt23^{1-689}$ (Hoppe *et al.*, 2000). Rsp5 is also involved in proteasomal degradation of Rpb1, the largest subunit of the RNA polymerase II (Harreman *et al.*, 2009). This protein is ubiquitinated in a two-step mechanism in which Rsp5 first adds monoubiquitin and then a second ubiquitin ligase produces a K48-linked polyubiquitin chain, which triggers proteasomal proteolysis (Harreman *et al.*, 2009). The *rsp5* mutants are hypersensitive to various toxic compounds, including hydrogen peroxide which causes oxidative stress (Hoshikawa *et al.*, 2003).

One of the proteins which binds to and is ubiquitinated by Rsp5 *in vitro* is Hem12 (Hesselberth *et al.*, 2006; Gupta *et al.*, 2007), a cytoplasmic uroporphyrinogen III decarboxylase (UROD), the fifth enzyme in the heme biosynthesis pathway. The *HEM12* gene is essential in yeast and *hem12*Δ cells do not grow unless the medium is supplemented with ergosterol and unsaturated fatty acids since Erg5 sterol desaturase and Ole1 fatty acid desaturase are hemoproteins. The *hem12*Δ mutant cells supplemented with those lipids are still respiratory-deficient and do not utilize glycerol, since they lack mitochondrial cytochromes. The heme biosynthesis pathway is conserved in humans and mutations in the human *UROD* gene resulting in UROD dysfunction cause a hereditary disease, porphyria cutanea tarda (PCT) (Frank & Poblete-Gutierrez, 2010), in which accumulation of porphyrins, phototoxic heme precursors, results in liver and skin damage (Mendez *et al.*, 2012). Some mutations in the *UROD* gene result in lower activity and instability of UROD. Mutations in other genes may affect UROD activity and predispose to sporadic PCT by unknown mechanisms (Garey *et al.*, 1993). In yeast cells, point mutations in the *HEM12* gene also lead to the accumulation of porphyrins (Zoladek *et al.*, 1996) and the *hem12* mutants mimic PCT (Kurlandzka *et al.*, 1988). Yeast strains with mutations in unknown genes that affect expression of *HEM12* have also been isolated, resembling the situation in humans (Zoladek *et al.*, 1995). Moreover, UROD was recently identified as a potential anticancer

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Abbreviations: ALA, 5-aminolevulinic acid; PCT, porphyria cutanea tarda; PY, LPEY motif; UROD, uroporphyrinogen III decarboxylase

target and UROD inhibitor combined effectively with radiation and other anticancer drugs (Yip *et al.*, 2014).

Heme, as the prosthetic group of numerous hemoproteins, is crucial in electron transport in the mitochondrial oxidative chain, in several metabolic pathways, and in the defense against reactive oxygen species (Heinemann *et al.*, 2008). Heme is essential for life but in excess it can be toxic to cells, therefore a crucial aspect of heme biosynthesis is its regulation. In mammals, the activity of 5-aminolevulinic acid (ALA) synthase, catalyzing the first step of heme biosynthesis pathway, is rate-limiting (Panda *et al.*, 2002). Recently, a negative feedback regulation of heme homeostasis has been identified with Rev-erbα heme receptor interacting with the NCoR protein to repress its target genes in human cells (Wu *et al.*, 2009). Yeast growth on glycerol medium results in a ~3-fold induction of heme biosynthesis compared with glucose-grown cells (Diflumeri *et al.*, 1993). In yeast cells, ALA synthesis is not rate-limiting since ALA is present in excess; instead the rate-limiting are other enzymes, including Hem12 UROD (Hoffman *et al.*, 2003). Hem12 protein could potentially be regulated post-translationally since it has been found to interact with WW domains of the Rsp5 ubiquitn ligase (Hesselberth *et al.*, 2006) and be ubiquitinated *in vitro* (Gupta *et al.*, 2007). Here, we analyzed a possible regulatory mechanism, ubiquitination of Hem12 protein *in vivo*, its dependence on the Rsp5 ubiquitin ligase, and significance for yeast physiology.

MATERIALS AND METHODS

Strains, media and growth conditions. The *Saccharomyces cerevisiae* strains used were MHY501 (Chen *et al.*, 1993), PC10 *rsp5-1* isogenic to MHY501 (P. Cholbinski IBB PAS), *rsp5*Δ (Hoppe *et al.*, 2000), BY4741 and its derivative Y23983 *HEM12/hem12*Δ*::kanMX4* (Euroscarf). The *hem12*Δ mutants bearing plasmids with *hem12-K8A* or *hem12-L36A,P37A* allele were obtained by sporulation and tetrad dissection of respective transformants of a *HEM12/hem12*Δ diploid strain.

Yeast were grown in YPD with 2% glucose, YPGly with 2% glycerol, SD or SC with 2% glucose (Sherman, 2002). YPD+G418 was used to select strains resistant to kanamycin, and sporulation medium (Sherman, 2002) for spore production. To monitor the effect of elevated temperature on the level of wild type and mutant HA-Hem12, the respective yeast cells were grown at 28°C in SC-leu medium, transferred to YPD to OD_{600} ~0.3, grown for two generations and shifted to 38°C for 2 or 4 hours. For cycloheximide-chase analysis of Hem12 degradation, yeast were grown in YPD medium to the logarithmic phase, and 0.5 ml aliquots were removed for immunoblotting at indicated times following the addition of 500 μg/ml cycloheximide (Sigma).

Plasmids and plasmid construction. Plasmid YEp-HIS-UBI was used (Stawiecka-Mirota *et al.*, 2007). Plasmid pBS-HA-HEM12 was constructed by transfer of NotI digested DNA fragment containing 3HA tag into the NotI site which was introduced after ATG codon of *HEM12* orf by PCR *in vitro* mutagenesis of pBS-HEM12 (Zoladek *et al.*, 1995). Then SacI-HindIII fragment containing *HA-HEM12* was transferred to SacI- and HindIII-digested pRS415 and pRS425 (Invitrogen) to obtain pRS415-HA-HEM12 and pRS425-HA-HEM12. Mutations in *HEM12* were introduced by PCR mutagenesis of pBS-HA-HEM12, respective fragments transferred to pRS415 and pRS425 and confirmed by sequencing. Also, the SacI-HindIII fragment of pBS-HEM12 was used to obtain pRS415-HEM12.

Purification of ubiquitinated proteins. Purification of His-tagged ubiquitinated protein was performed as described previously (Stawiecka-Mirota *et al.*, 2007). Total extracts, Ni-NTA-sepharose (Qiagen)-bound and unbound fractions were analyzed by Western blotting using anti-HA antibody.

Western blotting. Protein extracts were prepared and Western blotting was performed as described previously (Stawiecka-Mirota *et al.*, 2007). Anti-Nedd4 WW domain recognizing Rsp5 (Millipore), anti-Pgk1 (Molecular Probes), monoclonal anti-HA (12CA5, Covance), monoclonal anti-Vma2 (Molecular Probes) primary antibodies and secondary horseradish peroxidase-conjugated antibodies (DACO) were used and followed by enhanced chemiluminescence (Millipore). HA-Hem12 and Rsp5 levels were quantified using Image Quant and normalized to Vma2, Pgk1 or Ponceau Red stained proteins.

Real-time PCR. Real-time RT-PCR was performed using the LightCycler®480 System (Roche Laboratories) with SYBR Green detection. The primers' specificity was verified by melting curve analysis. Sequences of primers used are available upon request. RT-PCRs were performed in triplicate. cDNA was synthesized from 5 μg of total RNA using RevertAid™ H Minus M-MuLV Reverse Transcriptase kit (Fermentas). Average cycle thresholds were calculated and the Pfaffl method (Pfaffl, 2001) was used to calculate relative *HEM12* expression levels with respect to 5S rRNA.

Computational analysis. A protein multiple sequence alignment was obtained with the MAFT program (Katoh & Toh, 2008) using homologs identified with BLAST (Schaffer *et al.*, 2001). Several homologs of *S. cerevisiae* Hem12 with solved crystal structures have been indicated by BLAST and HHpred (Soding *et al.*, 2005) servers. The structure of human uroporphyrinogen decarboxylase (PDB entry 1URO; (Whitby *et al.*, 1998)) was chosen as a template to construct a model of Hem12 protein. Structural models of wild type and mutant Hem12 were obtained using the Sybyl-x1.2 package (TRIPOS, Inc., USA). The model structures were subjected to energy minimization using AMBERFF99 force field as implemented in Sybyl-x1.2.

RESULTS AND DISCUSSION

The Hem12 protein is monoubiquitinated by the Rsp5 ligase *in vivo*

Since *in vitro* experiments had identified Hem12 as a substrate of Rsp5 (Gupta *et al.*, 2007), we inquired if Rsp5 contributes to regulation of the heme biosynthesis pathway via *in vivo* ubiquitination and regulation of Hem12. To answer this question we used a HA-tagged version of *HEM12* expressed from a plasmid restoring viability of a *hem12*Δ strain on rich glucose medium at 28°C (Fig. 1A). The wild type or the *rsp5*Δ strain, where a plasmid encoding $Spt23^{1-689}$ ensured viability (Hoppe *et al.*, 2000), were transformed with single- or multicopy plasmids bearing *HA-HEM12*. Western blot analysis revealed that the level of HA-Hem12 overexpressed from a multicopy plasmid was increased 13–16 fold (Fig. 1B). To test if Hem12 is ubiquitinated *in vivo* we used strains expressing *HIS-UBI* encoding His-ubiquitin (Stawiecka-Mirota *et al.*, 2007) and *HA-HEM12* from the multicopy plasmid. His-ubiquitinated proteins were purified on Ni-sepharose beads and were analyzed by Western blotting with anti-HA antibody. Results shown in Fig. 1C document that HA-Hem12 is monoubiquitinated *in vivo*.

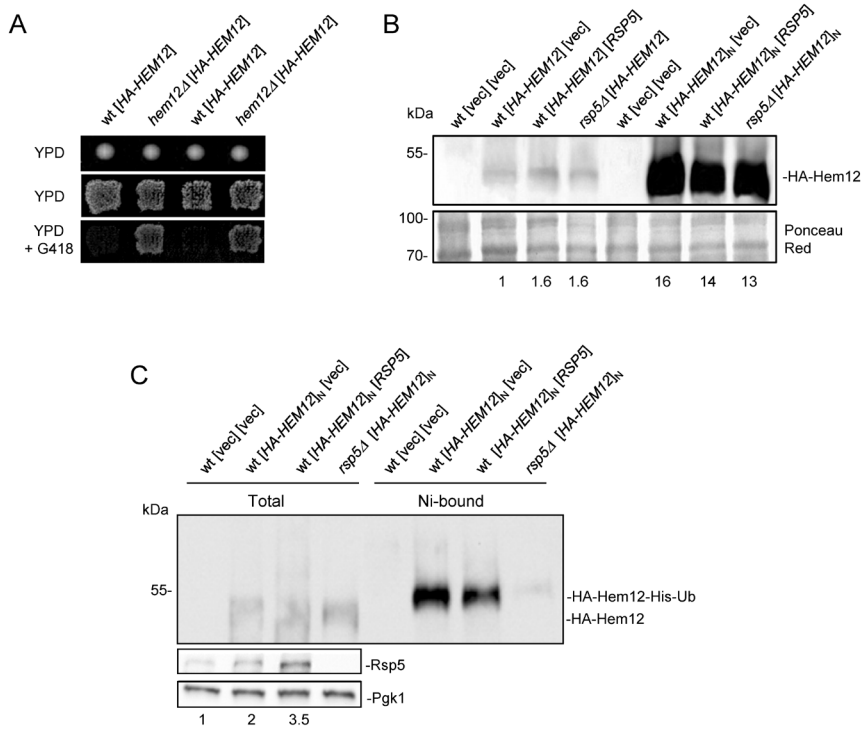

Figure 1. Hem12 protein is monoubiuqitinated *in vivo* and this ubiquitination depends on Rsp5.
(**A**) *HA-HEM12* complements lethality of *hem12*Δ. Diploid *HEM12/hem12*Δ*::kanMX4* was transformed with pRS415-HA-HEM12 plasmid, tetrads were obtained and spore clones tested for growth on YPD and YPD+G418. (**B**) Additional copy of *RSP5* or deletion of *RSP5* does not affect the level of Hem12. Wild type strain (MHY501) was transformed with an empty vector, pRS415-HA-HEM12 or multicopy pRS425-HA-HEM12 ([*HA-HEM12*]$_N$) and with YCp33-RSP5 or empty vector for control. Strain *rsp5*Δ bearing plasmid expressing *SPT23*$^{1-689}$ was transformed with pRS415-HA-HEM12 or pRS425-HA-HEM12. All of these strains also contained the YEp-HIS-UBI plasmid. Protein extracts were analyzed by Western blotting with anti-HA antibody. (**C**) Hem12 is ubiquitinated. Transformants expressing *HIS-UBI* and *HA-HEM12* from multicopy plasmids as in B, were grown, protein extracts were prepared, and His-ubiquitinated proteins were purified using Ni-sepharose resins. Total extracts and Ni-bound fractions were analysed by Western blotting with anti-HA antibody. Rsp5 was detected by anti-Nedd4 antibody and anti-Pgk1 was used to control for protein loading. HA-Hem12 and Rsp5 levels were quantified.

When the Rsp5 ubiquitin ligase is absent, ubiquitination of Hem12 is abolished to nearly background levels, which may indicate that in the absence of Rsp5 another ligase partially takes over. An additional copy of *RSP5* giving rise to a 3.5-fold elevated level of Rsp5 essentially did not increase the ubiquitination of Hem12. Thus, Rsp5 ubiquitinates Hem12 *in vivo* and some factors control the level of this ubiquitination tightly. The presence of an additional copy or deletion of *RSP5* essentially did not affect the steady state level of the Hem12 protein (Fig. 1B, C). Unlike polyubiquitination, monoubiquitination does not direct proteins to proteasomal degradation since proteasomes only recognize proteins tagged with K48-linked polyubiquitin chains containing more than four ubiquitins. Instead, monoubiquitination recruits proteins containing ubiquitin-binding domains (UBDs) and provides a signaling mechanism that regulates several cellular pathways (Ikeda & Dikic, 2008). Thus, the Rsp5-dependent monoubiquitination probably does not direct Hem12 for degradation but could affect its functioning through some other mechanism.

K8A substitution in Hem12 affects growth of cells on glycerol medium at an elevated temperature

Hem12 is highly conserved in evolution. UROD has a homodimeric structure with the active site clefts facing each other and all decarboxylation events occurring only in one active site (Martins *et al.*, 2001; Bushnell *et al.*, 2010). Assuming that the regulation of UROD by ubiquitination might be also conserved, we compared 34 UROD amino acid sequences to find conserved lysine residues which could be potential ubiquitination sites. Alignment of three sequences is presented in Fig. 2A. We found that five lysines: K8, K99, K174, K242 and K260 of Hem12 are conserved. Computer modeling of the Hem12 structure (Fig. 2B) showed that two of them, K174 and K260, are located on the dimer interface but three others are potentially available for ubiquitination. Since K8 is very close to the PY motif 36-LPEY-39 which binds the WW1 and WW3 domains of Rsp5 (Hesselberth *et al.*, 2006) we assumed that this lysine is likely the ubiquitination site. Computer modeling indicated that substitution of K8 with alanine should not affect the protein structure (not shown). Therefore, the *HA-hem12-K8A* mutant allele was constructed by *in vitro* mutagenesis and tested for complementation of *hem12*Δ inviability. A heterozygous *HEM12/hem12*Δ strain was transformed with a plasmid bearing the mutant allele, sporulated and tetrads were dissected. The *hem12*Δ [*HA-hem12-K8A*] spores were viable and their growth was comparable to that of wt [*HA-hem12-K8A*] and *hem12*Δ [*HA-HEM12*] strains on medium containing glucose or glycerol, a non-fermentable carbon source, at 28°C (Figure 3A and not shown). However, the *hem12*Δ [*HA-hem12-K8A*] mutant strain grew significantly slower on the glycerol medium at 38°C when compared with wt [*HA-hem12-K8A*] and *hem12*Δ [*HA-HEM12*] (Fig. 3A and not shown). We observed a similar steady state level of HA-Hem12 and HA-Hem12-K8A proteins in cells grown at 28°C, and a similar 5-fold decrease of the steady state level after 4 hours of incubation at 38°C (Fig. 3B). These results

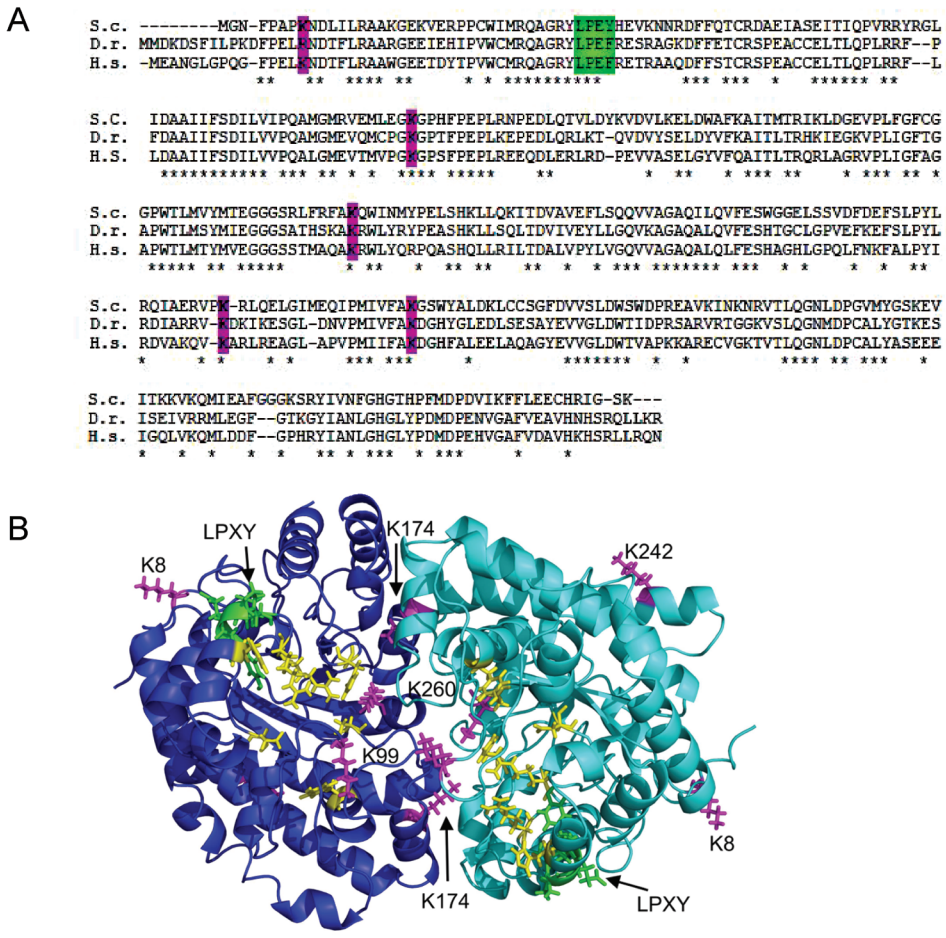

Figure 2. Five lysines are conserved in UROD.
(**A**) Alignment of amino acid sequences of UROD from *S. cerevisiae*, *Danio rerio* and *Homo sapiens*. Evolutionarily conserved lysines are purple, LPEY/F motif is in green. (**B**) Model of *S. cerevisiae* Hem12 structure. The active enzyme is a homodimer, individual monomers are shown in light and dark blue. Amino acid residues forming the active site are marked in yellow. Conserved lysines K8, K99, K174, K242 and K260 are in pink. Conserved LPEY motif which binds WW domains of Rsp5 is in green.

indicate that Hem12 is subject to downregulation upon heat stress and suggest that the *hem12-K8A* mutation does not affect the stability of Hem12 but negatively affects its activity or interaction with other proteins at the elevated temperature. Lysines other than K8 of Hem12 could be ubiquitinated and direct the wild type and mutant Hem12-K8A protein for degradation at an elevated temperature. In fact, Hem12-K8A mutant protein is still monoubiquitinated (not shown).

To investigate if Rsp5 is important for the degradation of Hem12 upon heat stress, the level of wild type Hem12 was monitored in wild type yeast and *rsp5-1* mutant with the L733S substitution in the catalytic HECT domain showing a temperature-sensitive growth defect (Wang *et al.*, 2001). Yeast were grown at 28°C, shifted for 2 or 4 hours to 38°C, and Western blotting was performed. The steady state level of Hem12 was lower in *rsp5-1* than in wild type cells but this protein was similarly degraded upon heat stress in both strains showing again that Rsp5 does not affect its degradation (Fig. 3C). This is in contrast to the recent finding that Rsp5 is the main ubiquitin ligase that targets cytosolic proteins following heat stress (Fang *et al.*, 2014). Thus, another ligase must be involved in forming polyubiquitin chains that direct Hem12 for proteasomal degradation. Previous *in vitro* experiments have revealed that Hem12 may be also a substrate of the cytosolic ligase Ubr1 (Lu *et al.*, 2008) which functions in quality control of cytoplasmic proteins (Eisele & Wolf, 2008; Xia *et al.*, 2008). It is possible that Rsp5 and Ubr1 function together in Hem12 ubiquitination *in vivo*. Moreover, Ubr1 could be responsible for the residual monoubiquitination of Hem12 observed in the *rsp5*Δ strain.

Double mutation *hem12-L36A,P37A* destroying the PY motif in Hem12 prevents cell growth on glycerol medium

To eliminate Rsp5 binding and ubiquitination of Hem12, L36A and P37A mutations which make the PY motif not functional (Merhi & Andre, 2012) were introduced into a plasmid carrying *HA-HEM12*. Computer modeling showed that these mutations should not affect the structure of the Hem12 catalytic center (not shown) and thus should not affect its catalytic activity. The heterozygous *HEM12/hem12*Δ strain was transformed with the above plasmid, sporulated and spore clones were isolated. The *hem12*Δ [*HA-hem12-L36A,P37A*] spores were viable but grew extremely slowly at 28°C on the glucose medium and not at all on the glycerol medium (Fig. 4A) suggesting that the mutant protein is barely active. This phenotype is much stronger than phenotype of *hem12-K8A* mutant suggesting that another lysine could be ubiquitinated when K8 is absent. Interestingly, after 2–3 passages on the glucose medium *hem12*Δ

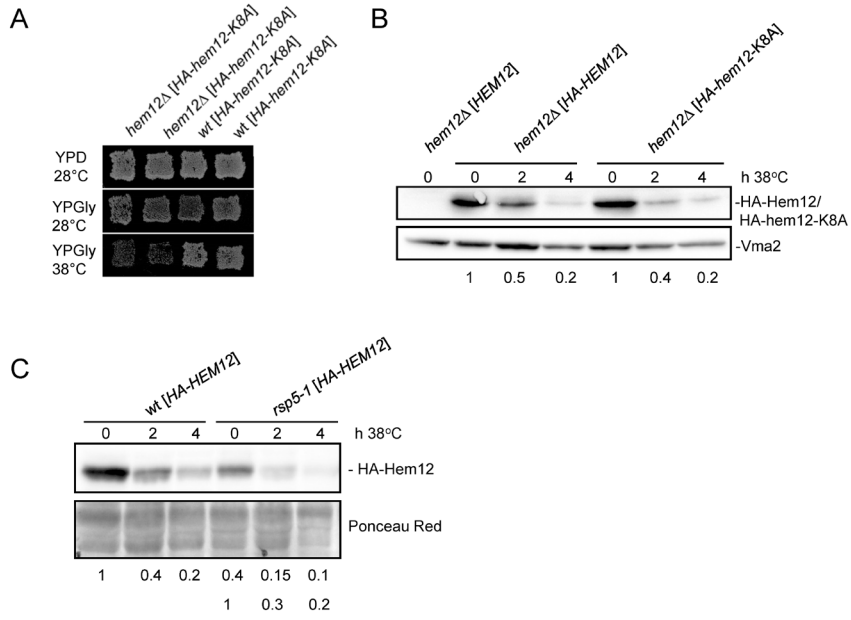

Figure 3. Effect of *hem12-K8A* mutation on cell growth on glycerol medium and the level of mutant HA-Hem12 protein.
(**A**) Complementation of *hem12*Δ by *HA-hem12-K8A*. *HEM12/hem12*Δ*::kanMX4* diploid was transformed with pRS415-HA-hem12-K8A, tetrads were obtained and growth of spore clones was compared. (**B**) The level of HA-Hem12 decreases at an elevated temperature but is not affected by *hem12-K8A* mutation. The *hem12*Δ [*HEM12*], *hem12*Δ [*HA-HEM12*] and *hem12*Δ [*HA-hem12-K8A*] strains were grown in YPGly at 28°C, shifted or not shifted to 38°C for 2 or 4 hours, extracts were analyzed by Western blotting using anti-HA and anti-Vma2 antibodies. (**C**) The *rsp5-1* mutation does not affect the degradation of Hem12 at an elevated temperature. Western blot using anti-HA antibody of wild type and *rsp5-1* cells expressing HA-Hem12 from single copy plasmid. HA-Hem12 levels were quantified.

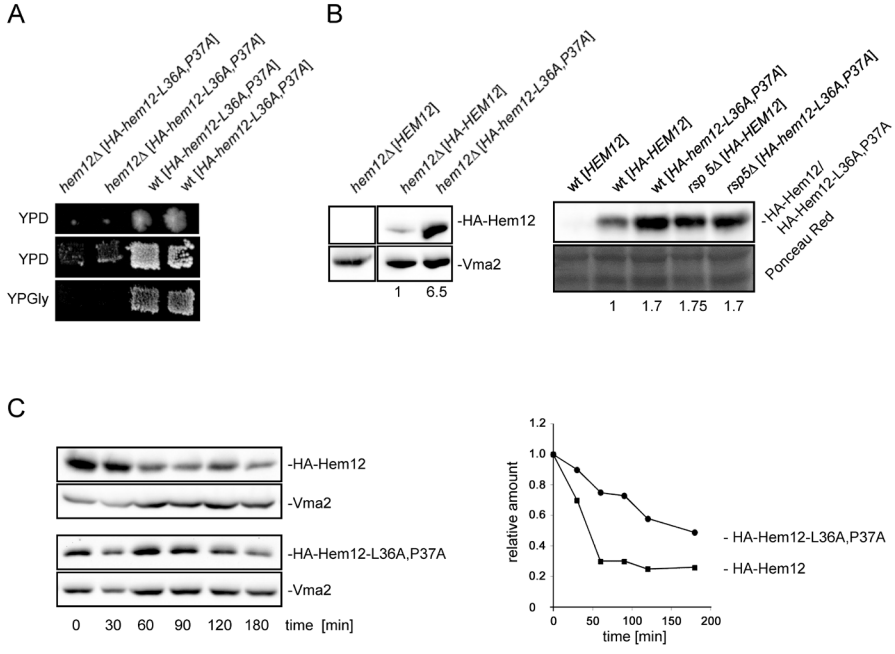

Figure 4. Effect of *hem12-L36A,P37A* mutation on cell growth and the level and stability of mutant Hem12 protein.
(**A**) Complementation of *hem12*Δ by *HA-hem12-L36A,P37A*. *HEM12/hem12*Δ*::kanMX4* diploid was transformed with pRS415-HA-hem12-L36A,P37A, tetrads were obtained and growth of spore clones was tested on YPD and YPGly at 28°C. (**B**) The level of HA-Hem12-L36A,P37A protein in various strains. The *hem12*Δ [*HEM12*], *hem12*Δ [*HA-HEM12*] strains were grown in YPD to log phase. The *hem12*Δ [*HA-hem12- L36A,P37A*] strain was reinoculated three times into fresh YPD and then grown to log phase. Extracts were analyzed by Western blotting using anti-HA and anti-Vma2 antibodies (left panel). The wt [*HA-HEM12*], wt [*HA-hem12- L36A,P37A*], *rsp5*Δ [*HA-HEM12*] and *rsp5*Δ [*HA-hem12- L36A,P37A*] strains were grown in SC medium, transferred to YPD and allowed to grow for two generations. Extracts were analyzed using anti-HA antibodies (right panel). One representative experiment of two is shown. (**C**) Cycloheximide chase analysis of wild type and mutant Hem12 levels in *hem12*Δ [*HA-HEM12*] and *hem12*Δ [*HA-hem12- L36A,P37A*] strains. HA-Hem12 turnover was monitored by Western blotting using anti-HA and anti-Vma2 antibodies at indicated chase times after addition of cycloheximide to cultures grown in YPD medium. HA-Hem12 levels were quantified.

[*HA-hem12-L36A,P37A*] spore clones achieved a normal rate of growth. Western blotting showed that the level of the mutant Hem12-L36A,P37A was greatly elevated in those strains, up to 6.5-fold over the wild type level (Fig. 4B, left panel). The glucose-adapted *hem12*Δ [*HA-hem12-L36A,P37A*] spore clones also grew on the glycerol medium (not shown). The level of HA-Hem12-L36A,P37A in wild type and *rsp5*Δ strain transformed with a plasmid bearing *HA-hem12-L36A,P37A* gene was similar to that of the wild type HA-Hem12 protein (Fig. 4B, right panel). This shows that HA-Hem12-L36A,P37A is present at increased levels only in the absence of wild type Hem12 protein and also that Rsp5 is not involved in its degradation.

The high steady state level of HA-Hem12-L36A,P37A protein in *hem12*Δ [*HA-hem12-L36A,P37A*] cells could result from an increased stability of the protein, from the induction of *HA-hem12-L36A,P37A* expression, or both. However, in our previous experiments (Fig. 1B) deletion of the *RSP5* gene did not significantly affect the level of HA-Hem12, suggesting that the mutation of Hem12 preventing its modification by Rsp5 should not have caused its stabilization. To clarify that, Hem12 stability was investigated. Cycloheximide was added to logarithmic phase cultures of wild type and *hem12*Δ [*HA-hem12-L36A,P37A*] spore clones to inhibit cytosolic protein synthesis and cell samples were taken at 30 or 60-min intervals. Western blot analysis of cell extracts showed that in fresh spore clones the level of mutant HA-Hem12-L36A,P37A was lower compared with that of the wild type (Fig. 4C, left panel time 0). The low level of the mutant protein cannot be solely responsible for growth defect of *hem12*Δ [*HA-hem12-L36A,P37A*] spore clones since the Sm39 mutant showing a similar low level of wild type Hem12 grows well on glycerol medium (Zoladek *et al*., 1995). The wild type Hem12 protein was rather stable and was degraded in two phases, a faster one with a half-life below 50 min and a slower phase with a half-life above 200 min (Fig. 4C, right panel). This result indicates that two pools of Hem12 protein are present in wild type cells, one that is prone to degradation and one protected from degradation, possibly by binding to other proteins. Degradation of the mutant HA-Hem12-L36A,P37A was slower than that of wild type Hem12, especially in the first phase. Thus, preventing the binding of Hem12 by Rsp5 resulted in Hem12 stabilization. Considering that Rsp5 does not affect the degradation of Hem12 this effect is probably indirect and unrelated to Hem12 ubiquitination by Rsp5.

To learn if changes in expression of the *HEM12* gene contribute to the observed high level of HA-Hem12-L36A,P37A mutant protein, the *HEM12* transcript was analyzed. RNA was isolated from wild type and *hem12*Δ [*HA-hem12-L36A,P37A*] spore clones propagated on glucose medium for three days, and quantified by RT-PCR. The level of *HA-hem12-L36A,P37A* mRNA was 2.15 fold higher than that of *HEM12* mRNA. These results collectively show that the *HA-hem12-L36A,P37A* mutant does not initially grow on the glycerol medium possibly because of low activity of the mutant HA-Hem12-L36A,P37A protein but easily adapts to that medium by increasing the level of the mutant protein through increased expression of the encoding gene and increased stability of the mutant HA-Hem12-L36A,P37A protein.

The obtained results led us to propose a model in which Hem12 is bound and mono-ubiquitinated by Rsp5, possibly at K8. The Rsp5-dependent ubiquitination does not direct Hem12 to degradation. The Rsp5 binding and the monoubiquitination of Hem12 independently help it to bind some unidentified protein which is required for efficient heme synthesis, cell survival and ability to grow on the glycerol medium.

Yeast Hem12 protein shows 50% identity and 67% similarity to the human UROD enzyme, which contains the LPEF motif. Therefore, human UROD could be also ubiquitinated and regulated by a human ubiquitin ligase of the Nedd4 family. Mutations affecting monoubiquitination or promoting degradation of UROD could possibly be one of the reasons of PCT in humans for which no mutation in the *UROD* gene has been found. Further work will be required to confirm the postulated ubiquitination of UROD and to establish its physiological role in humans.

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