A viable ubiquitin-activating enzyme mutant for evaluating ubiquitin system function in *Saccharomyces cerevisiae*

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Abstract Ligation of proteins to ubiquitin requires activation of ubiquitin by E1, the ubiquitin-activating enzyme. Mutant alleles of E1 in mammalian cells have been crucial for dissecting the contribution of the ubiquitin system to cell function. Comparable mutants have been unavailable for *Saccharomyces cerevisiae*. Here we describe the isolation and characterization of a hypomorphic allele of *S. cerevisiae* E1. Protein modification by ubiquitin is strongly impaired in the mutant, inhibiting degradation of ubiquitin–proteasome pathway substrates as well as ubiquitin-dependent but proteasome-independent degradation of membrane receptors. This allele will be a useful tool for evaluating the ubiquitin-dependence of cellular processes in yeast, even those in which the proteasome is not involved.

Key words: Ubiquitin; Proteasome; Cell cycle; Yeast; Mating type; Transposon mutagenesis

1. Introduction

Ubiquitin is a 76-residue polypeptide that has been implicated in a steadily increasing number of cellular processes [1–3]. The protein is synthesized in precursor form, being made either as a head-to-tail array of ubiquitin units or as a fusion to certain ribosomal proteins. The translational fusion of ubiquitin to these ribosomal proteins is required for normal ribosome biogenesis [4], although the ubiquitin moieties are cotranslationally removed by cellular deubiquitinating enzymes. All known post-translational functions of ubiquitin require its enzymatic ligation to target proteins. Covalent attachment occurs via an enzymatic cascade that begins with the activation of the C-terminal carboxyl group of ubiquitin by a ubiquitin-activating enzyme or E1 [3]. This ATP-dependent reaction results in a high-energy thioester bond between ubiquitin and E1. The ubiquitin is then transferred via transthiolation to a ubiquitin-conjugating enzyme or E2. With the aid of a ubiquitin–protein ligase, or E3, ubiquitin is attached to a target protein by an amide bond between the C-terminus of ubiquitin and an acceptor lysine side chain in the target protein.

Ligation of ubiquitin to a substrate can have a variety of consequences. For many proteins, a polyubiquitin chain is assembled on a substrate lysine residue(s); this chain binds with high affinity to the 26S proteasome [5], leading to the degradation of the substrate into small peptides and recycling of the ubiquitin moieties. Ubiquitination of over a dozen cell surface membrane proteins has also been reported, and the modification is necessary for their internalization and transport to the vacuole, where the proteins are destroyed in a proteasome-independent manner [6–8]. Interestingly, for ubiquitin-dependent endocytosis, either monoubiquitination or a short chain of ubiquitins linked by their Lys-63 side chains is sufficient for internalization [9,10]. Functional alteration by ubiquitin ligation of other proteins, e.g. certain histones, has been suggested as well, but the mechanistic consequences of these modifications are not well understood [11].

The first evidence for the physiological importance of the ubiquitin system came from seminal genetic studies with a temperature-sensitive mouse cell line that harbored a thermolabile E1 enzyme [12,13]. Although analysis of the *Saccharomyces cerevisiae* ubiquitin system has subsequently had a prominent role in uncovering the many physiological functions of protein ubiquitination [1,14,15], it has still often been difficult to link a particular metabolic process to protein ubiquitination in this organism. This limitation can be traced to the fact that ubiquitin–protein conjugates are often very difficult to detect and because no partial loss-of-function mutants are available in the essential yeast E1 gene, *UBA1*. Evaluating the effects of different E2 or *UBC* mutations on a protein or process is possible (e.g. [16]) but is laborious inasmuch as yeast cells have 11 different E2 enzymes that are targets for ubiquitin transfer from Uba1. At least as many E3 ligases are anticipated, so E3 mutants are also not an efficient route for determining the involvement of ubiquitin ligation in a process of interest.

Here we describe a new hypomorphic allele of *UBA1* that strongly impairs ubiquitin–protein ligation in *S. cerevisiae*. All tested ubiquitin-dependent processes were shown to be defective in this mutant. The pathways examined include ubiquitin-mediated receptor endocytosis, which does not require the proteasome. Therefore, this *uba1* allele should be of general utility in evaluating ubiquitin-dependence of a process in yeast, particularly in cases where the proteasome is not involved.

2. Materials and methods

2.1. Yeast and bacterial media and methods

Yeast rich (YPD) and minimal (SD) media were prepared as described, and standard yeast genetic methods were used [17]. Strains used for mutagenesis were MHY102 (*Matα ura3-52 lys2-801 leu2-3,112::LEU2-Δ68-210α2-lacZ*) [18] and the congenic MHY113 (*MAT***a** *ura3-52 lys2-801 leu2-3,112::LEU2-Δ68-210α2-lacZ*). MHY1409 (*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 uba1-2*) was made by backcrossing the original *uba1-2* strain to MHY501 (*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*) [16] four times.

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Escherichia coli strains used were JM101 and Electromax DH 10B, and standard techniques were employed for recombinant DNA work [19].

2.2. Isolation of uba1-2

The haploid yeast strains MHY102 and MHY113, which contain the integrated *Deg1*-β-galactosidase (*Deg1*-βgal)-encoding construct, were mutagenized using a mini-transposon library as described [20,21]. Briefly, mTn-3xHA/GFP transposon-mutagenized yeast fragments [21] were released from a holding vector by digestion with *Not*I and transformed into MHY101 and MHY113 using standard techniques. Mutagenized yeast was then selected on synthetic medium lacking uracil (SD−ura). After 2–3 days of growth, yeast was replica-plated onto SD−ura medium containing the chromogenic substrate X-gal [22] and grown for 2–3 days at 30°C. Colonies which rapidly turned dark blue were picked and re-screened on X-gal-containing plates. βGal activity was then quantitatively measured in these strains in liquid media using *ortho*-nitrophenyl galactoside (ONPG) as the substrate. Pulse-chase analysis [16] of *Deg1*-βgal degradation was done for the mutants that remained in order to verify directly a defect in *Deg1* fusion protein proteolysis.

The location of the mTn-3xHA/GFP insertions was ascertained by Vectorette PCR (http://genome-www.stanford.edu/group/botlab/protocols/vectorette.html). Briefly, yeast genomic DNA was isolated from the mTN-mutated strain and cut with *Alu*I. The restriction enzyme was heat-inactivated, and the digested genomic DNA was annealed to 'anchor bubbles' consisting of two annealed primers: 5′-GAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGAC-GAGAGAAGGGAGAG-3′ and 5′-GACTCTCCCTTCTCGAATC-GTAACCGTTCGTACGAGAACCGTTCGTACGAGAATCGCTG-TCCTCTCCTTC-3′. PCR amplifications were performed on the ligation mixture using the universal primer (5′-CGAATCGTA-ACCGTTCGTACGAGAATCGCT-3′) and a primer to the *GFP* sequence on the transposon (5′-CATCACCTTCACCCTCTCCACT-GAC3′). The isolated PCR product was then sequenced.

2.3. Measurements of protein half-life and immunoblot analysis

Pulse-chase analysis was carried out as described [16] except that quantitation was done on a phosphorimager. Immunoprecipitations of α2 were performed with anti-α2 antibodies [18], those of **a**1 with an anti-**a**1 antibody [23]. Immunoprecipitations of βgal were done using anti-βgal antibodies (Cappel, West Chester, PA, USA).

To follow the stability of Ste3, a 'cycloheximide-chase' protocol was used [24,25]. Cycloheximide was added to a concentration of 50 μg/ml to cells grown in minimal medium to an OD_{600} of 1. Extracts were then made from aliquots of cells taken at the desired time points by heating to 100°C in Laemmli SDS gel-loading buffer. After spinning out debris, samples were electrophoresed on SDS–polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Blots were incubated with anti-Ste3 antibodies [26] in TBST buffer containing 1% non-fat dried milk [25]. Antibody binding was detected using ECL reagents from Amersham (Arlington Heights, IL, USA). Western immunoblot analysis of cellular ubiquitin levels was performed as above using anti-ubiquitin antibodies [25].

2.4. Northern RNA hybridization analysis

Total RNA was purified from yeast cells using the Purescript RNA isolation kit (Gentra Systems; Research Triangle Park, NC, USA), and Northern RNA hybridization was performed as described [19]. 12 μg of RNA from each sample were loaded in each lane. The DNA probe used to detect *UBA1* was a 756-bp *Nsi*I fragment [27], which was radiolabelled with [α-^{32}P]dATP using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN, USA). The probe used to detect the actin mRNA was a radiolabeled 560-bp *Cla*I fragment from the yeast *ACT1* gene.

3. Results

3.1. Isolation of a viable uba1 mutant

As part of our studies on the mechanisms of ubiquitination and degradation of the yeast Matα2 repressor, the first identified ubiquitin–proteasome pathway substrate in yeast [18,28,29], we conducted a genetic screen to identify novel genes involved in its degradation. The first 67 amino acids of α2 include a ubiquitin-dependent degradation signal called *Deg1*, which when fused to the *E. coli* β-galactosidase (βgal) protein (Fig. 1A) caused rapid degradation of the resulting fusion protein in yeast. If yeast cells expressing the short-lived *Deg1*-βgal reporter were plated on medium containing the chromogenic substrate X-gal, the resulting colonies were white or pale blue. However, if the strain was compromised for *Deg1*-βgal degradation, the fusion accumulated to high levels, yielding dark blue colonies on X-gal. A similar screen had been conducted previously to identify the original *d*egradation *o*f *a*lpha2 (*doa*) mutants [18] (see Fig. 1B), but we had not identified any candidate E3 genes nor had we isolated several genes known to be required for *Deg1*-mediated ubiquitination,

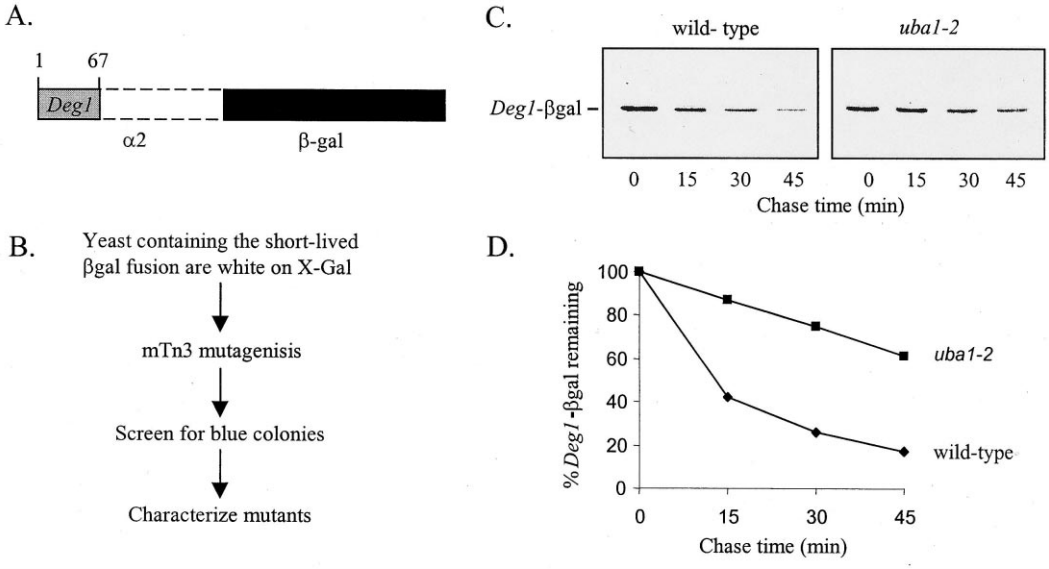

Fig. 1. Isolation of the *uba1-2* allele. A: Schematic of the $Deg1_{\alpha 2}$-βgal fusion construct used for the genetic screen described in B. B: Outline of the genetic screen used to isolate the *uba1-2* allele. C: Pulse-chase analysis of *Deg1*-βgal in wild-type and *uba1-2* cells. The proteins were precipitated by an antibody to βgal. D: Plot of *Deg1*-βgal degradation from C.

viz., *CUE1* and *UBC7*. The new screen was different in two ways. First, the reporter construct was integrated into the chromosome to provide more reliable color development on X-gal. Second, instead of using chemical mutagenesis, we used transposon mutagenesis, which facilitated subsequent identification of the affected genes.

Approximately 90 000 yeast cells that had been transformed with a mini-transposon (mTn3)-mutagenized yeast genomic DNA library [21] were screened for potential defects in *Deg1*-βgal degradation. Mutants that formed dark blue colonies on X-gal plates had their βgal activity measured quantitatively in liquid cultures with the ONPG substrate to eliminate false positives. The presence of single insertions and cosegregation of insertions with the apparent proteolytic defect were determined by backcrossing to a non-mutagenized strain and tetrad analysis. The mTn3 insertion sites were determined by PCR amplification of the genomic inserts and DNA sequencing (Table 1). Fifteen recessive mutants remained after these tests and were sequenced. Eleven had mTn3 insertions in the open reading frame of the non-essential *RPN4/SON1* gene; another *RPN4* insert was sequenced from a mutant with a dominant degradation defect, but linkage of the degradation defect to the mTn3 insertion was not verified. Rpn4 may be a subunit of the 19S regulatory particle of the proteasome but it also functions as a transcription factor that controls the expression of proteasome genes [30–32]. Two of the remaining mutants had transposon insertions in the *UBP14* gene, which encodes a deubiquitinating enzyme that disassembles unanchored ubiquitin chains and was known to be required for maximal α2 degradation rates [33]. Finally, two mutants had identical insertions 84 bp upstream of the translational start site of *UBA1*. By pulse-chase analysis, this mutation, which we call *uba1-2*, caused a ~four-fold decrease on *Deg1*-βgal degradation rate (Fig. 1C,D).

No viable yeast *uba1* mutant alleles had previously been identified. Because all post-translational functions of ubiquitin require its covalent attachment to specific proteins and because this modification universally requires the activation of ubiquitin by Uba1, the *uba1-2* mutant might provide a useful tool for research on the ubiquitin system and was therefore further characterized.

3.2. uba1-2 alters sensitivity to various environmental stresses

The ubiquitin system is involved in a broad range of cellular processes, many of which are essential for growth. Mutations compromising the ubiquitin system have been shown to lead to poor growth and to stress sensitivities. The *uba1-2* mutant had a marked growth defect at 30°C (Fig. 2A). Surprisingly, this growth defect is not only not exacerbated at higher temperatures, but the mutant strain actually grows as well as if not better than wild-type yeast at 37°C (Fig. 2B) and grows substantially better than wild-type at 38°C (Fig. 2C). Mutant *uba1-2* cells also had no discernible growth defect at 15°C relative to the congenic wild-type strain (Fig. 2D). It is important to note that the mutant growth rate at the low and high temperatures does not exceed the rate seen for the mutant at 30°C. The amino acid analog canavanine (1 μg/ml) did not worsen the *uba1-2* growth defect at 30°C (data not shown), but *uba1-2* cells were extremely sensitive to the heavy metal cadmium (Fig. 2E). Thus, *uba1-2* has a complex response to different environmental conditions (see Section 4).

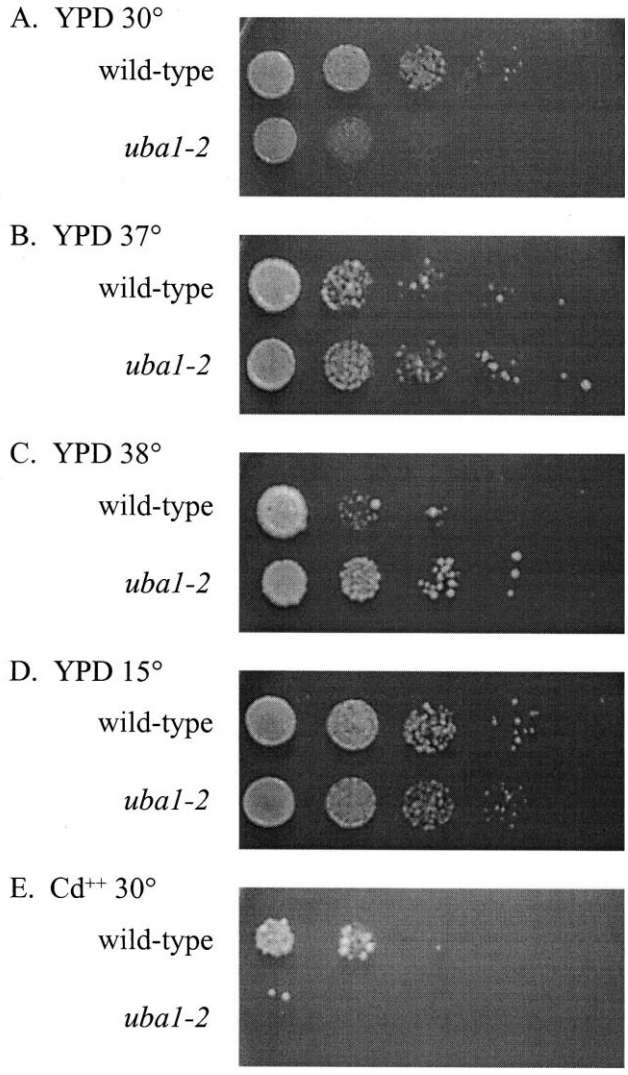

Fig. 2. The *uba1-2* mutation alters yeast growth and stress sensitivities. A: Wild-type and *uba1-2* cells were grown in liquid rich medium (YPD) to an OD_{600} of 1, spotted in 10-fold serial dilutions onto a YPD plate, and grown for 1 day at 30°C. B: As in A except cells were grown for 4 days at 37°C. C: As in A except cells were grown for 4 days at 38°C. D: As in A except cells were grown for 6 days at 15°C. E: As in A except cells were grown for 5 days on minimal medium containing 30 μM $CdCl_2$.

Table 1
Genes identified in mTn3 insertion screen

Gene	No. times isolated
RPN4/SON1/UFD5	12
UBP14	2
UBA1	2

3.3. uba1-2 reduces wild-type Uba1 protein function

The insertion of the mTn3 transposon upstream of the translational start site of *UBA1* in *uba1-2* cells should not alter the encoded Uba1 protein itself, but it may reduce Uba1 levels to a point where ubiquitin activation becomes rate-limiting for certain ubiquitin-dependent processes. We do not have antibodies against Uba1, so we compared the

UBA1 mRNA transcript in wild-type and mutant cells by Northern RNA hybridization analysis (Fig. 3A). In wild-type cells, *UBA1* mRNA was easily detected. Surprisingly, however, even prolonged exposure to film did not reveal the presence of a transcript in *uba1-2* cells that hybridized with the internal *UBA1* probe. The 5′ end of the UBA1 transcript has never been mapped, so it is conceivable that the transposon insertion alters the *UBA1* transcript itself, perhaps generating a very large transcript that does not transfer well during the Northern blotting procedure and/or has reduced metabolic stability.

Reduced Uba1 protein function should be detectable as a decrease in ubiquitin–protein ligation in *uba1-2* cells. To test this, wild-type and *uba1-2* cells were compared by anti-ubiquitin immunoblot analysis. Indeed, the *uba1* hypomorph showed a striking drop in bulk ubiquitin–protein conjugates and a corresponding increase in free ubiquitin compared to the wild-type strain (Fig. 3B). The only previously isolated yeast ubiquitin system mutant that showed such a general decrease in ubiquitin–protein conjugation was the *ubc4*Δ-*ubc5*Δ strain [34]. However, the latter strain is defective in only a limited set of ubiquitin-dependent pathways, e.g. it is not significantly impaired for *Deg1*-βgal degradation.

3.4. *uba1-2 is defective in multiple ubiquitin-dependent molecular processes*

For *uba1-2* to have broad utility in evaluating ubiquitin system function in yeast, the mutant should cause a general dysfunction in ubiquitin conjugation that is measurable by defects in a wide range of ubiquitin-dependent processes. We therefore measured the rate of degradation of a diverse series of ubiquitin system substrates. Leu-βgal is a short-lived model protein that is targeted via N-end rule-mediated ubiquitination to the proteasome (Fig. 4A) [15]. Ub-Pro-βgal is another short-lived test substrate that is ubiquitinated by E2 and E3 enzymes that are distinct from those that act on Leu-βgal or *Deg1*-βgal. Both Leu-βgal and Ub-Pro-βgal were stabilized ∼three-fold in the *uba1-2* strain (Fig. 4B). Finally, the short-lived transcriptional regulators α2 and **a**1 [35] were also modestly stabilized in *uba1-2* cells (data not shown).

We also asked whether defects in proteasome-independent but ubiquitin-dependent processes could be detected in the *uba1-2* mutant. Ste3 is the **a**-pheromone receptor which, when ubiquitinated, is internalized and transported by the endocytic pathway to the vacuole, where it is degraded by vacuolar proteases [26]. If *uba1-2* lowers the rate of Ste3 ubiquitination, Ste3 endocytosis should be impaired, which can be followed indirectly by measuring the rate of vacuolar hydrolase-dependent Ste3 degradation. Levels of total Ste3 were measured by anti-Ste3 immunoblotting at various times after protein translation had been blocked in wild-type and mutant cells (Fig. 4C). Ste3 was degraded significantly more slowly (2–3-fold) in the *uba1-2* strain than in the congenic wild-type strain.

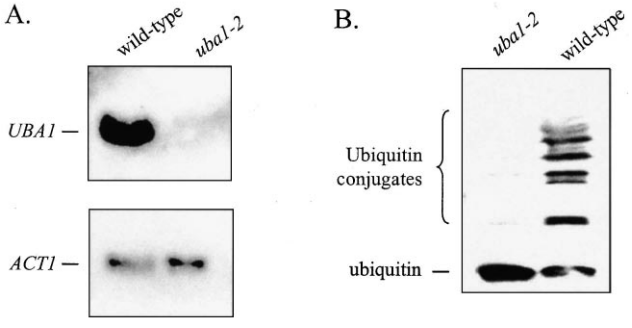

Fig. 3. *UBA1* transcript and function in *uba1-2* cells. A: Northern analysis of *UBA1* in wild-type cells and *uba1-2* cells. A probe recognizing the actin (ACT1) mRNA was used to verify similar RNA loading in each lane. B: Anti-ubiquitin immunoblot analysis of wild-type and *uba1-2* cells. The antibody used was a polyclonal antiserum that had been affinity-purified on a ubiquitin affinity resin.

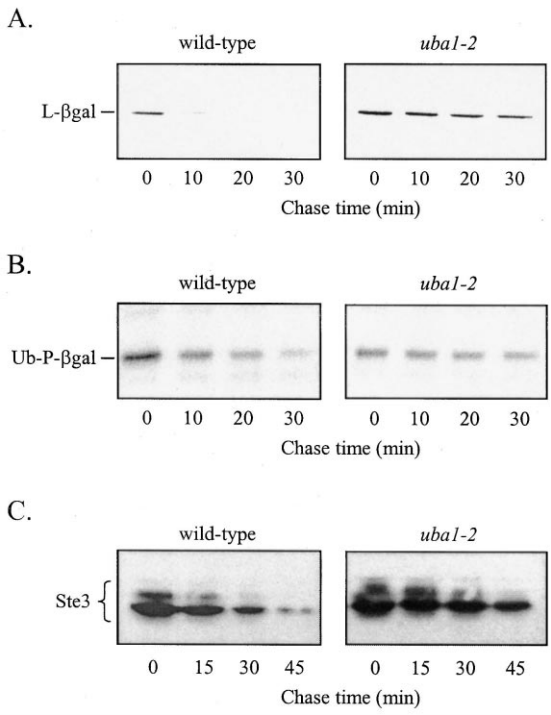

Fig. 4. *uba1-2* is impaired in a variety of ubiquitin related processes. A: Pulse-chase analysis of degradation of Leu-βgal in wild-type and *uba1-2* cells. B: Pulse-chase analysis of degradation of Ub-Pro-βgal in wild-type and *uba1-2* cells. C: Immunoblot analysis of Ste3 degradation in wild-type and *uba1-2* cells. Protein synthesis was blocked at $t = 0$ min by addition of 50 μg/ml cycloheximide.

4. Discussion

4.1. *Isolation of ubiquitin–proteasome pathway mutants*

The mutant screen described here has led to the identification of mutations in three yeast genes, all which were previously implicated in ubiquitin-dependent protein degradation. It is clear that there were strong biases in the types of mutants we were able to isolate. Three-fourths of the mutants were in a single gene, *RPN4*. Moreover, no inserts in novel genes or in other genes known to be important for *Deg1*-mediated degradation (e.g. *UBC6*, *UBC7*, *CUE1*, *DOA1*, *DOA4*; see [29]) were identified. We also identified numerous inserts in the ribosomal RNA locus, but these inserts did not cosegregate with the *Deg1*-βgal degradation. The mTn3 transposon libraries are now recognized to have strong insertion-site preferences.

Despite the failure to identify any new genes involved in *Deg1*-dependent proteolysis by the current screen, we have identified what should be a very useful hypomorphic allele

of the essential yeast gene *UBA1*. This allele, *uba1-2*, contains a mTn3-based transposon inserted 84 bp upstream of the translational start site of *UBA1*. Northern RNA hybridization analysis failed to detect the *UBA1* mRNA in *uba1-2* cells. It is unclear based on these experiments whether the transposon only lowers transcription rates of wild-type *UBA1* mRNA or also alters the *UBA1* mRNA in a way that reduces translation rates and/or mRNA stability. In either case, the coding sequence for the Uba1 protein is intact, suggesting that the *uba1-2* defects in ubiquitin-dependent pathways are due to insufficient amounts of otherwise normal Uba1 protein in the cell. This idea is consistent with the ubiquitin–protein conjugate profile in the mutant, which shows both increased monomeric ubiquitin and decreased levels of conjugated ubiquitin.

4.2. Paradoxical growth effects of uba1-2

The ubiquitin system is known to be essential for growth in yeast and to participate in the response to a variety of stresses such as heat shock. Under optimal growth conditions, *uba1-2* cells have a marked growth defect compared to wild-type cells (Fig. 2). At high temperatures, however, the mutant grows as well as or better than the wild-type strain. One explanation for this anomaly is that at high temperatures, proteins essential for yeast growth may partially unfold and be recognized as substrates for the ubiquitin system. Such proteins might be rapidly degraded in wild-type yeast, causing a significant slowing of growth. By this model, these proteins would be less prone to degradation in the *uba1-2* strain due to the scarcity of Uba1 protein, allowing their continued (partial) function or possibly, their refolding to functional form, particularly after heat shock-induced molecular chaperones have accumulated.

The *uba1-2* phenotype differs strikingly from that of the yeast *ubi4*Δ mutant, which lacks the stress-inducible polyubiquitin gene [36] and might have been expected to be fairly similar in phenotype. *ubi4*Δ strains grow well at 30°C but fail to form colonies at high temperature or in the presence of amino acid analogs, in contrast to *uba1-2* cells. Several explanations could, in principle, reconcile these seemingly contradictory data. One possibility is that non-covalently bound ubiquitin can function to some extent in place of conjugated forms in cells under stress conditions. The *uba1-2* strain has ample free ubiquitin, whereas the *ubi4*Δ strain is depleted for the unconjugated form. Alternatively, the *ubi4*Δ mutant may simply have a more severe loss of ubiquitin conjugation capacity, preventing the ubiquitination and degradation of toxic proteins that *uba1-2* cells can still degrade sufficiently rapidly. Finally, in the stress sensitivity measurements, the mutants are grown at 30°C and then shifted to stress conditions. The *uba1-2* mutant, unlike *ubi4*Δ, is strongly growth compromised at 30°C so it may undergo an adaptive response related to acquired thermotolerance, which would allow it to survive better upon subsequent exposure to certain environmental insults.

Interestingly, no discernible growth defect is seen at 15°C relative to wild-type cells. One possible explanation for this is that yeasts grow very slowly at this temperature. In optimal growth conditions, the ubiquitin conjugation system may be working close to capacity to support maximal growth rates. If Uba1 protein becomes scarce, as in the *uba1-2* strain, a significant growth defect would be observed. At lower temperatures, however, growth occurs much more slowly, and ubiquitin system activity may not need to be as vigorous as at optimal growth temperatures. In this case, the decreased amount of Uba1 protein in *uba1-2* would have little or no effect on the growth rates compared to wild-type yeast. These ideas remain to be examined experimentally.

4.3. Use of uba1-2 to study ubiquitin system function

All substrates of the ubiquitin-mediated, 26S proteasome-dependent degradation pathway that we tested showed decreased degradation rates in the *uba1-2* mutant. Moreover, the Ste3 **a**-factor receptor, which requires ubiquitination in order to be internalized and subsequently destroyed by vacuolar proteases, also was degraded more slowly in the mutant, implying that endocytosis of this protein is impaired by *uba1-2*. These data indicate that the ubiquitin-dependence of a cellular pathway can be reliably gauged in this strain. False-negative results are still possible with such a partial loss-of-function allele, but we have not yet observed this for any processes that are known to require ubiquitination.

We believe comparisons between wild-type and *uba1-2* mutant strains will be a logical first step in any test of ubiquitin system involvement in a particular physiological function in yeast. For example, the ornithine decarboxylase (ODC) protein in mammalian cells is degraded by the 26S proteasome, but this does not require ubiquitination [37]. In yeast, the 26S proteasome is also required for ODC proteolysis [38], but the ubiquitin-dependence of this degradation could not be readily tested. The *uba1-2* strain would be ideal for this. Similarly, processes such as protein secretion, nuclear transport, or chromatin remodeling, which have not been linked to the ubiquitin system, might be tested for ubiquitin-dependence by examining these pathways in *uba1-2* cells. Any protein whose degradation mechanism comes under scrutiny could also be profitably analyzed, at least as an initial test, in the hypomorphic *uba1* strain. Suspicion of ubiquitin system involvement often begins with the observation of additional bands on SDS–polyacrylamide gels that are of higher molecular mass than the protein under investigation. A loss or reduction in such bands in *uba1-2* cells provides a rapid and sensitive test to determine whether they are likely to be ubiquitinated species.

It is useful to compare the potential utility of the *uba1-2* mutant to two other general strategies that have been developed to study ubiquitin system involvement in various processes in yeast. In the first of these, strains were constructed that had a single ubiquitin gene under the control of a regulatable promoter [39]. Ubiquitination could then be measured at various times after synthesis of ubiquitin is turned off as ubiquitin is gradually depleted from cells. The disadvantages of this approach are that the strain is cumbersome to manipulate, and, more importantly, the measurements are made as cells are gradually losing viability. The second strategy that was previously employed was to examine a process of interest in *doa4*Δ cells [25]. Doa4 is a deubiquitinating enzyme [40]. Loss of this enzyme results in significantly reduced levels of cellular ubiquitin even under conditions where cell viability is not reduced. One drawback of using *doa4*Δ, however, is that the mutant is not only defective for ubiquitin homeostasis but is also impaired for proteasome function, so the basis of a specific physiological defect in this mutant is not always easy to decipher. Hence, hypomorphic alleles of *UBA1*, such as *uba1-2*, will permit approaches complementary to and in

some cases superior to those just described for the investigation of ubiquitin system function.

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