Curr Genet (1998) 33: 412–419

ORIGINAL PAPER

Bonnie K. Baxter · Elizabeth A. Craig

Isolation of *UBP3*, encoding a de-ubiquitinating enzyme, as a multicopy suppressor of a heat-shock mutant strain of *S. cerevisiae*

Received: 9 January / 11 March 1998

Abstract Yeast strains lacking functional copies of the two genes *SSA1* and *SSA2*, which encode cytosolic molecular chaperones, are temperature-sensitive. In this report, we describe the isolation of a high-copy suppressor of this temperature sensitivity, *UBP3*, which encodes a de-ubiquitinating enzyme. We show that *ubp3* mutant yeast strains have a mild slow-growth phenotype and accumulate ubiquitin-protein conjugates. We propose a model in which Ubp3p acts in vivo to reverse the ubiquitination of substrate proteins, allowing temporarily misfolded proteins an opportunity to fold correctly.

Key words *UBP3* · *ssa1 ssa2* · Hsp70 · Ubiquitin

Introduction

The 70-kilodalton heat-shock proteins, or Hsp70s, are among the most highly conserved proteins in biology; Hsp70s are found in all organisms studied, from bacteria to humans. In the budding yeast *Saccharomyces cerevisiae*, 14 Hsp70s have been identified, which can be grouped into subfamilies according to localization and functional complementation. The *SSA* subfamily of Hsp70s is essential; at least one of the four *SSA* genes must be expressed at high levels for cells to remain viable (Werner-Washburne et al. 1987). Roles have been identified for the Ssa proteins in such diverse cellular functions as protein folding (Levy et al. 1995), protein translocation from the cytosol into organelles (Chirico et al. 1988; Deshaies et al. 1988; Becker et al. 1996), thermotolerance (Sanchez et al. 1993), and autoregulation of the heat-shock response (Stone and Craig 1990); it is not yet clear which of these functions contribute to the absolute requirement of cells for Ssa. The four gene family members, although very similar in coding sequence, are regulated differently. *SSA1* and *SSA2* are normally expressed at high levels during steady state logarithmic growth; *SSA1* expression is further induced by heat shock. Expression of *SSA3* or *SSA4* requires induction by heat shock or a variety of other stressors (Werner-Washburne et al. 1989). Strains lacking functional copies of *SSA1* and *SSA2* survive by inducing expression of *SSA3* and *SSA4*, but are nonetheless slow-growing and temperature-sensitive (Craig and Jacobsen 1984; Werner-Washburne et al. 1987). In the present study, we report the isolation and characterization of a high-copy suppressor, *UBP3*, of *ssa1 ssa2* strains.

UBP3 encodes a de-ubiquitinating enzyme, or *ub*iquitin processing *p*rotease (Baker et al. 1992). *UBP3* was isolated in a screen designed to identify yeast genes which, when co-expressed in *Escherichia coli* with a gene encoding a protein fusion of ubiquitin to β-galactosidase, led to the specific removal of the ubiquitin moiety. This screen mimics a situation which occurs in vivo in eucaryotic cells, in which polyprotein fusions constitute the source of ubiquitin. For example, ubiquitin is encoded by a family of four genes in yeast. *UBI1*, *UBI2*, and *UBI3* each encode a fusion of ubiquitin to a ribosomal protein; *UBI4* encodes a pentameric ubiquitin repeat. Ubp activity is required to generate functional ubiquitin from each of these precursors.

Other cellular roles exist for de-ubiquitinating enzymes in addition to the generation of free ubiquitin from its precursors (for a recent review see Hochstrasser 1996). When a proteolytic substrate has been multi-ubiquitinated and then destroyed by the proteasome, ubiquitin is recycled. Ubp activity is required to free ubiquitin from substrate fragments; a role which has been suggested for Doa4p/Ubp4p in yeast (Papa and Hochstrasser 1993). Ubp activity is also needed for the trimming of isopeptide-linked polyubiquitin chains which are unanchored to a substrate, an activity of Ubp14p in yeast and isopeptidase T in human cells (Amerik et al. 1997). Finally, there are data to suggest that cells have Ubp activity which acts to de-ubiquitinate substrate proteins and thus prevent their proteo-

B. K. Baxter · E. A. Craig (✉)
Department of Biomolecular Chemistry, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA
e-mail: ecraig@facstaff.wisc.edu
Fax: +1-608-262 5253

Communicated by S. W. Liebman

Table 1 Yeast strains used in this study

Description	Name	Genotype	Source
ssa1 ssa2	BB129A	*MAT**a** ssa1-3::HIS3 ssa2-2::URA3*[a]	This study
ubp3	BB178	*MATα ubp3::LEU2*[a]	This study
	BB179	*MAT**a**/α SSA1/ssa1-3::HIS3 SSA2/ssa2-2::URA3 ubp3::LEU2/UBP3*[a]	This study
ssa2 ubp3	BB180	*MATα ssa2-2::URA3 ubp3::LEU2*[a]	This study
ssa1 ubp3	BB181	*MATα ssa1-3::HIS3 ubp3::LEU2*[a]	This study
ssa2	BB182	*MAT**a** ssa2-2::URA3*[a]	This study
ssa1	BB183	*MAT**a** ssa1-3::HIS3*[a]	This study
WT	BB185	*MAT**a*** (wild-type at *SSA1 SSA2 UBP3*)[a]	This study
ssa1 ssa2	BB189	*MAT**a** ssa1-3::HIS3 ssa2-2::URA3*[a]	This study
ssa1 ssa2	BB191	*MATα ssa1-3::HIS3 ssa2-2::URA3*[a]	This study
ubp3	BB193	*MATα ubp3::LEU2*[a]	This study
WT	DS10	*MAT**a*** (wild-type at *SSA1 SSA2 UBP3)*[a]	David Stone
WT	DS13	*MATα* (wild-type at *SSA1 SSA2 UBP3)*[a]	David Stone
ssa1 ssa2	SL314-B1	*MATα ssa1::HIS3 ssa2::LEU2*[b]	Susan Lindquist
sir4Δ	CFY374	*MAT**a** sir4Δ::LEU2*[b] *[pJR368]*[c]	Catherine Fox
ssa1 ssa2 sir4Δ	BB370	*MAT**a** sir4Δ::LEU2 ssa1::HIS3 ssa2::LEU2*[b]	This study
ssa1 ssa2	BB371	*MATα ssa1::HIS3 ssa2::LEU2*[b]	This study

[a] S288C-derived strains. Markers (homozygous in diploid strains) are *his3-11,15 leu2-3,112 lys1 lys2 trp1-Δ1 ura3-52*
[b] W303-derived strains. Markers are *his3-11,15 leu2-3,112 lys2 trp1-1 ura3-1*
[c] pJR368: wild-type *SIR4* cloned into YCp50 (CEN, *URA3*)

lytic destruction (see for example Hershko et al. 1984; Hough and Rechsteiner 1986). This activity has been postulated for the *Drosophila* Fat facets (FAF) protein (Huang et al. 1995). It has been suggested that the role of such an activity would be to serve as a proofreader for the system in order to prevent the destruction of proteins which had been mistakenly ubiquitinated. The yeast genome encodes 17 proteins known, or believed, to have Ubp activity (Hochstrasser 1996). The size of this protein family suggests that its members will have distinct cellular roles and specificities. With the exceptions of Doa4p/Ubp4p and Ubp14p, however, the nature of these in vivo roles has yet to be clearly established.

Data in this report demonstrate that Ubp3p has an important role in vivo. The growth of *ssa1 ssa2* strains is improved by *UBP3* over-expression and impaired by *UBP3* disruption. In a wild-type background, disruption of *UBP3* causes a mild growth impairment as well as dramatic alterations in the profile of ubiquitin-conjugated substrate proteins. Models for the cellular function of Ubp3p and its ability to confer improved growth to *ssa1 ssa2* strains are presented.

Materials and methods

Strains, media, and genetic techniques. Escherichia coli cells were grown in LB (0.5% yeast extract, 1% tryptone, 1% NaCl) supplemented with 100 μg of ampicillin per ml as necessary for plasmid selection. *E. coli* was transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions or by a calcium chloride-based protocol (Maniatis et al. 1982). Restriction enzymes and buffers were from New England Biolabs (Beverly, Mass.), Promega (Madison, Wis.), or Boehringer Mannheim (Indianapolis, Ind.) and were used according to the manufacturer's instructions. The yeast genomic library employed for the selection of high-copy suppressors was constructed in the cloning vector YEp351 (Hill et al. 1986). The strain used for library construction was an S288C-derived *ssa1 ssa2* strain carrying the extragenic dominant suppressor mutation *EXA1-1* (Nelson et al. 1992). The library was designed to enable the cloning of *EXA1-1*, but also provided an opportunity to isolate high-copy suppressors. *UBP3* is not allelic to *EXA1-1* (data not shown) and an independent plasmid carrying wild-type *UBP3* confers suppression (see Fig. 2A), indicating that *UBP3* is a true high-copy suppressor. Yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or in selective media (0.67% yeast nitrogen base without amino acids, 2% dextrose, supplemented with required amino acids as necessary). Yeast were transformed by electroporation using the Gene Pulser apparatus (Bio-Rad laboratories, Hercules, Calif.) or according to a modified lithium acetate protocol (Gietz et al. 1995). Matings were done on YPD, with selection of zygotes by micromanipulation. Sporulation plates contained 1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, and amino-acid supplements. Growth tests were routinely conducted by growing cells overnight at the permissive temperature and then diluting into sterile water as a series of 10-fold serial dilutions. Aliquots of each dilution were spotted to agar plates which were incubated at the temperatures indicated. For each analysis, multiple independent transformants were tested in parallel; representative results are presented. For the genomic disruption of *UBP3*, a 2-kb *Hin*dIII fragment from the *UBP3* coding region was subcloned into cloning vector pIC20H. The resulting construct was digested with *Bgl*II and ligated to a 2.9-kb *Bgl*II fragment from YEp13 carrying *LEU2*. The disruption plasmid was digested with *Hin*dIII and transformed into wild-type strain DS13 as a one-step disruption construct to create BB178. Integration was confirmed by Southern blotting. BB178 was mated to *ssa1 ssa2* strain BB129A to create the diploid strain BB179, heterozygous at all three loci. This diploid strain is the parent of the haploid strains shown in lanes 2–9 of Fig. 3. The *ssa1 ssa2* strain BB129A, which was used in the initial phases of this study, was later found to be unable to grow on non-fermentable carbon sources, indicating that it had acquired a secondary mutation which interfered with mitochondrial function and thus rendered it petite. To ensure that this mutation was not confounding the results, growth experiments were repeated with two newly obtained, non-petite *ssa1 ssa2* strains, BB189 and BB191. No differences were discovered in the ability of any plasmid tested to confer improved or impaired growth. In the latter phases of this work, experiments were routinely conducted in duplicate using both BB189 and BB191. To test whether *SIR4* is required for suppression by *UBP3*, *sir4Δ* strain CFY374 (which carries wild-type *SIR4* on a centromeric plasmid) was crossed with *ssa1 ssa2* strain SL314-B1. Diploids were isolated by double selection and sporulated, and haploid progeny were obtained by micromanipulation. Haploid strains BB370 (*ssa1 ssa2 sir4*) and BB371 (*ssa1 ssa2*) were obtained from

the same ascus, both carrying the centromeric *SIR4* plasmid. This plasmid was cured by counterselection on 5-FOA, and the *sir4* genotype of BB370 was confirmed by its inability to mate with tester strains of either genotype. We have discovered that the two primary yeast strain backgrounds in use in our laboratory sometimes differ in regard to phenotypes associated with heat-shock gene mutations (for example, see Baxter et al. 1996). The majority of this work was conducted in strains derived from S288C (see Table 1, first 13 strains). To ensure that the phenomena described here are not dependent on strain-background differences, many of these experiments were repeated in strains derived from W303 (e.g., SL314-B1, Table 1). These include the suppression analysis of *UBP3* (Fig. 1A), the growth phenotype of a *ubp3* deletion (see Fig. 3A), and the Northern analysis of the ubiquitin system gene expression in wild-type vs *ssa1 ssa2* mutant cells (see Fig. 4). All results were essentially the same in the two backgrounds.

Northern analysis. Cells were grown to mid-log phase at the temperature indicated and harvested by centrifugation. Total cellular RNA was isolated by the heat-freeze extraction method as previously described (Schmitt et al. 1990) and quantitated spectrophotometrically. RNA fractions were separated on an agarose/5.5% formaldehyde gel and transferred to a GeneScreen membrane (DuPont Co., Boston, Mass.) by capillary transfer. Probes were labeled by random priming and hybridizations were carried out at 42 °C overnight in hybridization buffer (50% formamide, 10% dextran, 5×Denhardt's, 1% SDS, 2.5 mM sodium pyrophosphate, 50 mM Tris pH 7.5, 1 M NaCl). The results were visualized by autoradiography and quantitated by densitometric scanning. For re-probing, the membrane was first stripped by repeated washing with a boiling solution of 0.1×SSC and 0.1% SDS, allowed to air dry, and incubated at 42 °C with hybridization buffer for at least 6 h before the new probe was added.

Immunoblot analysis. For immunoblot analysis, yeast cells were lysed by vortexing with glass beads in breaking buffer (2% Triton-X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), boiled in SDS sample buffer (Bollag and Edelstein 1991), and clarified by centrifugation. Lysates were separated by SDS-PAGE, stained with Coomassie brilliant blue, and quantitated by densitometric scanning to normalize loadings. Equivalent loadings were then separated by SDS-PAGE and transferred to nitrocellulose (Hybond-C, Amersham Corp., Arlington Heights, Ill.). For detection of ubiquitin, nitrocellulose membranes were routinely autoclaved in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) for 10 min after transfer. Membranes were then washed twice with water, once with TBST (0.5 M NaCl, 20 mM Tris pH 7.5, 0.1% Tween 20), and once with HST (1 M NaCl, 20 mM Tris pH 7.5, 0.5% Tween 20) before a 60-min incubation with primary antibody (a generous gift of Steven VanNocker and Richard Vierstra, University of Wisconsin) in HST. Membranes were then washed twice with HST and twice with TBST, incubated for 30 min with secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Amersham, diluted 1:40 000 in TBST), and washed four times with TBST. The results were visualized by ECL (Amersham).

Results

Isolation of *UBP3* as a multicopy suppressor of *ssa1 ssa2*

A high-copy plasmid was isolated from a yeast genomic library which confers improved high-temperature growth to *ssa1 ssa2* strains; an example of the growth improvement is shown in Fig. 1A. Suppression was plasmid-dependent: transformants which lost the plasmid during non-selective growth no longer showed improved growth at high temperature (data not shown). The suppressor conferred a marked

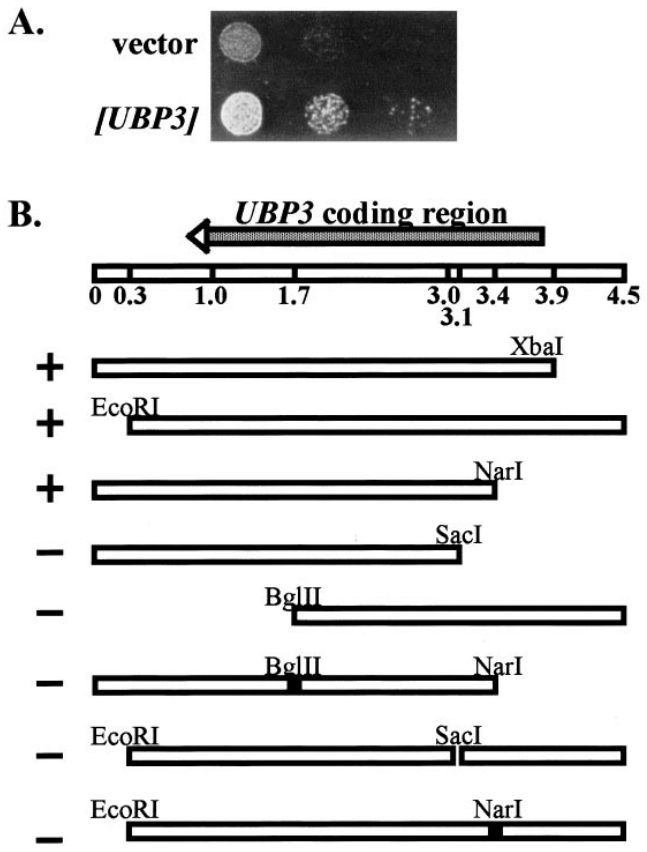

Fig. 1A, B *UBP3* is a multicopy suppressor of *ssa1 ssa2*. **A** *ssa1 ssa2* mutant strain BB189 was transformed with pRS424 ("vector") or pRS-UBP3 ("*[UBP3]*"). Transformants were selected at the permissive temperature (23 °C) and equal numbers of cells were spotted onto a selective medium and incubated for 3 days at 35 °C. The spots shown are 10-fold serial dilutions from left to right. **B** the yeast insert of suppressor plasmid p6 contains the coding region of *UBP3*. The 4.5-kb insert of plasmid p6 was subcloned as diagrammed and fragments were tested for the ability to give the suppression shown in panel **A**. The results are shown as a "+" or "–" to the left of each fragment. The last three subclones were cut at the restriction site shown and either filled in with Klenow fragment (*filled-in boxes*) or excised with T4 DNA polymerase (*gap*) and re-ligated. Either treatment causes a frameshift in the *UBP3* coding region

improvement of growth at temperatures ranging up to 35 °C, although transformants were not restored to wild-type growth rates and remained unable to form colonies at 37 °C. The yeast insert contained on the suppressor plasmid is diagrammed in Fig. 1B. A large open reading frame (ORF) within this insert encodes Ubp3p, a de-ubiquitinating enzyme (Baker et al. 1992). Disruption of the *UBP3* reading frame at any of three internal restriction sites eliminated suppression (Fig. 1B, last three constructs), indicating that *UBP3* is responsible for suppression. A low-copy (centromeric) *UBP3* construct conferred no growth improvement, suggesting that high-level expression of *UBP3* is necessary for suppression (data not shown).

Surprisingly, a multicopy subclone carrying the *UBP3* ORF with a 5′ truncation at the *Nar*I site was able to suppress (Fig. 1B); Northern analysis revealed that this subclone expresses a truncated *UBP3* mRNA at a similar abun-

dance to the full-length mRNA expressed from the original clone (data not shown). Translation beginning at the first methionine after the *Nar*I site would result in a Ubp3p protein lacking its first 123 amino acids (out of 912 total for the longest ORF in the full-length clone). It is interesting to note in this context that another de-ubiquitinating enzyme from yeast, Ubp2p, can be severely truncated at its amino terminus and still retain function (Baker et al. 1992).

Effect of other ubiquitin-system components on the growth of *ssa1 ssa2* strains

There are many distinct types of in vivo roles for proteins with Ubp activity, ranging from the processing of ubiquitin polyprotein precursors to the regeneration of ubiquitin after substrate proteolysis. As a first step toward elucidating the in vivo function of Ubp3p and the mechanism of its suppression of the growth defect of an Hsp70 mutant strain, genes encoding other components of the ubiquitin-dependent proteolytic pathway were tested for the ability to improve the growth of *ssa1 ssa2* strains at elevated temperatures. Multicopy plasmid constructs expressing *UBP1*, *UBP2*, or *UBP3* under control of the highly expressed *ADH1* promoter were transformed into *ssa1 ssa2* strains and tested for suppression. *UBP1* conferred some growth improvement, although less than that conferred by *UBP3* (Fig. 2A). Although the growth improvement conferred by *UBP1* was slight, the effect was reproducible in several independent transformants of the *ssa1 ssa2* strain shown in Fig. 2A, as well as in a separate, isogenic *ssa1 ssa2* strain. *UBP2* transformants showed a slight improvement of growth, but efforts to repeat this result in another *ssa1 ssa2* strain failed to show any effect. When a multicopy vector carrying *DOA4/UBP4* or the dominant negative allele $DOA4^{Ser571}$ was tested, neither *DOA4* nor $DOA4^{Ser571}$ had any consistently detectable effect (Fig. 2B). This finding suggests that Ubp3p has an in vivo function which is distinct from those of Doa4p and Ubp2p, and perhaps from Ubp1p as well.

Two of the ubiquitin-conjugating enzymes in yeast, Ubc4p and Ubc5p, have been implicated in the heat-shock response and are believed to be responsible for the conjugation of ubiquitin to stress-damaged proteins (Seufert et al. 1990). A plausible explanation for suppression by *UBP3* is that misfolded proteins accumulate in an *ssa1 ssa2* strain because of the lack of Ssa1p and Ssa2p, and that Ubp3p facilitates the clearance of misfolded proteins through ubiquitin-mediated proteolysis. In this case, overexpression of *UBC4* or *UBC5* might be expected to confer suppression as well. Transformation of *ssa1 ssa2* strains with *UBC4* (Fig. 2C) or *UBC5* (data not shown) failed to suppress the high-temperature growth defect of these cells. In fact, the presence of *UBC4* on a multicopy plasmid caused a further impairment of growth, particularly at high temperature.

As a second test of the idea that increasing ubiquitin-dependent proteolysis might cause suppression, we transformed an *ssa1 ssa2* strain with a multicopy plasmid carrying the ubiquitin-encoding gene *UBI4*. We reasoned that the extra ubiquitin provided by this construct could increase the activity of the ubiquitin pathway and thus speed the clearance of misfolded proteins. As assayed by immunoblot analysis (Fig. 2D), introduction of this plasmid re-

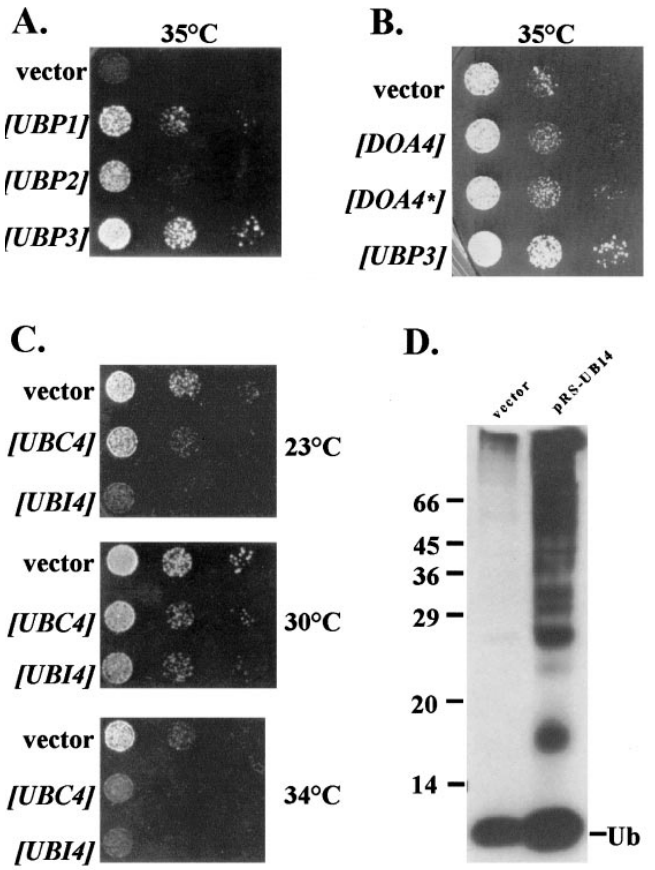

Fig. 2A–D Effect of other ubiquitin-system components on *ssa1 ssa2* mutant strains. **A** *ssa1 ssa2* mutant strain BB189 was transformed with vector YEplac181 alone or carrying the *UBP1*, *UBP2*, or *UBP3* gene under control of the *ADH1* promoter (a gift of Rohan Baker). Transformants were selected at the permissive temperature (23°C) and equal numbers of cells were spotted to a selective medium and incubated at 35°C for 4 days. **B** as in **A**. *ssa1 ssa2* mutant strain BB191 was transformed with the vector plasmid pRS424 alone or carrying a wild-type or dominant negative allele of *DOA4* (a gift of Mark Hochstrasser) *"DOA4": $DOA4^{Ser571}$*. The plate shown was incubated at 35°C for 3 days. As seen in the difference between **A** and **B**, slight variations from experiment to experiment at temperatures close to 35°C strongly affect the growth of *ssa1 ssa2* carrying a vector alone, making it important to compare the growth of any given transformant with a vector-transformed control grown on the same plate. **C** As in panels **A** and **B**, with the vector plasmid pRS424 alone or carrying the *UBC4* or *UBI4* coding region and promoter. BB189 transformants were selected at 23°C, spotted to a selective medium, and incubated at the temperature shown for 3 days. For all of the comparisons in panels **A** through **C**, essentially identical results were obtained with multiple transformants in at least two independent *ssa1 ssa2* strains. The only exceptions were that the mild suppression seen for *UBP2* (panel **A**) was not consistently observed, and that $DOA4^{Ser571}$ conferred some growth improvement to one out of four transformants. **D** protein extracts were prepared from *ssa1 ssa2* strain BB129A carrying vector plasmid pRS424 or pRS-UBI4 and subjected to immunoblot analysis as described in Materials and methods, using an antiserum reactive against ubiquitin. An equal amount of total protein was loaded in each lane

sulted in a dramatic increase in proteins reactive with an antiserum against ubiquitin. Immunoreactive proteins ranged in size from free ubiquitin to greater than 70 kDa, demonstrating that the extra ubiquitin provided by this construct can be processed and covalently conjugated to substrates. Incidentally, this finding shows that the Ubp activity required for processing ubiquitin precursors such as Ubi4p is not limiting in *ssa1 ssa2*, which suggests that suppression via Ubp3p is not operating at this step. Importantly, *UBI4* does not confer improved growth to an *ssa1 ssa2* strain. In fact, strains carrying the *UBI4* construct grew markedly more poorly than those carrying a vector control (Fig. 2C). The finding that over-expression of either *UBC4* or *UBI4* impedes, rather than improves, the growth of *ssa1 ssa2* suggests that *UBP3*-mediated suppression is not operating by simply increasing ubiquitin-dependent proteolysis.

Disruption of *UBP3* causes slow growth and an accumulation of ubiquitin-protein conjugates

To analyze the function of Ubp3p more directly, we constructed a strain in which the genomic copy of *UBP3* is disrupted by the insertion of the *LEU2* marker at the internal *Bgl*II site; subcloning analysis had demonstrated that disruption of *UBP3* at this site eliminates function, as assessed by the ability to suppress *ssa1 ssa2* (Fig. 1B). Disruption of *UBP3* in an otherwise wild-type strain caused a mild slow-growth phenotype, particularly at elevated temperatures; normal growth was restored by a plasmid carrying *UBP3* (Fig. 3A). These findings demonstrate that *UBP3*, while not essential, is necessary for optimal growth.

To assess the genetic interactions of *SSA1*, *SSA2* and *UBP3*, a *ubp3* mutant strain was crossed with an *ssa1 ssa2* mutant to form the diploid strain BB179, heterozygous at all three loci. Sporulation of this diploid yielded pinpoint colonies at a frequency of about 1:8; however, these colonies remained barely discernible even after extended incubation (for example, see Fig. 3B). In cases where the genotype could be determined either through the testing of auxotrophic markers after propagation of the colony (successful less than half of the time) or by deduction from the gen-

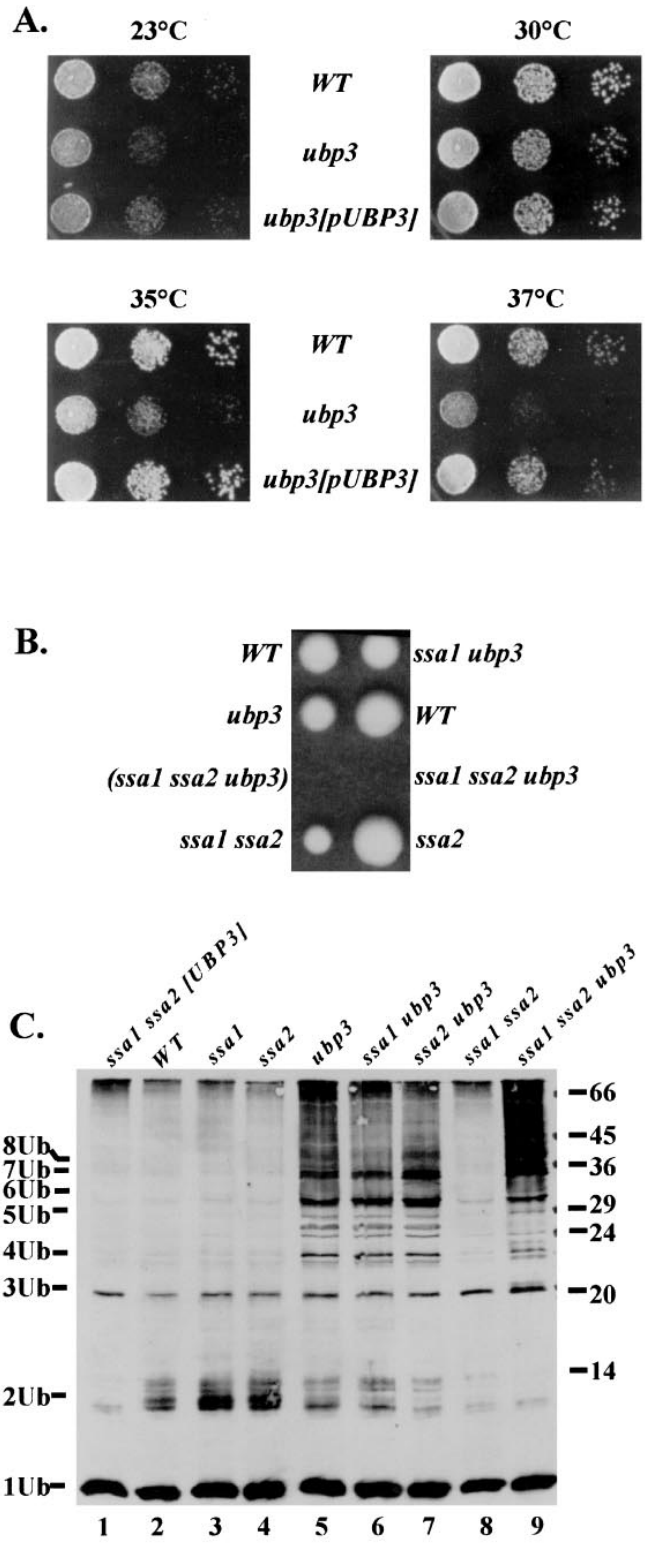

Fig. 3A–C Disruption of *UBP3*. **A** wild-type strain BB185 and the *ubp3* disruption strain BB193 ("*WT*" and "*ubp3*") were transformed with vector plasmid pRS424 (no plasmid indicated) or the multicopy plasmid pRS-UBP3 ("*[pUBP3]*"). Transformants were spotted in equal numbers to a selective medium and incubated at the temperatures shown for 2 days. Restoration of wild-type growth was also seen with a low-copy (centromeric) plasmid carrying *UBP3* (data not shown). **B** progeny of diploid strain BB179. A tetrad dissection plate is shown, with the four progeny of each tetrad aligned vertically. Genotypes, as determined by subsequent testing of auxotrophic markers, are indicated (parentheses indicate a strain that could not be propagated; its genotype is inferred from those of the other members of the tetrad). Even after extended incubation, *ssa1 ssa2 ubp3* triple mutants formed pinpoint colonies too small to be visible in this photograph. **C** extracts were prepared from *ssa1 ssa2* mutant strain BB129 A carrying pRS-UBP3 (*lane 1*) and from haploid progeny of diploid strain BB179, heterozygous for disruption of *SSA1*, *SSA2*, and *UBP3* (*lanes 2–9*). Strains in *lanes 2–9* are as follows: BB185, 183, 182, 193, 181, 180, 189, and 186. The genes which are disrupted in each strain are shown at the top. Proteins were separated on a 15% polyacrylamide gel, transferred to nitrocellulose, and probed with a polyclonal antiserum against ubiquitin. Migration of a set of isopeptide-linked ubiquitin chains and of molecular weight markers is indicated at the left and right, respectively; numbers at the right refer to the size of each marker protein in kDa

otype of other members of the same tetrad, these pinpoint colonies represented triple *ssa1 ssa2 ubp3* mutants; no triple mutants were obtained which formed healthy colonies on the dissection plate. As seen in Fig. 3B, the severe growth impairment of *ssa1 ssa2 ubp3* triple mutants was much more dramatic than that exhibited by either *ssa1 ssa2* double mutants or *ubp3* single mutants. Growth of *ssa1 ssa2* strains is thus both impaired by disruption of *UBP3* and improved by *UBP3* over-expression, suggesting a physiologically relevant genetic interaction between *SSA* and *UBP3*.

In an effort to assess the nature of the defect seen in strains lacking a functional copy of *UBP3*, we performed an immunoblot analysis with polyclonal antisera raised against ubiquitin. Analysis of cell extracts from a representative set of strains revealed that levels of free ubiquitin did not vary measurably (Fig. 3C). The fact that free ubiquitin does not seem to be deficient in strains lacking *UBP3* again suggests that Ubp3p is not required for the generation of ubiquitin from its polypeptide precursors. Further, there were no discernible differences between the wild-type strain and strains lacking functional copies of *SSA1* and/or *SSA2*, with or without a plasmid bearing *UBP3* (lanes 1, 2, 3, 4, and 8; the apparent differences in the range of 10–15 kDa were not consistently observed.) Strikingly, however, all of the strains lacking a functional copy of *UBP3* showed an accumulation of immunoreactive proteins in the high-molecular-weight range (lanes 5, 6, 7, and 9). The majority of these immunoreactive proteins did not co-migrate with isopeptide-linked ubiquitin chains used as markers, whose migration is indicated at the left of the figure. The abundance of high-molecular-weight immunoreactive bands indicates an elevated steady state level of ubiquitin-protein conjugates in *ubp3* mutant strains.

Transcription of ubiquitin-system genes is altered in *ssa1 ssa2* mutant strains

It has been shown previously that *ssa1 ssa2* mutant strains have elevated levels of expression of heat-shock genes under steady state growth conditions (Craig and Jacobsen 1984; Werner-Washburne et al. 1987; Stone and Craig 1990) and that some ubiquitin system genes are heat-responsive (e.g., *UBC4* and *UBC5*, Seufert et al. 1990; and *UBI4*, Finley et al. 1987). We reasoned that our observation that over-expression of *UBP3* can improve the growth of *ssa1 ssa2* strains might reflect an altered regulation of ubiquitin-system genes in this background. For example, over-expression of *UBI4* and *UBC4* could lead to the conjugation of ubiquitin to improper substrates, a conjugation which perhaps could be reversed by an over-produced Ubp3p. To examine this question, we studied the expression of ubiquitin-system genes in an *ssa1 ssa2* mutant background by Northern analysis.

UBI4, which encodes polyubiquitin, is responsible for providing ubiquitin in vivo under conditions of stress. When cells are subjected to heat shock or exposed to various other stressors, expression of the other three ubiquitin genes, *UBI1–3*, decreases while expression of *UBI4* is

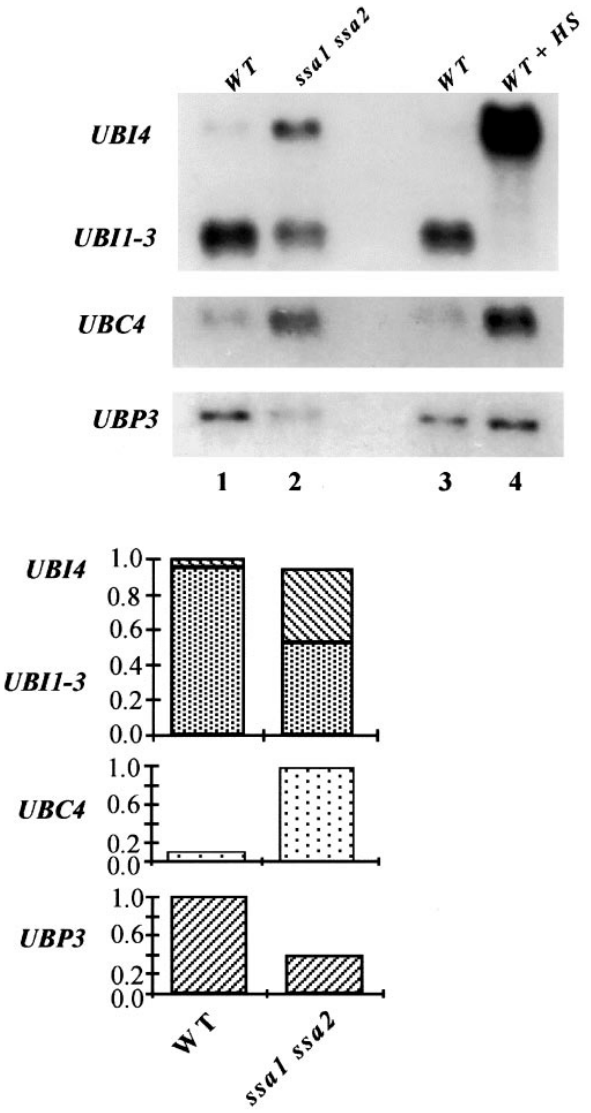

Fig. 4 Transcription of ubiquitin system genes in an *ssa1 ssa2* mutant strain. Total RNA was prepared from wild-type strain DS10 and *ssa1 ssa2* mutant strain BB129 A grown to mid-log phase at 30°C in YPD (*lanes 1 and 2*). For comparison, a culture of DS10 was grown at 23°C and shifted to 39°C for 30 min (*lanes 3 and 4*). RNA was separated in 1% (*UBI1-3*, *UBI4* and *UBP3*) or 1.5% (*UBC4*) agarose/formaldehyde gels, transferred to nylon membranes, and probed with random-primed DNA fragments from the coding regions indicated at the left (for *UBI1-3* and *UBI4*, which share almost complete identity in the ubiquitin-encoding regions, a single *UBI4*-derived probe was used). Bands were detected by autoradiography and quantitated by densitometric scanning. Quantitation of *lanes 1 and 2*, normalized to *ACT1* probings of the same membranes, is shown at the bottom of the figure. For the ubiquitin mRNA quantitation, *UBI4* message is represented by *hatched boxes* and *UBI1-3* message by *stippled boxes*

induced (Finley et al. 1987, and Fig. 4A, lanes 3 and 4). We found this situation mimicked in an *ssa1 ssa2* mutant strain. In this background, expression of *UBI4* mRNA is induced under normal growth conditions, while expression of *UBI1*, *UBI2*, and *UBI3* is decreased when compared to a wild-type strain (Fig. 4A, lanes 1 and 2). When the results were quantitated by densitometric scanning, however, the overall level of ubiquitin message was shown to be

quite similar in the two strains. This is consistent with the results of our anti-ubiquitin immunoblot results (Fig. 3C), in which we did not detect any difference in the levels of either free or conjugated ubiquitin between wild-type and *ssa1 ssa2* mutant strains.

As described above, *UBC4* and *UBC5* encode ubiquitin-conjugating enzymes which are thought to be responsible for most conjugation of ubiquitin to substrate proteins under conditions of stress (Seufert et al. 1990). *UBC5* mRNA levels were not elevated in an *ssa1 ssa2* mutant strain when compared to wild-type, and did not show heat-shock induction in these experiments (data not shown). *UBC4*, however, did show measurable heat-shock induction (Fig. 4B, lanes 3 and 4) and was also somewhat elevated in an *ssa1 ssa2* mutant strain when compared to a wild-type control (Fig. 4B, lanes 1 and 2). Thus, there appears to be at least one ubiquitin-system component, Ubc4p, which is over-expressed in an *ssa1 ssa2* background. In its original characterization, *UBP3* was reported to be a heat-shock gene (Baker et al. 1992). In our hands, *UBP3* did not show dramatic heat-shock induction (Fig. 4C, lanes 3 and 4). Unexpectedly, *UBP3* expression was slightly lower in an *ssa1 ssa2* mutant strain than in a wild-type control (Fig. 4C, lanes 1 and 2). The mechanisms which might effect this alteration in expression are unclear.

Suppression by *UBP3* does not require Sir4p

Recently, the observation has been made that Ubp3p interacts in vitro with Sir4p [silent *i*nformation *r*egulator 4, (Moazed and Johnson 1996)]. Sir4p functions as part of a multiprotein complex to bring about the transcriptional silencing of telomeres and of the silent mating-type loci *HML* and *HMR* in yeast (Rine and Herskowitz 1987; Aparicio et al. 1991; Moazed and Johnson 1996). Cells carrying a deletion of *SIR4* show wild-type growth but are completely deficient in telomeric and *HML/HMR* silencing (Rine and Herskowitz 1987; Aparicio et al. 1991). Interestingly, cells lacking a functional copy of *UBP3* show enhanced silencing of marker genes inserted either close to telomeres or at the silent *HML* mating-type locus, suggesting that Ubp3p may function in vivo to oppose the function of the silencing complex (Moazed and Johnson 1996).

To explore whether transcriptional silencing via the Sir complex is relevant to *UBP3*-mediated suppression of *ssa1 ssa2* cells, we constructed a diploid strain heterozygous for loss-of-function alleles of *SSA1*, *SSA2*, and *SIR4* (see Materials and methods); *ssa1 ssa2* and *ssa1 ssa2 sir4* haploid progeny were obtained and transformed with a multicopy vector carrying *UBP3*. Over-expression of *UBP3* confers suppression regardless of the presence or absence of a functional copy of *SIR4*. This finding clearly demonstrates that interaction with Sir4p is not necessary for the in vivo role of Ubp3p which is relevant to suppression.

Discussion

The data presented here offer evidence that the protein product of *UBP3* has a functional role in vivo which is particularly important for the growth of cells lacking functional copies of genes encoding the molecular chaperones Ssa1p and Ssa2p. Over-expression of *UBP3* in this genetic background leads to a marked improvement of growth at elevated temperatures. Furthermore, disruption of *UBP3* in the context of *ssa1 ssa2* deletions results in strains that grow extremely poorly, even at the permissive temperature. Disruption of *UBP3* in an otherwise wild-type background leads to a mild slow-growth phenotype and a dramatic accumulation of ubiquitin-protein conjugates.

When we tested other de-ubiquitinating enzymes for the ability to suppress the growth defect of an *ssa1 ssa2* mutant, none of the genes we tested was able to confer the degree of growth improvement conferred by *UBP3*. Two possible explanations for these findings are: (1) *UBP3* is expressed at higher levels than the other *UBP* genes tested, or (2) the role of Ubp3p which is important for suppression cannot be completely filled by Ubp1p, Ubp2p, or Doa4p/Ubp4p. Given that *UBP1*, *UBP2*, and *UBP3* were all expressed from the high-level *ADH1* promoter in the growth experiments shown in Fig. 2A, and that the *DOA4* plasmid constructs used here have been shown to have significant effects on ubiquitin-mediated degradation of the short-lived protein MATα2 (Papa and Hochstrasser 1993), we favor the latter explanation.

Further suppression tests of ubiquitin-system genes gave an intriguing result: two genes which encode enzymes known to facilitate ubiquitin-dependent proteolysis, *UBI4* and *UBC4*, actually further impaired the growth of an *ssa1 ssa2* mutant strain. The simplest interpretation of this result is that Ubp3p acts in opposition to Ubi4p and Ubc4p, to slow ubiquitin-mediated proteolysis of substrate proteins. This would be the case if Ubp3p were acting as a proofreading enzyme for the ubiquitin system, removing ubiquitin from substrates before they could be destroyed by the proteasome. Such a role for Ubp3p would account for the accumulation of ubiquitin-protein conjugates in *ubp3* disruption strains, assuming that such conjugates accumulate faster than they can be destroyed. The existence of a ubiquitin "proofreading" activity in reticulocyte extracts was noted in early biochemical analyses of the ubiquitin system: ubiquitin-lysozyme conjugates, upon incubation with reticulocyte lysate fractions in the absence of ATP, are processed to release both free ubiquitin and free lysozyme (Hershko et al. 1984; Hough and Rechsteiner 1986). More recently, the Fat facets protein (FAF) of *Drosophila* has been proposed to have ubiquitin proofreading activity, preventing the degradation of a particular regulatory protein (or proteins) important in eye development (Huang et al. 1995). While FAF is thought to be important for a specific protein or small set of proteins, the accumulation of a large number of immunoreactive proteins in *ubp3* disruption strains as detected by an anti-ubiquitin immunoblot suggests a broad range of specificity for Ubp3p.

The implications of these findings for understanding the physiology of *ssa1 ssa2* mutant strains are complex. The Ssa protein family has been implicated in a variety of functions, including protein folding and the translocation of proteins into organelles. A deficiency in Ssa might be expected to lead to an accumulation of misfolded or mislocalized proteins in the cytosol. An increase in ubiquitin-mediated proteolysis should facilitate clearance of these abnormal proteins. Indeed, we have observed that at least one ubiquitin-system component, Ubc4p, is up-regulated in an *ssa1 ssa2* background. However, we have shown that the introduction of multicopy plasmids carrying either *UBC4* or *UBI4*, which encode components of the ubiquitin system known to be involved in the clearance of abnormal or damaged proteins, leads to impaired, rather than improved, growth of *ssa1 ssa2*.

A possible explanation for this somewhat puzzling result is that the ubiquitin system is destroying something in *ssa1 ssa2* mutant cells which is necessary for optimal growth. Over-expression of *UBP3* could rescue this putative substrate or substrates through de-ubiquitination, while further over-expression of *UBI4* or *UBC4* might well exacerbate the problem. This putative substrate(s) could be a molecule(s) which is (are) normally short-lived and subject to ubiquitin-mediated degradation, as has been postulated in the case of FAF discussed above. Alternatively, it is possible that the absence of the molecular chaperones Ssa1p and Ssa2p results in a number of cellular proteins which take an abnormally long time to fold, assemble, or localize correctly, and thus become targets of ubiquitination. Destruction of these abnormal proteins would be adaptive if they were irreversibly misfolded and thus useless. However, if some or all of these proteins had the potential to fold and function correctly given sufficient time, they would need to be protected from ubiquitin-dependent degradation in the interim. Over-expression of *UBP3* could accomplish this task.

Acknowledgements We thank Mark Hochstrasser, Rohan Baker, Steven VanNocker, and Richard Vierstra for reagents, advice, and many helpful discussions, and Julie Davis for critical reading of the manuscript. John Halladay and other members of the Craig laboratory provided a stimulating scientific environment and many helpful thoughts and critiques. The *ssa1 ssa2* strains in the W303 strain background were a gift of Susan Lindquist; the *sir4* strain CFY374 was a gift of Catherine Fox. Marisela De Leon-Viera and Elizabeth Krainer were instrumental in the early subcloning analysis of suppressor plasmid p6. This work was supported by Graduate Research Fellowships to B. K. B. from the Cremer Basic Science Fellowship Fund, the National Science Foundation and the Wisconsin Alumni Research Foundation, and by NIH grant #GM31107 to E. A. C.

References

Amerik AY, Swaminathan S, Krantz BA, Wilkinson KD, Hochstrasser M (1997) In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. EMBO J 16: 4826–4838

Aparicio OM, Billington BL, Gottschling DE (1991) Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. Cell 66: 1279–1287

Baker R, Tobias J, Varshavsky A (1992) Ubiquitin-specific proteases of *Saccharomyces cerevisiae*. J Biol Chem 267: 23364–23375

Baxter BK, James P, Evans T, Craig EA (1996) *SSI1* encodes a novel Hsp70 of the *Saccharomyces cerevisiae* endoplasmic reticulum. Mol Cell Biol 16: 6444–6456

Becker J, Yan W, Craig EA (1996) Functional interaction of cytosolic Hsp70 and DnaJ-related protein, Ydj1p, in protein tranlocation in vivo. Mol Cell Biol 16: 4378–4386

Bollag DM, Edelstein SJ (1991) Protein methods. Wildy-Liss, Inc., New York

Chirico W, Waters MG, Blobel G (1988) 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 332: 805–810

Craig EA, Jacobsen K (1984) Mutations of the heat-inducible 70 kilodalton genes of yeast confer temperature-sensitive growth. Cell 38: 841–849

Deshaies R, Koch B, Werner-Washburne M, Craig E, Schekman R (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature 332: 800–805

Finley D, Ozkaynak E, Varshavsky A (1987) The yeast polyubiquitin gene is essential for resistance to high temperature, starvation and other stresses. Cell 49: 1035–1046

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11: 355–360

Hershko A, Leshinsky E, Ganoth D, Heller H (1984) ATP-dependent degradation of ubiquitin-protein conjugates. Proc Natl Acad Sci USA 81: 1619–1623

Hill JE, Myers AM, Koerner TJ, Tzagoloff A (1986) Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast 2: 163–167

Hochstrasser M (1996) Ubiquitin-dependent protein degradation. Annu Rev Genet 30: 405–439

Hough R, Rechsteiner M (1986) Ubiquitin-lysozyme conjugates. J Biol Chem 261: 2391–2399

Huang Y, Baker RT, Fischer-Vize JA (1995) Control of cell fate by a deubiquitinating enzyme encoded by the *fat facets* gene. Science 270: 1828–1831

Levy E, McCarty J, Bukau B, Chirico W (1995) Conserved ATPase and luciferase refolding activities between bacteria and yeast Hsp70 chaperones and modulators. FEBS Lett 368: 435–440

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Moazed D, Johnson AD (1996) A deubiquitinating enzyme interacts with SIR4 and regulates silencing in *S. cerevisiae*. Cell 86: 667–677

Nelson RJ, Heschl M, Craig EA (1992) Isolation and characterization of extragenic suppressors of mutations in the *SSA* hsp70 genes of *Saccharomyces cerevisiae*. Genetics 131: 277–285

Papa FR, Hochstrasser M (1993) The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human *tre-2* oncogene. Nature 366: 313–319

Rine J, Herskowitz I (1987) Four genes responsible for a position effect on expression from HML and HMR in *Saccharomyces cerevisiae*. Genetics 116: 9–22

Sanchez Y, Parells DA, Taulien J, Vogel JL, Craig EA, Lindquist S (1993) Genetic evidence for a functional relationship between Hsp104 and Hsp70. J Bacterial 175: 6484–6491

Schmitt ME, Brown TA, Trumpower BL (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res 18: 3091–3092

Seufert W, McGrath JP, Jentsch S (1990) *UBC1* encodes a novel member of an essential subfamily of yeast ubiquitin-conjugating enzymes involved in protein degradation. EMBO J 9: 4535–4541

Stone DE, Craig EA (1990) Self-regulation of 70-kilodalton heat shock proteins in *Saccharomyces cerevisiae*. Mol Cell Biol 10: 1622–1632

Werner-Washburne M, Stone DE, Craig EA (1987) Complex interactions among members of an essential subfamily of *hsp70* genes in *Saccharomyces cerevisiae*. Mol Cell Biol 7: 2568–2577

Werner-Washburne M, Becker J, Kosics-Smithers J, Craig EA (1989) Yeast Hsp70 RNA levels vary in response to the physiological status of the cell. J Bacteriol 171: 2680–2688