Rsp5 ubiquitin ligase modulates translation accuracy in yeast *Saccharomyces cerevisiae*

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ABSTRACT

Rsp5p is an essential yeast ubiquitin protein ligase that ubiquitinates multiple proteins involved in various processes. Recent studies indicate that ubiquitination also affects translation. Here, we show that the strain with the *rsp5-13* mutation exhibits altered sensitivity to antibiotics and a slower rate of translation. Using a sensitive dual-gene reporter system, we demonstrate that stop codon readthrough efficiency is decreased in the *rsp5-13* mutant, while both +1 and –1 frameshifting were unaffected. The effect of the *rsp5-13* mutation on readthrough could be reversed by increased expression of ubiquitin and partially suppressed by overproduction of the elongation factor eEF1A. As assessed by fluorescence in situ hybridization, the *rsp5-13* mutant cells accumulate tRNA nuclear pools, perhaps depleting tRNA from the cytoplasm. Nuclear accumulation of tRNA is observed only when *rsp5-13* cells are grown in media with high amino acid content. This defect, also reversed by overproduction of the elongation factor eEF1A, may be the primary reason for altered translational decoding accuracy.

Keywords: yeast; Rsp5 ubiquitin ligase; translation; tRNA export

INTRODUCTION

Ubiquitination is a post-translational modification of proteins that rapidly and reversibly changes in response to environmental stimuli or to programmed changes in cell state. Ubiquitination results from a cascade of reactions involving numerous enzyme components enabling a high degree of specificity and flexibility. In the first step of the cascade, an ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent manner. In the second step, ubiquitin is transferred to a cysteinyl group on one of the ubiquitin-conjugating enzymes (E2s). Finally, through the action of a ubiquitin–protein ligase (E3), an isopeptide bond links ubiquitin to a particular substrate. The E3 proteins generally play a dominant role in substrate recognition and binding (for reviews, see Hershko and Ciechanover 1998; Schwartz and Hochstrasser 2003).

Distinct variants of ubiquitin chains regulate various processes: polyubiquitination involving ubiquitin lysines

48 and 29 directs short lived, damaged, misfolded, or misassembled proteins to the 26S proteasome for degradation (Hochstrasser 1996; Hershko and Ciechanover 1998). In contrast, polyubiquitination involving lysine 63 (K63) regulates post-replicative DNA repair, transcription, cell cycle transitions, and endocytosis of plasma membrane proteins; monoubiquitination affects endocytosis/lysosomal degradation, meiosis, and chromatin remodeling (for reviews, see Hicke 2001; Weissman 2001; Lindsten et al. 2002).

Recent results indicate that ubiquitination also affects translation. Spence and colleagues (2000) have shown that active ribosomes are multiubiquitinated and that the L28 protein, located in the peptidyltransferase center, is ubiquitinated by the K63 multiubiquitin chain when incorporated in the ribosome. Moreover, a mutant strain solely expressing UbK63R and unable to form these variant chains showed altered sensitivity to translational inhibitors and a reduced translational rate in vitro. Proteomic studies revealed that many other ribosomal proteins, of both the small and large subunits, as well as translation elongation factor eEF1A, are ubiquitinated (Hitchcock et al. 2003; Peng et al. 2003), indicating that the regulatory effect of ubiquitination on translation might be quite complex.

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Rsp5p is an essential HECT domain containing ubiquitin–protein ligase in yeast. In addition to the catalytic HECT domain, Rsp5p possesses a C2 domain responsible for binding Ca^{2+} , lipids, and proteins, and three WW domains that mediate protein–protein interactions (Harvey and Kumar 1999). Rsp5p interacts with itself in a two-hybrid system and, therefore, is likely to be a multimeric protein (Dunn and Hicke 2001a). Rsp5p is localized in uniformly distributed punctate complexes in cells and cofractionates with a nonnuclear, nonmitochondrial, organellar subcellular fraction (Gajewska et al. 2001).

Rsp5p forms mono- or multi-ubiquitin chains linked via K63 of ubiquitin. Rsp5p impacts a wide variety of physiological processes, including regulation of endocytosis and lysosomal degradation of plasma membrane permeases such as Fur4p (Galan et al. 1996), Gap1p (Springael et al. 1999), Tat2p (Beck et al. 1999), and receptors such as Ste2p and Ste3p (Dunn and Hicke 2001b). Rsp5p also ubiquitinates a component(s) of the endocytic machinery (Dunn and Hicke 2001a; Gajewska et al. 2001; Kamińska et al. 2002; Stamenova et al. 2004). Moreover, Rsp5p-dependent ubiquitination is involved in sorting of amino acid permeases at the Golgi apparatus (Helliwell et al. 2001) and in the multivesicular bodies (Katzmann et al. 2004; Morvan et al. 2004). Rsp5p plays less well-characterized roles in other processes, including transcription, mitochondrial inheritance, the mitochondrial–cytoplasmic distribution of proteins, minichromosome maintenance, response to anesthetics, regulation of cellular pH, biosynthesis of unsaturated fatty acids, and nuclear export of RNA (Huibregtse et al. 1995; de la Fuente et al. 1997; Wang et al. 1999; Wolfe et al. 1999; Hoppe et al. 2000; Kamińska et al. 2000; Neumann et al. 2003; Rodriguez et al. 2003; Shcherbik et al. 2003, 2004; Gwizdek et al. 2005). The mechanisms by which Rsp5p affects these various processes remain to be elucidated.

We previously identified *rsp5* mutations in a genetic selection for alterations in nonsense suppression, which indicated that Rsp5p might affect translation (Żołądek et al. 1995). In the present study we assessed the role of Rsp5p in this process. We showed that the *rsp5–13* mutation alters cell sensitivity to antibiotics that act on translation and that it also affects the fidelity of translation. Additional copies of *UBI1* encoding ubiquitin reverse the effect of the *rsp5–13* mutation on translation. Moreover, we show that an additional copy of *TEF2*, encoding eEF1A, suppresses the *rsp5–13* growth defects and translational phenotypes. Defects in fidelity of translation could be explained, at least in part, by tRNA nuclear accumulation displayed by cells with the *rsp5–13* allele.

RESULTS

The *rsp5–13* mutation affects translation

The *rsp5–13* mutation results in G(707)D substitution in the catalytic domain of Rsp5p, which causes changes in

nonsense suppression and temperature-sensitive growth (Żołądek et al. 1997). We constructed isogenic strains to analyze the effect of *rsp5–13* mutation on translation and growth on media containing translational antibiotics. The *rsp5–13* mutant grows slower than wild type, with a doubling time of 2 h, 43 min versus 1 h, 39 min for the wild type at 30°C at the beginning of the exponential growth (t_0 to t_{345}), although growth resumes at a normal rate during the late exponential phase and reaches the same plateau. Consistent with slower growth, [^{35}S]-methionine incorporation into proteins in cells grown in YPD at 30°C shows that the *rsp5–13* mutant exhibits about a 70% decrease in the rate of total protein synthesis (Fig. 1). Slower protein synthesis could be due to defects in any step of translation: initiation, elongation, or in the generation or stability of ribosomes. To distinguish between these, we analyzed polysome profiles for wild-type and *rsp5–13* cells. Lysates were obtained from the wild type and the *rsp5–13* mutant strain grown at 30°C, as well as from these strains grown at 30°C and subsequently transferred for 3.5 h to 37°C to partially deactivate mutant Rsp5p (data not shown). The polysomes from wild-type and *rsp5–13* cells were quite similar, indicating that the synthesis of ribosomes and polysomes stability is not disturbed in the *rsp5–13* mutant strain, and therefore may not be responsible for the slower and thermosensitive growth phenotypes.

The *rsp5–13* mutant strain exhibits altered sensitivity to translational inhibitors

Analysis of sensitivity of mutants to translational drugs can uncover underlying defects in translation (Masarekar et al. 1981). Our studies of the *rsp5–13* mutant revealed its high sensitivity to the transpeptidylase inhibitor, anisomycin

FIGURE 1. The *rsp5–13* mutation affects the rate of protein synthesis. Wild-type cells (black circles) and *rsp5–13* mutant cells (triangles) were grown at 30°C to early exponential growth phase ($A_{600} = 0.5$) in methionine-free synthetic medium, followed by [^{35}S]-methionine labeling. Incorporation of radioactive methionine was measured at the indicated time points.

FIGURE 2. The *rsp5-13* mutant exhibits altered sensitivity to translational drugs. Wild-type (MK1) and *rsp5-13* (MK5) cells were grown to exponential phase. Serial 10-fold dilutions were spotted on YPD and media containing indicated antibiotics and incubated for 3 to 6 d at 30°C.

(Fig. 2), which targets 25S rRNA and the L3 protein (Rodriguez-Fonseca et al. 1995), and to cycloheximide (Fig. 2), an inhibitor of protein synthesis, which interferes with tRNA translocation when bound to the large subunit of the ribosome (Abraham and Pihl 1983). The *rsp5-13* mutant was also resistant to paromomycin and moderately sensitive to the G418 and gentamicin (Fig. 2), aminoglycoside antibiotics that bind to the decoding region of the ribosomal A site and decrease decoding accuracy (Moazed and Noller 1987).

Sensitivity of the *rsp5-13* strain to several translational antibiotics supports other data showing that Rsp5p affects translation. Addition of gentamicin, G418, or paromomycin causes lower fidelity of translation. Resistance to paromomycin usually correlates with the phenotype of higher fidelity of translation, antisuppression, or lower readthrough of stop codons, but there are exceptions to that rule (Dinman and Kinzy, 1997; Valente and Kinzy 2003). The different responses of the *rsp5-13* to these antibiotics indicate that the role of Rsp5p in translation may be complex.

Readthrough of stop codons is reduced in the *rsp5-13* strain and is restored by overexpression of *UBI1*

To test whether defects in Rsp5p-dependent ubiquitination influence the fidelity of translation, readthrough of stop codons and frameshifting were measured in the *rsp5-13* mutant and in the wild-type strains using a dual-gene reporter system. This system consists of vectors containing readthrough or frameshift-promoting signals inserted between the *lacZ* and *luc* sequences, encoding β -galactosidase and luciferase, respectively (Stahl et al. 1995). Luciferase activity reflects the readthrough or frameshifting efficiency, while β -galactosidase activity serves as a general control of expression level, integrating a number of possible sources of variability (plasmid copy number, transcriptional activity, mRNA stability, and translation rate). Our results showed that the Rsp5p defect has no effect on the frequency of -1 and $+1$ frameshift events (data not presented). However, the *rsp5-13* mutant showed an almost twofold lower level of UAA (p -value = 0.00001), UGA (p -value = 0.00217), and UAG (p -value = 0.0002) stop codon readthrough than the parental strain (Fig. 3A).

Maintenance of proper free ubiquitin levels is critical for cell viability, particularly when cells are challenged by stress or drug treatment (Hanna et al. 2003). Ubiquitin is encoded by four genes, *UBI1*, *UBI2*, and *UBI3*, supplying the bulk ubiquitin in favorable growth conditions, and *UBI4*, expressed under stress conditions (Ozkaynak et al. 1984; Finley et al. 1987, 1989). These genes have been previously found to act as multicopy suppressors of the temperature-sensitive growth of *rsp5* mutants (Żołądek et al. 1997; Kabir et al. 2005). Here we checked whether suppression of growth is correlated with suppression of translational defects.

We monitored translational readthrough for *rsp5-13* [*UBI1*]_N and wild-type [*UBI1*]_N transformants expressing ubiquitin from a multicopy plasmid. Only a small difference in readthrough between the wild type and the *rsp5-13* mutant overexpressing ubiquitin was detected (Fig. 3A,B), indicating that an excess of ubiquitin produced from *UBI1* gene partially suppresses the *rsp5-13* effect on readthrough of stop codons. Neither overexpression of ubiquitin nor empty vector (not shown) has an effect on readthrough in the wild-type strain. An additional copy of the *RSP5* gene or its overexpression from a multicopy plasmid had no effect on readthrough of the wild-type strain (data not shown). Our data support the notion that both Rsp5p ubiquitin ligase and an active ubiquitination pathway are necessary for the maintenance of normal fidelity of translation.

rsp5-13 defect of readthrough is suppressed by an additional copy of *TEF2*

The elongation factor eEF1A, which delivers tRNAs to the A-site of the ribosome, is encoded by two nearly identical genes, *TEF1* and *TEF2*, and mutations in these genes affect fidelity of

FIGURE 3. Effect of *rsp5-13* mutation and extra copies of *UBI1* and *TEF2* genes on stop codon readthrough. (A) *rsp5-13* mutant shows a twofold lower level of stop codon readthrough than the parental strain. Measurements were made in wild-type (MK1) and *rsp5-13* (MK5) strains. Details are given in Materials and Methods. (B) Overexpression of *UBI1* gene suppresses the phenotype of low stop codon readthrough in the *rsp5-13* strain. Measurements were made in wild type (MK1) and *rsp5-13* (MK5) transformed with YEp96 plasmid expressing ubiquitin. (C) Overexpression of *TEF2* gene suppresses the phenotype of low stop codon readthrough in *rsp5-13* strain. The level of readthrough was measured in wild type (MK1) and *rsp5-13* (MK5) transformed with YCp-TEF2 plasmid.

translation (Song et al. 1989; Dinman and Kinzy 1997; Carr-Schmid et al. 1999). To investigate a possible functional interaction between eEF1A and Rsp5p, *TEF2* was expressed from a centromere containing plasmid (maintenance in about 1 copy/cell) in wild-type and *rsp5-13* mutant strains and the level of readthrough was measured. The difference between these strains was smaller than that between the wild-type and *rsp5-13* strains lacking YCp-*TEF2*. This indicates that *TEF2* functions as a partial suppressor of the readthrough defect in *rsp5-13* cells (Fig. 3C). Expression of an additional copy of the *TEF2* gene had no effect on readthrough in the wild-type strain (Fig. 3, cf. C and A). As anticipated from these results, an additional copy of the *TEF2* gene also corrected the thermosensitive growth of *rsp5-13* cells, since the *rsp5-13* [*TEF2*] transformants grew on YPD and minimal medium at 34°C better than *rsp5-13* transformed with the vector alone (Fig. 4). Moreover, the additional copy of the *TEF2* gene enabled the *rsp5-13* mutant to grow better in the presence of cycloheximide. However, there was little effect of *TEF2* additional copy expression on growth on anisomycin and G418-containing medium (Fig. 4). The genetic interactions suggest that the *rsp5-13* mutation may affect translation at the elongation step. However, we did not detect differences in polysome profiles in the *rsp5-13* mutant compared to wild type, perhaps because polysome analysis is not sensitive enough to detect the changes uncovered by the drugs and readthrough assay.

An additional copy of *TEF2* suppresses tRNA nuclear accumulation caused by *rsp5-13*

Readthrough of stop codons depends on ribosomal proteins, translation factors, and many proteins associated with the ribosome, such as chaperones and actin cytoskeleton (for reviews, see Kandl et al. 2002; Valente and Kinzy 2003). The distribution of tRNA in the cell, its quality, aminoacylation, and modifications, association with eEF1A, and/or accessibility to the translational apparatus also affect the fidelity of protein synthesis and, in particular, the termination step.

We analyzed the subcellular distribution of tRNA by fluorescence in situ hybridization (FISH). The *rsp5-13* mutant cells, in contrast to the wild-type strain, accumulate large amounts of nuclear tRNAs. Nuclear accumulation of tRNA in *rsp5-13* mutant cells was observed after 3.5 h of

incubation at 37°C or 33.5°C using digoxigenin (DIG)-labeled probes specific for tRNA^{Tyr}, encoded by intron-containing genes, or tRNA^{Met}, encoded by intronless genes (Fig. 5A and see 5C). These findings are in agreement with previous observations showing Rsp5p involvement in nuclear export of tRNA (Neumann et al. 2003). In agreement with other published data (Rodriguez et al. 2003), poly(A)-containing RNA was also found to accumulate in the nucleus of *rsp5-13* cells at the nonpermissive temperature (Fig. 5A).

We discovered a surprising effect of nutritional conditions upon tRNA subcellular distribution. Nuclear accumulation of both types of tRNAs was observed in *rsp5-13* mutant cells grown in rich YPD or SD + casamino acids media, but nuclear tRNAs were not detected when these cells were grown to logarithmic phase in SC-ura medium, which contained all amino acids in concentrations described by Sherman (2002) (Fig. 5B). The second and last media differ only in concentrations of amino acids, indicating that these are components important for Rsp5p action in tRNA transport. These data indicate further that wild-type Rsp5 ligase activity is necessary for appropriate tRNA nucleus/cytosol distribution only when amino acids are abundant.

An additional copy of *TEF2* suppresses the defect in the translational readthrough level provoked by *rsp5-13* mutation and improves thermosensitive growth of mutant strain; thus we checked whether the tRNA nuclear accumulation is ameliorated in *rsp5-13* cells overexpressing the *TEF2* gene. Wild type with vector alone [–], *rsp5-13* [–], and *rsp5-13* [*TEF2*] strains were pregrown in SD + casamino acids medium, grown in YPD at 23°C overnight, and shifted to 33.5°C for 3.5 h. FISH was performed using a probe hybridizing to intron-containing pre-tRNA^{Tyr} and processed tRNA^{Tyr}. In contrast to *rsp5-13* [–], no nuclear accumulation of tRNA was observed in *rsp5-13* [*TEF2*] (Fig. 5C). The data indicate that overproduced eEF1A suppresses the *rsp5-13* defect in subcellular distribution of tRNA as well as translational readthrough and growth defects in the absence and presence of antibiotics.

DISCUSSION

Several, mostly unexplained, relationships between the ubiquitin system and protein synthesis have been previously

FIGURE 4. An additional copy of *TEF2* gene improves growth of *rsp5-13* mutant at elevated temperature and on media containing translational antibiotics. Growth of wild-type and *rsp5-13* strains transformed with the vector and *rsp5-13* strain transformed with YCp-*TEF2* was compared after plating serial dilutions of cultures on YPD or YPD containing indicated antibiotics and incubation for 3–6 d at 30°C or 34°C.

FIGURE 5. *rsp5-13* mutation causes accumulation of tRNA in the nucleus and an additional copy of *TEF2* gene reverses this effect. (A) Wild-type and *rsp5-13* strains were grown at 23°C in YPD medium and shifted for 3.5 h to 37°C. FISH was performed using specific digoxigenin-labeled probes recognizing intron-containing pre-tRNA and mature tRNA ($tRNA^{Tyr}$), intronless $tRNA^{Met}$, and probes specific for polyA. DNA was stained with DAPI. (B) Wild-type and *rsp5-13* strains were grown at 23°C in SC-ura or SD + casamino acids medium and shifted for 3.5 h to 37°C. FISH was performed using specific digoxigenin-labeled probes recognizing $tRNA^{Tyr}$. DNA was stained with DAPI. (C) Wild-type [vector], *rsp5-13* [vector] and *rsp5-13* [*TEF2*] strains were grown at 23°C and shifted for 3.5 h to 33.5°C. FISH was performed using specific digoxigenin-labeled probes recognizing $tRNA^{Tyr}$. DNA was stained with DAPI.

observed. In this report we show that a mutant deficient in Rsp5 ubiquitin–protein ligase is hypersensitive to drugs that inhibit translation and resistant to a drug that causes misreading. Mutant *rsp5-13* cells grow slower and exhibit a lower rate of translation compared to the parental strain. We also observed a significant decrease of translational readthrough in the *rsp5-13* mutant, although both +1 and –1 frameshifting remained unaltered, suggesting that competition between natural suppressor tRNAs and release factors favors the latter. Furthermore, the *rsp5-13* mutation also causes tRNA nuclear accumulation, which may account for its antisuppressor phenotype.

The *rsp5-13* mutant cells exhibit a broad spectrum of sensitivity to translational inhibitors, but are particularly resistant to paromomycin, which strongly stabilizes the A-site binding of near-cognate peptidyl-tRNAs and increases misreading (Ogle et al. 2001; Vicens and Westhof 2001). This correlates with an almost twofold decrease of readthrough at all three stop codons in *rsp5-13* mutant cells compared to wild-type cells.

There is no effect of ubiquitin levels on nonsense suppression in the wild-type cells (Chernova et al. 2003; see Results). However, in the presence of the prion form of the translational termination complex component, Sup35p [*PSI⁺*], changes in the free ubiquitin pool affect suppression efficiency. Moreover, the ubiquitin pool also affects cellular responses to drugs that influence translation. For example, *ubp6Δ* mutant cells, lacking the de-ubiquitinating enzyme, have a lowered ubiquitin pool and are hypersensitive to cycloheximide and anisomycin, drugs affecting translation (Keeven et al. 2002; Chernova et al. 2003; Hanna et al. 2003). Thus, *ubp6Δ* resembles the *rsp5-13* mutant in this respect. However, the altered antibiotic sensitivity of ubiquitin pathway mutants may reflect effects on various pathways, not only translation. Recently, it was demonstrated that the pool of ubiquitinated proteins and free ubiquitin levels are highly reduced in the *rsp5-1* (L733S) mutant, especially at elevated temperature (Krsmanovic and Kolling 2004). Accordingly, the low readthrough levels in *rsp5-13* cells might be related to a diminished ubiquitin pool that appears to be about 75% of wild-type levels (M. Kwapisz, J.-P. Rousset, and T. Żoładek, unpubl. results), although the pool of ubiquitinated proteins is similar in *rsp5-13* and in wild type (data not shown). Supporting the role of the ubiquitin pool is our data showing that the non-

sense codon readthrough phenotype of *rsp5-13* cells is suppressed by overexpressed ubiquitin.

At least two mechanisms may account for the effect of free ubiquitin and the *rsp5-13* mutation on stop codon readthrough. (1) Ubiquitination may directly affect an element of the translational apparatus functioning in the termination process. Indeed, several such factors have been found to be ubiquitinated (Hitchcock et al. 2003; Peng et al. 2003). However, we were unable to demonstrate ubiquitination of eRF3/Sup35p and the cellular level of this factor was similar in wild-type and *rsp5-13* strains (our unpubl. observations). (2) Alternatively, and the model we favor, changes in other processes requiring ubiquitination may indirectly affect stop codon recognition.

There are previous reports documenting that translation rate and/or nonsense codon suppression can be affected indirectly via alterations in tRNA distribution, accessibility, and properties, by altered tRNA modification, and/or by factors regulating tRNA levels (Dihanich et al. 1987; Hurt

et al. 1987; Beier and Grimm 2001; Kwapisz et al. 2002; Leconte et al. 2002). For example, the yeast Los1p was first identified by its loss of nonsense suppression phenotype (Hopper et al. 1980). Los1p functions as a nuclear exportin for tRNA, providing one of two or more pathways to deliver tRNA from the nucleus to the cytosol (Hurt et al. 1987; Hellmuth et al. 1998; Sarkar and Hopper 1998; for reviews, see Grosshans et al. 2000b; Hopper and Phizycky 2003). Thus, the altered nucleus/cytosol distribution of tRNA evidenced by the *rsp5-13* mutant cell (shown previously for *rsp5-3* by Neumann et al. 2003) might be the cause of its defects in translation efficiency and fidelity. If this is the case, then suppression of *rsp5-13* by additional eEF1A might result either from more efficient utilization of the residual cytoplasmic pool of tRNA or by a role of eEF1A in tRNA nuclear export, as has been previously proposed (Grosshans et al. 2000a). Neumann et al. (2003) reported also that *rsp5* mutants show defects in pre-rRNA processing, and this may possibly contribute to the changes in translation rate and fidelity we observe.

Why does mutation of *RSP5* cause altered distribution of tRNA between the nucleus and the cytoplasm? Direct modification by Rsp5p of Los1p and/or other components of the machinery governing RNA nuclear transport could provide one such mechanism. Indeed, a genome-wide study identified Los1p as an ubiquitinated protein (Peng et al. 2003). Alternatively, Rsp5p may be involved in a nutrient-dependent signaling process that affects the distribution of tRNA between the nucleus and the cytoplasm. Retrograde tRNA movement of tRNA has been demonstrated (Shaheen and Hopper 2005; Takano et al. 2005) and has been shown to be responsive to nutrient deprivation (Shaheen and Hopper 2005). Moreover, as shown by Grosshans and colleagues (2000a), amino acid deprivation results in tRNA nuclear accumulation. Although Rsp5p alters cellular amino acid permease sorting (Hein et al. 1995; Galan et al. 1996; Gajewska et al. 2001), others have shown that such inappropriate permease sorting need not dramatically alter internal amino acids pools (Crespo et al. 2004). Thus, Rsp5p unlikely affects tRNA distribution via altered cellular amino acid pools. Since Rsp5p functions in regulation of the transcription factors, Spt23p, Mga2p, and Gln3p (Hoppe et al. 2000; Shcherbik et al. 2003; Crespo et al. 2004), tRNA nuclear accumulation in *rsp5-13* cells, only when grown in media with high amino acids concentrations, could be rather a response to an altered transcriptional program or a result of direct involvement of Rsp5p in amino acid signaling.

During the cell life translation maintains a balance between speed and accuracy (Kurland 1992) and cell may gain in fitness if translational accuracy varies in response to growth conditions and nutrient accessibility. The translational accuracy is sensitive to tRNA accessibility and its distribution in the cell. The role of Rsp5p-dependent ubiquitination could be general and complex, and would facilitate the traffic of RNA and various proteins in the cell in

the response to changing physiological conditions, especially amino acid accessibility. It is likely that several Rsp5p substrates are involved in the regulation of nuclear export systems, but that remains to be determined.

MATERIALS AND METHODS

Strains, media, and genetic procedures

Yeast strains used in this study are: MHY501 *MAT α his3- Δ 200 leu2-3,112 ura3-52 lys2-801 trp1-1* (Chen et al. 1993), MK1 (MHY501 but *HA-RSP5*; this work), and MK5 (MHY501 but *HA-rsp5-13*; this work). *Escherichia coli* strain DH5 α F' (*F' supE44 lacU169 [80 lacZM15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for cloning and plasmid propagation. For replacement of the *RSP5* gene, integration plasmids YIpHA-RSP5/*rsp5-13* bearing the *RSP5* or *rsp5-13* mutant version and the triple hemagglutinin (HA)-epitope tag (see below; Gajewska et al. 2001) were linearized with PstI (Promega) or AgeI (Promega) and transformed into the MHY501 strain. Integrants were selected on SC-ura plates and then incubated on SD + 5'fluorouracil (5'FOA) plates (see below) to select for cells that had lost the *URA3* marker. Allele replacement was confirmed by PCR, and Rsp5p HA-tagging was confirmed by Western blot using antibodies against the HA epitope (clone 12CA5, BabCo).

E. coli cells were grown in LB medium supplemented with appropriate antibiotics to maintain the plasmids. The following media were used for yeast growth: YPD (2% glucose, 1% peptone, 1% yeast extract), SD (2% glucose, 0.67% yeast nitrogen base without amino acids), SD + casamino acid (SD supplemented with 1% casamino acids and 20 mg/L tryptophan) used for FISH experiments, SD + CSM (SD supplemented with 0.57% complete synthetic media lacking leucine or lacking leucine and uracil or lacking leucine and tryptophan, from ICN) used for fidelity of translation measurements, and SC-ura (SD supplemented with all amino acids and adenine 40 mg/L but lacking uracil, according to Sherman 2002), used for FISH experiments and strain construction. Solid media contained 2% agar. The amounts of drugs used were as follows: 0.22% 5'FOA, 0.1 μ g/mL cycloheximide, 1.0 mg/mL paromomycin, 10 μ g/mL anisomycin, 50 μ g/mL G418, 10 μ g/mL gentamicin. Paromomycin- and gentamicin-containing plates were buffered with 100 mM potassium phosphate buffer (pH 7.5–8.0). In all cases, growth of wild-type cells was not inhibited at the drug concentrations used.

Suppression of both the temperature-sensitive phenotype and antibiotics sensitivity was monitored by a drop test. An appropriate strain was suspended in water and serial 10-fold dilutions of cells were spotted onto solid media and incubated for 2–6 d at indicated temperatures. The growth rate in liquid cultures was monitored by A_{600} measurements. Standard yeast genetic methods were employed (Sherman 2002). Cells were transformed by the lithium acetate method (Chen et al. 1992). The *CUP1* promoter was induced with 0.1 mM CuSO₄.

Plasmids and plasmid construction

The plasmids used are: YIpHA-RSP5 (this work), YIpHA-*rsp5-13* (this work), YCp33HA-RSP5 (Gajewska et al. 2001), YEp-NP11/RSP5 (from B. Andre, Universite Libre de Bruxelles, Belgium),

YCp-TEF2/JWB2828 (from T. Kinzy, University of Medicine and Dentistry of New Jersey, Piscataway), YEplac2 (Ecker et al. 1987), pACTQ, pAC1789, pACTy, pACTMV (Stahl et al. 1995), pACTAA, and pACTGA (Bidou et al. 2000). The YIpHA-RSP5 plasmid used for integration into the MHY501 strain was constructed by cloning the EcoRI-SphI DNA fragment obtained by digestion of YCp33HA-RSP5 (Gajewska et al. 2001) into vector YIpplac211 (Gietz and Sugino 1988). The YIpHA-rsp5-13 was obtained by substituting the AgeI-MunI fragment in YIpHA-RSP5 with the DNA fragment, synthesized in a PCR reaction using specific primers and genomic DNA from T23 *rsp5-13* strain as the template (Żóładek et al. 1997) and digested by AgeI and MunI enzymes.

DNA restriction and electrophoresis were carried out according to standard procedures (Sambrook and Russell 2001). PCR reactions and sequencing were performed on double-stranded DNA, and sequencing was carried out using an automatic sequencer ABI310 Perkin Elmer in the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. Oligonucleotides used were synthesized at the above laboratory and their sequences are available upon request.

Readthrough frequency analysis

The reporter plasmids, pACTy (+1 frameshifting), pAC1789 (−1 frameshifting), pACTMV (UAG readthrough), pACTGA (UGA readthrough), pACTAA (UAA readthrough), and pACTQ (in frame control), were transformed into yeast strains (Stahl et al. 1995; Bidou et al. 2000). At least two transformants, cultivated at 30°C in SD + CSM, were assayed in a given experiment. Cells were harvested at an OD₆₀₀ of 1.5–3 and disrupted using glass beads. Luciferase and β-galactosidase activities were assayed as described (Stahl et al. 1995). The recoding efficiency, expressed in percentages, was calculated by dividing the luciferase/β-galactosidase ratio obtained using each test plasmid by the same ratio obtained with the in-frame control plasmid (Bidou et al. 2000). Results are the mean of at least five independent experiments and were tested by a Mann–Whitney *u*-test (Lowry 2000).

Fluorescence in situ hybridization

For FISH, published procedures and oligonucleotides were employed (Sarkar and Hopper 1998; Sarkar et al. 1999; Feng and Hopper, 2002). Strains were grown at 23°C to log phase in YPD or selective media SC-ura or SD + casamino acids and subsequently shifted to 37°C for the 3.5 h. For the *TEF2* suppression experiment strains were grown at 23°C to log phase and subsequently shifted to 33.5°C for the 3.5 h. Digoxigenin-labeled oligonucleotide probes specific for intronless tRNA (tRNA^{Met}) or recognizing intron-containing pre-tRNA and processed tRNA (tRNA^{Tyr}) were used. Fluorescence images were observed by using a Nikon Microphot-FX microscope, captured with a SenSys charge-coupled device camera (Photometrics) with QED software (QED Imaging) and assembled with Adobe Photoshop 5.0.

In vivo [³⁵S]-methionine incorporation

An overnight YPD culture at OD₆₀₀ 0.8–1.1 was spun down, suspended in P buffer (40 mM potassium phosphate buffer at pH 7.4, 0.45% glucose, 0.0077% SD + CSM-methionine), and

incubated with shaking at 30°C for 30 min. Forty microcuries of [³⁵S]-methionine were added and samples were withdrawn at the time points indicated. Incorporation of labeled methionine was stopped by adding 1/10 volume of 0.2 M unlabeled methionine. Cells were filtered under vacuum onto GF/A glass microfiber filters (Whatman), washed with 10% TCA, 95% ethanol, and acetone, and dried. Radioactivity was measured in a scintillation counter (Ciechanover et al. 1984, with modifications).

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Rsp5 ubiquitin ligase modulates translation accuracy in yeast *Saccharomyces cerevisiae*

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The strain that was used in this study designated as MK5 contains mutation *rsp5–19*, resulting in P418L substitution in WW domain of Rsp5, and not of *rsp5–13* mutation resulting in substitution G707D in Hect domain of Rsp5, as published. The name of this strain should be MK15. All other published data in this paper are correct. The authors apologize any confusion these errors may have caused. The errors do not change the conclusions of the paper.

Specifically, the changes in the paper are listed below:

- In Results, page 1711, the last sentence in the first column should begin: “The *rsp5–19* mutation results in P418L substitution in WW3 domain of Rsp5 ...”
- In Materials and Methods, page 1715, Materials and Methods, the first sentence beneath the first subheading after the citation (Chen et al. 1993) should read: “MK15 (MHY501 but *rsp5–19*; this work).” In addition, the name of the mutation should be *rsp5–19* and the name of the strain should be MK15 throughout the publication (not *rsp5–13* and MK5, as published).
- In Materials and Methods, page 1715, beneath the subhead **Plasmids and plasmid construction**, the name of the plasmid at the end of the third line from the bottom of the page should be YIpHA-*rsp5–19* (not YIpHA-*rsp5–13*). This is also the case on page 1716, line 8.
- In Materials and Methods, page 1716, line 11, the name of strain should be TZ29 *rsp5–19* (not TZ23 *rsp5–13*).

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