

# Critical roles for a genetic code alteration in the evolution of the genus *Candida*

Raquel M Silva<sup>1</sup>, João A Paredes<sup>1</sup>,  
Gabriela R Moura<sup>1</sup>, Bruno Manadas<sup>2</sup>,  
Tatiana Lima-Costa<sup>1</sup>, Rita Rocha<sup>1</sup>,  
Isabel Miranda<sup>1</sup>, Ana C Gomes<sup>1</sup>,  
Marian JG Koerkamp<sup>3</sup>, Michel Perrot<sup>4</sup>,  
Frank CP Holstege<sup>3</sup>, Hélian Boucherie<sup>4</sup>  
and Manuel AS Santos<sup>1,\*</sup>

<sup>1</sup>Department of Biology and CESAM, University of Aveiro, Aveiro, Portugal, <sup>2</sup>Centre for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal, <sup>3</sup>Department of Physiological Chemistry, University Medical Center Utrecht, Utrecht, The Netherlands and <sup>4</sup>Institut de Biochimie et Génétique Cellulaires, CNRS, Bordeaux, France

During the last 30 years, several alterations to the standard genetic code have been discovered in various bacterial and eukaryotic species. Sense and nonsense codons have been reassigned or reprogrammed to expand the genetic code to selenocysteine and pyrrolysine. These discoveries highlight unexpected flexibility in the genetic code, but do not elucidate how the organisms survived the proteome chaos generated by codon identity redefinition. In order to shed new light on this question, we have reconstructed a *Candida* genetic code alteration in *Saccharomyces cerevisiae* and used a combination of DNA microarrays, proteomics and genetics approaches to evaluate its impact on gene expression, adaptation and sexual reproduction. This genetic manipulation blocked mating, locked yeast in a diploid state, remodelled gene expression and created stress cross-protection that generated adaptive advantages under environmental challenging conditions. This study highlights unanticipated roles for codon identity redefinition during the evolution of the genus *Candida*, and strongly suggests that genetic code alterations create genetic barriers that speed up speciation.

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## Introduction

The discovery of alterations and expansions to the standard genetic code abolished the hypothesis of a frozen and universal genetic code (Crick, 1968) and raised important new questions, namely '(i) how do organisms survive codon identity redefinition? (ii) how and why do certain codons disappear while

others alter their identity? and (iii) what is the evolutionary and physiological impact of codon identity redefinition?' Neutral (Codon Capture) evolutionary mechanisms postulate that highly biased G + C pressure can force codons to disappear and such unassigned codons can be captured by natural or mutant tRNAs from non-cognate codon families (Osawa and Jukes, 1989). This neutral mechanism explains some mitochondrial genetic code changes and unassignment of the CGG and AGA/AUA codons in *Mycoplasma capricolum* and *Micrococcus luteus*, which have 25% and 74% GC, respectively (Ohama *et al*, 1990; Oba *et al*, 1991).

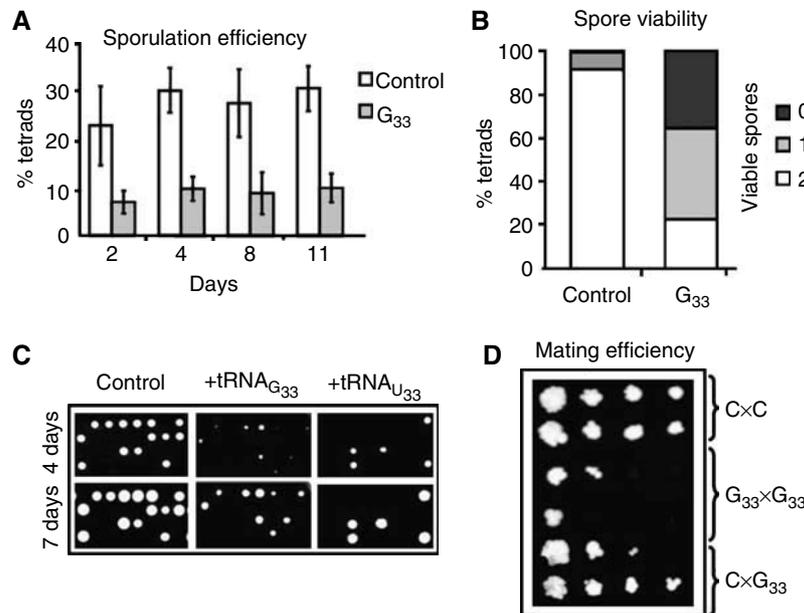
Codon identity redefinition can also be driven by selection through codon misreading (Schultz and Yarus, 1994). This requires structural change of the translational machinery, in particular of tRNAs, aminoacyl-tRNA synthetases and release factors, which decrease codon decoding fidelity and generate codons with more than one identity (Schultz and Yarus, 1994). These selection-driven mechanisms support the expansion of the genetic code to selenocysteine (twenty-first amino acid) and pyrrolysine (twenty-second amino acid) (Chambers *et al*, 1986; Hao *et al*, 2002). Selenocysteine is used in both prokaryotic and eukaryotic selenoproteins and its insertion into the genetic code required reprogramming of UGA stop codons by novel translation elongation factors (SelB-prokaryotes; EF-sec and SBP2-eukaryotes), a new tRNA (tRNA<sup>Sec</sup>) and a selenocysteine mRNA insertion element (SECIS) (Namy *et al*, 2004). L-Pyrrolysine is used in the archaeon *Methanosarcina barkeri* through reprogramming of UAG-stop codons in methylamine methyltransferase genes (Theobald-Dietrich *et al*, 2005). Codon ambiguity also explains the identity alterations of several sense and non-sense codons in bacteria, mitochondria and in the cytoplasm of eukaryotes. For example, the decoding of leucine CUN (N any nucleotide) codons as threonine in yeast mitochondria and the decoding of leucine CUG codons as serine in the cytoplasm of various *Candida* species (Schultz and Yarus, 1994; Massey *et al*, 2003; Miranda *et al*, 2006).

Genetic code alterations driven by codon ambiguity are most interesting because they should block lateral gene transfer and sexual reproduction. The former is highlighted in *Candida albicans*, where serine CUG decoding prevents the expression of functional wild-type green fluorescent protein (GFP), whose gene contains a single CUG codon, and of many other CUG-containing reporter genes, namely *Escherichia coli*  $\beta$ -galactosidase, *Renilla reniformis* luciferase, and *Saccharomyces cerevisiae* orotidine-5'-phosphate decarboxylase (Ura3p) (Cormack *et al*, 1997). Additionally, codon identity redefinition reshapes protein primary structure and may create novel protein functionalities, which on one hand may speed up the evolution of new phenotypes, but on the other may generate proteome and genetic incompatibilities to sexual reproduction. The latter is highlighted by the inability of using *S. cerevisiae* genes to complement *C. albicans* homologous gene disruptions (Sugiyama *et al*, 1995).

\*Corresponding author. Department of Biology, University of Aveiro, Santiago Campus, Aveiro 3810-193, Portugal. Tel.: +351234370771; Fax: +351234426408; E-mail: msantos@bio.ua.pt

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**Figure 2** Genetic code alterations act as a barrier for sexual reproduction. The ability of *S. cerevisiae* cells expressing *C. albicans* G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> to reproduce sexually was determined by their sporulation and mating efficiencies. (A) The number of tetrads produced by diploid ambiguous cells decreased by 30% when compared with control cells, indicating that sporulation efficiency was lower in CUG ambiguous cells. (B) Spore viability was reduced in ambiguous cells, since most tetrads yielded only one (42%) or no viable spores (35%), while tetrads from control cells produced mainly two viable spores (92%). (C) Ambiguous cells produced fewer viable spores and spores grew slower, as shown by growth on solid selective medium. The U<sub>33</sub> tRNA spores shown had mutations in the tRNA<sup>Ser</sup><sub>CAG</sub> gene. (D) Haploid control and ambiguous cells with opposite mating types were mixed and serial dilutions of the mixtures were plated onto selective media. C × C, G × G and C × G indicate the crosses between the control cells, or ambiguous G<sub>33</sub> tRNA cells or control and ambiguous G<sub>33</sub> tRNA cells, respectively. The reduced amount of diploids produced by crossing ambiguous (G<sub>33</sub> tRNA) cell lines showed that mating efficiency was also negatively affected by CUG ambiguity. In all cases, the U<sub>33</sub> tRNA<sub>CAG</sub> was lethal in haploid backgrounds. Alternatively, its gene acquired mutations that inactivated the U<sub>33</sub> tRNA.

(Santos *et al*, 1996). Here, we used these plasmid-transformed strains to elucidate the impact of CUG identity redefinition on *S. cerevisiae* gene expression and physiology. The tRNA<sup>Ser</sup><sub>CAG</sub> gene was also integrated into the genome of *S. cerevisiae* to evaluate the impact of this genetic code alteration on sexual reproduction. These *S. cerevisiae* clones expressed their own tRNA<sup>Leu</sup><sub>UAG</sub> plus the *C. albicans* tRNA<sup>Ser</sup><sub>CAG</sub> and incorporated leucine or serine randomly at CUG positions on a genome-wide scale. This mimicked the CUG ambiguity present in the *Candida* ancestor, where a cognate tRNA<sup>Leu</sup> plus the novel mutant tRNA<sup>Ser</sup><sub>CAG</sub> also competed for CUG codons (Figure 1A; Massey *et al*, 2003). Two versions of the *C. albicans* tRNA<sup>Ser</sup><sub>CAG</sub> were used: the *C. albicans* wild-type tRNA<sup>Ser</sup><sub>CAG</sub> containing G<sub>33</sub>, which is an inefficient decoder that appeared late in the evolutionary pathway of the genetic code alteration, plus a mutant tRNA<sup>Ser</sup><sub>CAG</sub> containing the canonical U at position 33 (U<sub>33</sub>), which is an efficient decoder and represents the primordial tRNA (Santos *et al*, 1996; Perreau *et al*, 1999). The latter allowed us to confirm that the higher decoding efficiency of U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> was not an impediment to CUG identity redefinition. The levels of serine misincorporation *in vivo* at CUG positions were determined by mass spectrometry using a CUG-reporter system (Figure 1C; Supplementary Figures 1–3). In diploid cells, serine incorporation was 1.4% and 2.31% for the G<sub>33</sub> and U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub>, respectively (Figure 1D). Considering that background decoding error *in vivo* in yeast is in the order of 0.001% (Stansfield *et al*, 1998), those values represent 1400- and 2310-fold increase in decoding error and provide important insight into the level of ambiguity experienced by the *Candida*

ancestor during the initial stages of the CUG identity change (Massey *et al*, 2003).

We have investigated the impact of partial CUG identity redefinition in sexual reproduction using *S. cerevisiae* clones expressing U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> and G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub>. The inefficient G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> decoder decreased sporulation efficiency by 30% (Figure 2A), while clones expressing the efficient U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> decoder showed very high sporulation variability; 55% of the clones sporulated normally, 25% did so very poorly and 20% did not sporulate at all (data not shown). The impact of CUG ambiguity on fertility was also tested by germinating spores of dissected asci. In selective media (YEPD–geneticin), 95% and 44% of control and G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> spores were viable, respectively, but 42% of G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> asci had one viable spore and 23% had two viable spores (Figure 2B and C). In 80% of U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> clones that sporulated, 76% of the asci produced spores that did not germinate, 9% had one viable spore and only 15% had two viable spores (data not shown). Since CUG ambiguity is toxic and creates genetic instability (see below), the integrity of G<sub>33</sub> and U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> genes was verified by PCR amplification and resequencing of the respective DNA fragments (8 G<sub>33</sub> and 17 U<sub>33</sub> in total), isolated from the colonies of germinated spores. Spores expressing G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> had the correct tRNA gene sequence, while all the spores expressing U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> contained mutations in positions that mapped to the extra-loop and anticodon stem of the mature tRNA (data not shown). That is, U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> is lethal in haploid backgrounds, since only cells containing mutations in U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> gene could sporulate. These mutations also

explained the fast growth of the spores (Figure 2C) and the very high sporulation variability observed in diploid cells expressing  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> (see above). It also confirmed previous studies showing that haploid *S. cerevisiae* cannot be transformed with plasmids carrying  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> (Santos *et al*, 1996).

Since  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> was lethal in haploid backgrounds, the impact of ambiguous CUG decoding on mating was evaluated using clones expressing the  $G_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> gene. Crosses of  $G_{33}$  (MAT $\alpha$ )  $\times$   $G_{33}$  (MAT $\alpha$ ) displayed low mating efficiency, which increased slightly for  $G_{33}$  (MAT $\alpha$ )  $\times$  control (MAT $\alpha$ ) crosses (Figure 2D). These mating differences are explained by gene dosage effects because diploids of  $G_{33}$  (MAT $\alpha$ )  $\times$   $G_{33}$  (MAT $\alpha$ ) crosses had two copies of the  $G_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> gene, while diploids of control (MAT $\alpha$ )  $\times$   $G_{33}$  (MAT $\alpha$ ) crosses had a single copy of this tRNA gene and tRNA copy number determines tRNA abundance and CUG ambiguity levels.

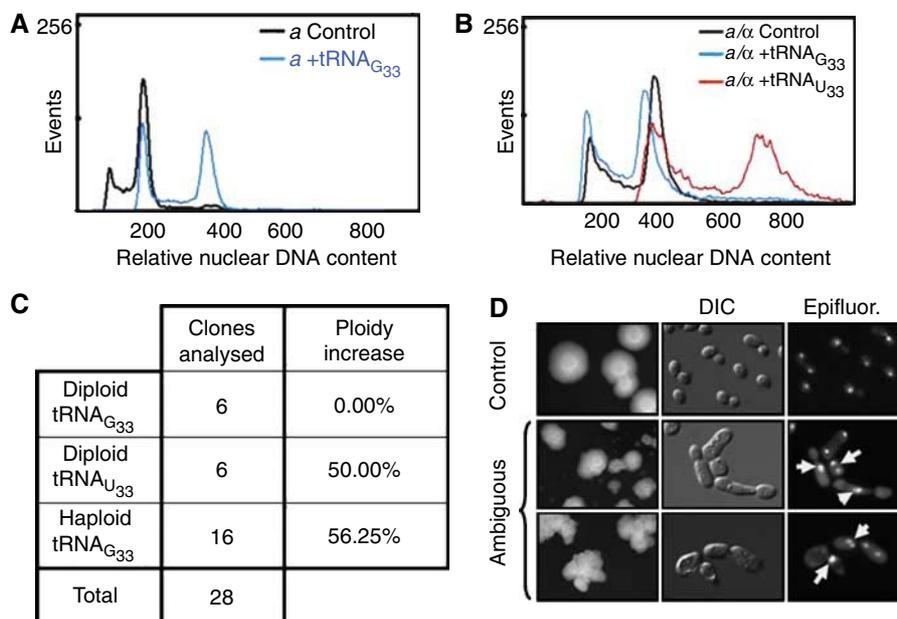
Interestingly, flow cytometry analysis showed that expression of  $U_{33}$  and  $G_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> in *S. cerevisiae* increased ploidy (Figure 3A and B).  $G_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> induced ploidy increase from N to 2N in 56% of the haploid clones, while  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> increased it from 2N to 4N in 50% of diploid clones (Figure 3C). Expression of  $G_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> in diploid cells also shifted DNA content peaks slightly, suggesting that the cell population contained a significant number of aneuploid cells. Similar DNA peak shifts were observed in clones expressing  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> (Figure 3B). Expression of  $G_{33}$  and  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> also generated highly heterogeneous colony and cell morphologies and increased cell size significantly, which is consistent with ploidy increase (Figure 3D). Nuclear DAPI staining showed the presence of micronuclei and two or more large nuclei in cells expressing  $G_{33}$  and  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub>. Some daughter cells did not have nuclei, suggesting disruption of

chromosome segregation or aberrant nuclear division during mitosis (Figure 3D).

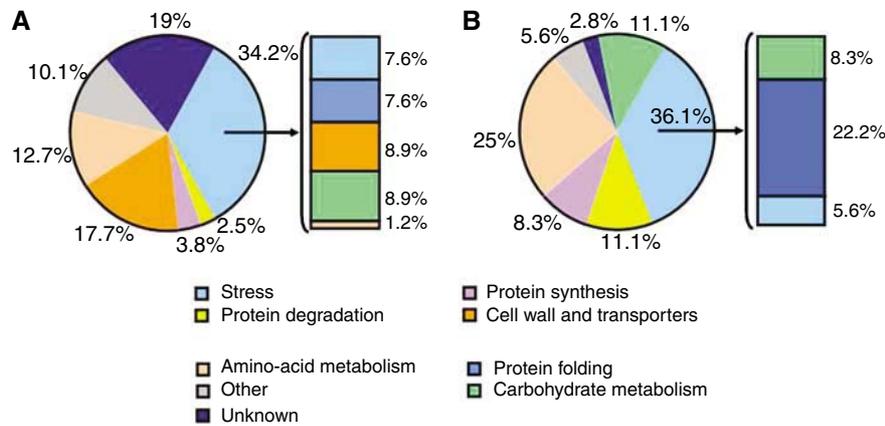
### CUG ambiguity altered gene expression and physiology

To shed further light on the impact of codon identity redefinition on physiology and evolution, gene expression was monitored in cells expressing  $G_{33}$  and  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> using DNA microarrays. Similar results were obtained for both tRNAs, although differences in the magnitude of changes were observed in some cases (Supplementary Table 1). Overall, DNA microarray profiling uncovered alterations in the expression of genes belonging to the stress response, carbohydrate and amino-acid metabolism, cell wall structure, protein synthesis and degradation (Figure 4A).

Expression of 58 genes was upregulated, whereas that of 21 genes was downregulated (Supplementary Table 1). Most upregulated genes were stress-response genes (34%), namely molecular chaperones *HSP12* (12.4-fold), *HSP26* (5.8-fold), *HSP70* (*SSA4*) (3.9-fold) and *HSP104* (2.4-fold). CUG ambiguity also resulted in the upregulation of drug-resistance genes, namely copper-binding metallothionein genes *CRS5* (3 fold) and *CUP1* (2.9-fold), as well as the membrane ABC (ATP-binding cassette) transporter gene *PDR5* (2.2-fold). Other changes involved general stress-response genes, namely the multistress response protein genes *DDR2* (9 fold), *HSP42* (3.9-fold), *HSP30* (3.7-fold), the stress-induced methylglyoxal reductase gene *GRE2* (2.1-fold), the GPI-anchored cell-wall glycoprotein genes *SED1* and *SPI1* (2.8-fold) and the cell-wall genes *TIP1* (6.2-fold), and *CWP2* (4 fold), which encode major structural mannoproteins (Table 1). These gene expression alterations are in line with our previous results showing that CUG ambiguity increases tolerance to arsenite, cadmium, cycloheximide, ethanol, oxidants and



**Figure 3** Genetic code alterations induce ploidy variation. Flow cytometry analysis of haploid (A) and diploid (B) *S. cerevisiae* cell lines expressing the *C. albicans*  $G_{33}$  or  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> had a general increase of the nuclear DNA content, providing evidence of polyploidy and aneuploidy events in CUG ambiguous cells. (C) Ploidy shift was observed in 56% of the haploid  $G_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> clones and 50% of diploid  $U_{33}$  tRNA<sup>Ser</sup><sub>CAG</sub> clones tested by flow cytometry analysis. (D) Heterogeneity of the ambiguous cell population is shown by the variability in colony, cell and bud size and shape. The increase in cell volume is consistent with polyploidization of the ambiguous clones. DAPI staining highlights ambiguous cells with two nuclei or without nucleus, suggesting the presence of polyploid and aneuploid cells.



**Figure 4** Genetic code alterations reprogramme gene expression. **(A)** Transcriptome analysis indicates the percentage of genes with altered expression levels in ambiguous strains. Genes whose expression was both up- and downregulated by CUG ambiguity were grouped according to their functions. The genes that are included in the stress group from the pie-chart were further divided into the functional categories displayed on the adjacent column. **(B)** Proteome data show the percentage of proteins whose expression was altered in *S. cerevisiae* cells expressing *C. albicans* U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub>, distributed by functional categories. The proteins that are included in the stress group from the pie-chart were further divided into the functional categories displayed on the adjacent column. Both analyses indicate that genetic code ambiguity extensively remodelled gene expression, altered the expression of genes and proteins belonging to the stress response, protein synthesis, folding and degradation pathways, and general metabolism.

**Table I** Selected genes whose expression was altered by CUG ambiguity

Function	Gene	Fold	Function	Gene	Fold
Chaperones	HSP12	12.4	Carbohydrate metabolism	HXK1	6.2
	HSP26	5.8		TSL1	3.2
	SSA4	3.9		GLK1	2.3
	HSP104	2.4		TPS1	2.1
Stress response	CRS5	3.0	Protein synthesis	RPL22B	-2.1
	CUP1	2.9		YDR341C	-2.1
	PNC1	2.1		RPL9B	-2.0
Cell wall and transporters	PHO84	17.6	Amino acid metabolism	PUT1	3.7
	PIR3	7.5		LYS9	-2.4
	TIP1	6.2		SAM4	-2.0
	CWP2	4.0			
	PDR5	2.2			

Genes induced in ambiguous cells display positive fold variation and genes repressed are indicated by negative fold variation (FDR = 0.001).

salt (Santos *et al*, 1999). Overexpression of the high-affinity inorganic phosphate transporter (*PHO84*; 17.6-fold); the acid phosphatases *PHO12* (3.4-fold), *PHO11* (3.1-fold) and *PHO5* (2.9-fold); the hexokinase I (*HXK1*; 6.2-fold); glucokinase (*GLK1*; 2.3-fold); glycogen phosphorylase (*GPH1*; 4.7-fold) and the components of the trehalose-6-phosphate synthetase/phosphatase complex (*TSL1* and *TPS1*; 3.2- and 2.1-fold) indicated that CUG ambiguity affected phosphate, glucose, glycogen and trehalose metabolism (see below).

Since expression of G<sub>33</sub> and U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> generated highly heterogeneous cell populations (Figure 3) and induced the stress response, which altered gene expression at the post-transcriptional level (DiDomenico *et al*, 1982), we wondered whether DNA microarray profiling was providing a complete view of the gene expression alterations induced by ambiguous CUG decoding. To clarify this question, quantification of protein expression was carried out using phosphorimaging of [<sup>35</sup>S]methionine-labelled proteins fractionated onto 2D-PAGE (Figure 4B; Supplementary Figure 4 and Table 2). Up-regulation of 43 proteins belonging to the stress response and

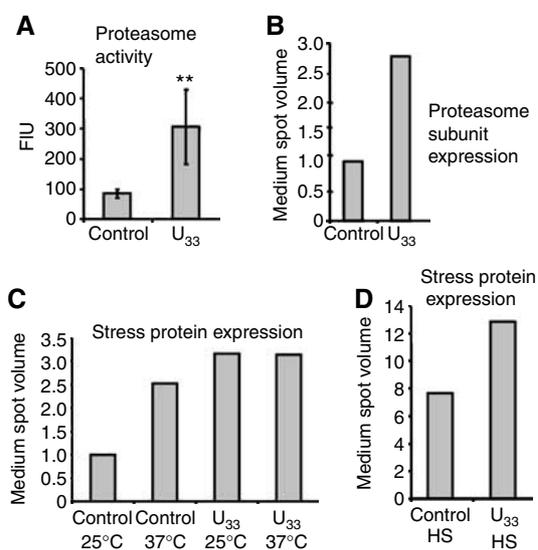
protein degradation, and down-regulation of 34 proteins involved in amino-acid metabolism and protein synthesis was observed (Figure 4B). These data confirmed the DNA microarray data, but fold-induction differences were observed for several genes (Table II). For example, Hsp104p and Pnc1p were up-regulated 13.1- and 29.5-fold (Table II), while their mRNAs increased 2.4- and 2.1-fold only, respectively (Tables I and II). Two additional differences were found between the microarray and proteomics data. The latter, but not the former, showed up-regulation of several proteasome subunits, namely Rpn10p, Rpn12p, Pup2p and Scl1p (Table II), which was confirmed by increased proteasome activity (3.6-fold) in ambiguous cells (Figure 5A and B).

Strong up-regulation (29.5-fold) of the enzyme nicotinamide (PNC1), which converts nicotinamide into nicotinic acid in the NAD<sup>+</sup> salvage pathway (Anderson *et al*, 2003), was unanticipated, as NAD<sup>+</sup> is a cofactor of the histone deacetylase Sir2p. In yeast, Sir2p is involved in silencing chromatin at telomeres, ribosomal DNA (rDNA) and mating-type loci, and deletion of the *SIR2* gene promotes aging by

**Table II** Selected proteins whose expression was altered by CUG ambiguity

Function	Protein	Fold	P-value	Function	Protein	Fold	P-value
Chaperones	Hsp104	13.1	—	Amino-acid metabolism	Met17	-5.3	0.0004
	Ssa1	4.7	0.0007		Aro8	-5.1	0.000007
	Ssa2	2.5	0.0091		Arg1	-3.4	0.005
	Ssa4	N	—		Lys9	-2.8	0.006
Stress Response	Pnc1	29.5	0.0154	Leu2	-2.3	0.0001	
	Ahp1	3.4	0.0021				
Carbohydrate metabolism	Glk1	5.0	0.0091	Protein synthesis	Krs1	-2.7	0.0011
	Hxk1	N	—		Ssb1	-2.1	0.0167
	Hor2	N	—		Ssb2	-2.1	0.0052
Protein degradation	Rpn12	5.2	0.0230				
	Rpn10	3.6	0.0065				
	Pup2	3.6	0.0010				
	Sc11	3.6	0.0228				

Proteins whose expression was altered in ambiguous cells, indicating the respective fold variation and statistical significance. Proteins induced display positive fold variation and proteins repressed are represented by negative fold variation; N stands for new spots (proteins that were not expressed in the control condition and, therefore, their fold variation could not be accurately determined). An average fold represents proteins that are present in the 2D gel by more than one spot.



**Figure 5** Genetic code alterations reprogramme the stress response. (A) Proteasome activity increased 3.6-fold in *S. cerevisiae* cells expressing *C. albicans* tRNA<sup>Ser</sup><sub>CAG</sub> (U<sub>33</sub>), as shown by enhanced proteolysis of the chymotrypsin-like substrate SucLLVY-AMC. The results are expressed as mean  $\pm$  s.d. of 4–6 independent experiments (\*\**P* < 0.01 by Student's *t*-test). Fluorescence intensity (FIU) is shown in arbitrary units. (B) Expression of proteasome subunits (Supplementary Table 3) was threefold induced by CUG ambiguity, as measured by proteome analysis. Control (C) and ambiguous (U<sub>33</sub>) cells were grown at 25°C (25), 37°C (37) or heat shocked (HS). Proteins were labelled *in vivo* with L-[<sup>35</sup>S]methionine and separated by 2D-PAGE as described in Materials and methods. The medium expression level of the selected proteins was calculated and normalized to the control to deduce general folds. (C) Ambiguity pre-adapted cells to tolerate adverse growth conditions. Expression of stress proteins (Supplementary Tables 3 and 4) increased twofold in control cells at 37°C, but not in ambiguous cells that already had increased amounts (3 fold) of these stress-protective proteins. (D) Ambiguous cells retained the capacity to respond to additional stress. Expression of stress proteins (Supplementary Tables 3 and 4) was induced in both strains under heat-shock (8- and 13-fold for the control and ambiguous cells, respectively).

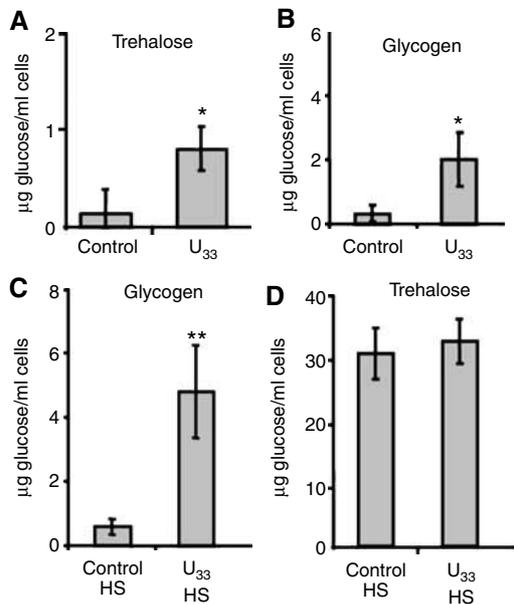
increasing recombination at the rDNA locus (Sinclair and Guarente, 1997). Since *PNC1* integrates multiple stimuli and modulates environmental adaptation through Sir2p

(Anderson *et al*, 2003), its strong upregulation by codon misreading (Table II) suggests that aberrant proteins activate conserved mechanisms that stabilize the genome. Indeed, *PNC1* may counteract genome-destabilizing effects caused by CUG ambiguity by stabilizing the rDNA locus.

#### Constitutive CUG ambiguity does not impair long-term adaptation

Redefinition of codon identity evolved gradually over millions of years ( $272 \pm 25$  million years in *Candida*) and results in constitutive synthesis of aberrant proteins, generating a unique form of intracellular stress. An important question came to mind, namely 'would ambiguous cells lose their capacity to respond to additional environmental challenges?' In order to shed light on this question, various stress proteins, including proteasome subunits, were labelled with [<sup>35</sup>S]Met and quantified by phosphorimaging of 2D protein maps, as before (Supplementary Tables 3 and 4). Ambiguous cells had higher proteasome activity and displayed increased expression of proteasome subunits (Figure 5A and B) and increased levels of stress proteins at 25°C, in particular of molecular chaperones (Figure 5C). However, the latter were not upregulated at 37°C (Figure 5C) and their expression only increased when cells were exposed to heat shock (Figure 5D). This capacity of ambiguous cells (permanently stressed) to respond to heat stress by further upregulating critical components of the stress response supports the hypothesis that codon identity redefinition did not compromise adaptation to environmental change.

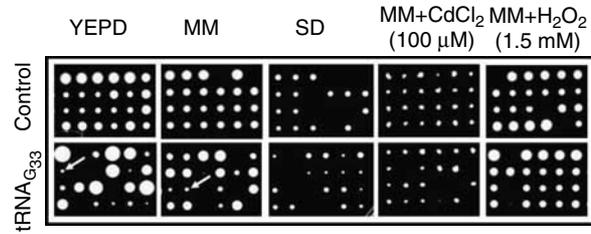
Glycogen and trehalose are reserve carbohydrates that accumulate under stress as energy reserves. Trehalose also stabilizes protein structure at high temperatures and decreases the aggregation of unfolded or heat-denatured proteins (Singer and Lindquist, 1998; Ueda *et al*, 2001). Accordingly, ambiguous cells accumulated them at higher levels than control cells (Figure 6A and B), due to upregulation of the components of the trehalose synthase complex, namely *TPS1* (2 fold) and *TSL1* (3 fold) (Table I). Under heat shock, ambiguous cells had higher levels of both glycogen and trehalose (Figure 6C and D), and proteome analysis under heat shock confirmed that both control and U<sub>33</sub>



**Figure 6** Genetic code alterations increase trehalose and glycogen accumulation. C and U<sub>33</sub> represent *S. cerevisiae* control cells and cells expressing *C. albicans* U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub>, respectively. The results are expressed as mean ± s.d. of four independent experiments (\**P* < 0.05 and \*\**P* < 0.01 by Student's *t*-test). Trehalose (A) and glycogen (B) content were 6 fold higher in exponentially growing CUG ambiguous cells (OD = 0.5), when compared with control cells. (C, D) Glycogen and trehalose accumulation were induced in ambiguous cells after a 30-min heat shock; glycogen content increased 8 fold under heat shock when compared with control cells, while trehalose accumulation increased in both ambiguous and control strains under heat stress.

tRNA<sup>Ser</sup><sub>CAG</sub> cells upregulated proteins belonging to the trehalose biosynthesis pathway (Supplementary Tables 4 and 5). Previous studies showed that glycogen concentration plateaus after 60 min under heat shock and its level is maintained up to 120 min (Parrou *et al*, 1997). In our experimental conditions (30 min of heat shock) control cells accumulated small amounts while ambiguous cells already had significant amounts of glycogen (Figure 6B and C). Genes encoding enzymes of the glycogen biosynthesis pathways, namely *GSY2* and *GLC3*, were not significantly upregulated in cells expressing U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> (Supplementary Table 5); however *GPH1* involved in glycogen degradation was upregulated 4 fold, pointing towards increased simultaneous recycling of this reserve carbohydrate, as previously described (Parrou *et al*, 1997).

Since codon-decoding ambiguity causes major proteome disruption and reduces fitness, genetic code alterations should have been eliminated by natural selection. The gene and protein expression profiling data described herein provide an explanation for this apparent paradox. Up-regulation of proteasome activity, induction of stress proteins, cell-wall remodelling and accumulation of trehalose and glycogen contributed to elimination or recovery of aberrant proteins. Furthermore, gene expression and physiological remodelling created stress cross-protection and pre-adapted cells to severe environmental change, allowing them to explore new ecological niches. Indeed, previous studies have shown that ambiguous *S. cerevisiae* were able to outcompete wild-type cells



**Figure 7** Ambiguous cells have selective advantage under stress. Spores of *S. cerevisiae* control cells and cells expressing the G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> were grown for 7 days in agar plates of rich medium (YEPD), synthetic medium (SD) and minimal medium (MM) containing 100 µM CdCl<sub>2</sub> or 1.5 mM H<sub>2</sub>O<sub>2</sub>. Spores from ambiguous cells grew much slower than non-ambiguous spores in rich medium, but recovered competitive capacity when growing under stress conditions, as shown by a similar colony size of control and ambiguous cells.

under stress (Santos *et al*, 1999). In this study, spores of *S. cerevisiae* expressing G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> plated in rich glucose media (YEPD) grew much slower than non-ambiguous spores (Figure 7), but stress severity reduced colony size differences between control and ambiguous clones, namely in synthetic media (SD) and in minimal media (MM) containing 100 µM CdCl<sub>2</sub> or 1.5 mM H<sub>2</sub>O<sub>2</sub> (Figure 7). This supports the hypothesis that, to a certain extent, stress tolerance and stress cross-protection overcome the negative impact of proteome disruption caused by mistranslation.

## Discussion

### A role for CUG identity redefinition in *Candida* evolution

New species may arise from ecological differentiation, hybridization, chromosomal and ploidy alterations and gradual evolution of genetic incompatibilities (Otto and Whitton, 2000; Storchova and Pellman, 2004). Our data show that, beyond blocking lateral gene transfer, genetic code alterations remodel gene expression and physiology and are able to create immediate genetic barriers to sexual reproduction. Indeed, partial redefinition of CUG identity in *S. cerevisiae* had a strong negative impact on sporulation efficiency, fertility and mating. Only half of the spores (44%) expressing G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> were viable and U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> was lethal in haploid backgrounds. Mating was severely affected in clones expressing G<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> and completely disrupted in clones expressing U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub> (Figure 2). More importantly, tRNA<sup>Ser</sup><sub>CAG</sub> generated high cell heterogeneity and created diploid and tetraploid lineages with nuclear aberrations (micro-nuclei) (Figure 3). Since diploid *S. cerevisiae* cells tolerated U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub>, which mimics the ancestral tRNA<sup>Ser</sup><sub>CAG</sub> (Massey *et al*, 2003), and haploid cells did not, it is likely that CUG identity redefinition locked the *Candida* ancestor in a diploid or polyploid state and blocked sexual reproduction altogether. This is supported by diploidy and lack of sexual reproduction in extant *Candida* species, and by the observation that polyploid plants and animals are normally parthenogenic (asexual) (Otto and Whitton, 2000). Ploidy increase and population heterogeneity induced by codon identity redefinition is of further evolutionary consequence, as ploidy differences alter gene expression, developmental, physiological, cell size and other unique morphological characteristics in yeast and polyploid tumour cells (Galitski *et al*, 1999).

Intriguingly, in *E. coli*, codon-decoding ambiguity generated translational stress-induced mutagenesis (TSM) and created hypermutagenic cells (Al Mamun *et al*, 2002). If TSM occurred in the *Candida* ancestor, it is likely that diploidy and polyploidy may have buffered the genome against rapid accumulation of degenerative mutations. Alternatively, ploidy increase may have allowed rapid evolution of new gene functions by freeing one copy of duplicated genes for evolutionary experimentation. This hypothesis is strongly supported by very high genome heterozygosity and rapid functional divergence of a number of gene families in most *Candida* species (Zhao *et al*, 2003; Jones *et al*, 2004).

### Genetic code alterations remodel gene expression

Yeast cells respond to sudden exposure to environmental stress by transiently (60 min) changing the expression of an array of genes (approximately 600 downregulated and 300 upregulated). As cells adapt to stress, gene expression recovers to a new steady state and transcript abundance differences between normal and stress conditions become much lower (Gasch *et al*, 2000). This so-called environmental stress response (ESR) is mediated by the transcription factors Msn2p and Msn4p through the general stress-responsive element (STRE; CCCCT) (Gasch *et al*, 2000; Causton *et al*, 2001). Unicellular organisms also respond to specific stresses by activating unique cellular programs that are controlled by other transcription factors, namely Yap1p (oxidative), Hog1p (osmotic) and Hsf1p (Heat shock) (Brewster *et al*, 1993; Kuge *et al*, 1997; Boy-Marcotte *et al*, 1999). Exposure to mild stress activates ESR, creating stress cross-protection and resistance to severe stress conditions (Mizzen and Welch, 1988; Gasch *et al*, 2000). Redefinition of codon identity resulted in constitutive stress due to permanent synthesis of aberrant proteins (misfolded) over millions of years, but how this stress response was (de)regulated remains to be studied.

Partial CUG identity redefinition in *S. cerevisiae* induced a stress response similar to that induced by heat shock and the proline analogue azetidine-2-carboxylic acid (AZC), two stress agents that also generate protein misfolding (Trotter *et al*, 2002). The 81 genes altered in response to CUG mistranslation were a subset of the 217 and 293 genes up- and downregulated by AZC, respectively (Trotter *et al*, 2002). However, AZC induced genes whose expression is controlled by the heat-shock factor (HSF) through the heat-shock element (HSE), while CUG ambiguity induced genes whose expression is controlled by Fkh1p, Fkh2p, Hsf1p, Msn2p and Msn4p (Supplementary Table 6). The latter (but not AZC) also induced the accumulation of trehalose and glycogen, which are under the control of Msn2p and Msn4p (Boy-Marcotte *et al*, 1999; Trotter *et al*, 2002). The transcription factors Fkh1p and Fkh2p regulate the yeast cell cycle, pseudohyphal growth and are involved in chromatin silencing at HML and HMR (Hollenhorst *et al*, 2000; Zhu *et al*, 2000; Sun *et al*, 2002). Interestingly, mistranslation of threonine ACA codons as alanine in *Schizosaccharomyces pombe* resulted in late mitotic defects, abnormal chromosome segregation, aneuploidy and decreased cell viability (Kimata and Yanagida, 2004). Therefore, it is now important to elucidate how CUG mistranslation triggered cell morphology and ploidy alterations and clarify how it created cell-cycle defects and mating impairment in *S. cerevisiae*.

The stress responses induced by CUG ambiguity and heat shock were rather similar, since the latter is also mediated by HSF, Msn2p and Msn4p. HSF activation by heat shock is due to titration by misfolded proteins of its chaperone HSP70, while Msn2p and Msn4p are activated by phosphorylation by protein kinase A (PKA) (Thevelein and de Winde, 1999). Also, like heat shock, CUG ambiguity induced thermotolerance (Santos *et al*, 1996), which is dependent on the activation of the protein kinase C-mitogen-activated protein kinase pathway (PKC1-MAP) (Kamada *et al*, 1995). Therefore, CUG identity redefinition may have interfered with important signalling pathways that modulated the general stress response in the *Candida* ancestor. If so, it is likely that such pathways have been remodelled in *Candida* over the last  $272 \pm 25$  million years of CUG identity redefinition. This hypothesis is strongly supported by major differences in the *S. cerevisiae* and *C. albicans* stress responses. For example, the former has a general stress response while the latter does not. *C. albicans* does respond to stress, but each stress response is unique and there is no cross-protection (Enjalbert *et al*, 2003). Furthermore, CaMsn4p and CaMnl1p (homologues of *S. cerevisiae* Msn2p) do not play an active role in the *C. albicans* stress response and their functions and those of their *S. cerevisiae* homologues diverged significantly (Nicholls *et al*, 2004). Also, *C. albicans* does not accumulate trehalose and glycogen in response to stress (Enjalbert *et al*, 2003).

The mobilization of molecular chaperones to correct misfolded proteins was prominent, but it did not compromise the capacity to respond to additional stress. Indeed, *S. cerevisiae* cells expressing *C. albicans* tRNA<sup>Ser</sup><sub>CAG</sub> were able to further induce the expression of heat-shock proteins when exposed to a temperature up-shift from 25 to 37°C during 30 min (Figure 5D). In other words, ambiguous cells were not impaired in their capacity to respond to different unfavourable conditions that require gene expression reprogramming. However, *S. cerevisiae* cells expressing *C. albicans* tRNA<sup>Ser</sup><sub>CAG</sub> grown under an additional permanent stress did not show further induction of the stress-protective proteins (Figure 5C), as observed for control cells, suggesting that these cells are pre-adapted to adverse environments.

### Conclusions

Genetic code alterations create major proteome chaos and should be eliminated by natural selection. The robust cellular response to codon-decoding ambiguity and the selective advantages observed under stress in yeast (Santos *et al*, 1999), explain this apparent paradox. Despite this, genetic code changes have a major impact on protein primary structure on a proteome-wide scale, and one would expect that they create genetic barriers to sexual reproduction on an evolutionary time scale. This is of relevance to the early evolution of the genetic code, since incorporation of new amino acids into the primordial code would have also created codon ambiguity and generated genetic barriers to exchange of genetic information between 'old code' and 'new code' lineages, contributing to the elimination of less competitive 'old code' lineages. Why code expansion stopped at the twenty-second amino acid remains to be elucidated, but it is likely that increased proteome complexity may have been the critical factor that prevented further expansion.

## Materials and methods

### Strains and growth conditions

*S. cerevisiae* strains used were based on the CEN.PK2 background (*MAT $\alpha$ /MAT $\alpha$ ; ura3-52/ura3-52; trp1-289/trp1-289; leu2-3\_112/leu2-3\_112; his3 $\Delta$ 1/his3 $\Delta$ 1*). CUG ambiguous cells were obtained by expressing *C. albicans* tRNA<sup>Ser</sup><sub>CAG</sub> either by transformation, performed according to the lithium acetate method (Gietz and Woods, 1994), or by integration in the genome, carried out by PCR-based gene disruption method (Lorenz *et al.*, 1995). Unless otherwise stated, control cells are *S. cerevisiae* cells transformed with the single-copy vector pRS315 (vector alone), and ambiguous strains carry the plasmids pUKC715 and pUKC716, containing G<sub>33</sub> and U<sub>33</sub> tRNA<sup>Ser</sup><sub>CAG</sub>, respectively (Santos *et al.*, 1996). Cells were grown to OD<sub>600</sub> 0.5 at 25°C, in minimal medium containing 0.67% yeast nitrogen base without amino acids, buffered at pH 5.8 with 1% succinate and 0.6% NaOH, and 2% glucose, supplemented with 100  $\mu$ g/ml of the required amino acids. For heat-shock experiments, cells were grown at 25°C, transferred to new flasks preheated at 37°C and grown for additional 30 min at 37°C. For sporulation, mating and stress plate assays, DNA fragments containing *S. cerevisiae* *HIS3* and *URA3* genes were integrated in the genome, and this strain was used to integrate the PCR products of the gene *KanMX* (control) or *KanMX*+tRNA<sup>Ser</sup><sub>CAG</sub> (G<sub>33</sub> or U<sub>33</sub>) at the *LEU2* locus. All integrations were confirmed by PCR and sequencing.

### CUG ambiguity reporter system

To quantify CUG ambiguity in *S. cerevisiae* cell lines expressing the *C. albicans* tRNA<sup>Ser</sup><sub>CAG</sub> G<sub>33</sub> and U<sub>33</sub>, a reporter protein was engineered. Briefly, this protein is based on the *CaPGK1* gene, has one CUG codon flanked by thrombin cleavage sites and a 6-histidine tail for purification by affinity chromatography. The *CaPGK1* promoter and ORF were cloned on pRS425 between the *Hind*III and *Pst*I restriction sites. External *Xho*I and *Sac*II restriction sites were eliminated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and the CUG-reporter cassette was inserted between internal *Xho*I and *Sac*II restriction sites, generating the plasmid pUA202. *S. cerevisiae* cells transformed with pUA202 were grown to OD<sub>600</sub> between 1 and 1.5 and harvested by centrifugation. The pellet was washed with mQ water and frozen at -80°C. Cells were lysed in ice-cold buffer A (6 M urea, 15 mM Na-HEPES, 150 mM NaCl, 0.01% Triton X-100, 10% glycerol, pH 7.8) supplemented with protease inhibitors (Complete EDTA-free protease inhibitor cocktail tablets (Roche) and 2 mM PMSF). Cells were broken by agitation in the presence of glass beads and lysates were clarified by centrifugation. The supernatant was transferred to a new, clean tube and incubated with 1 ml of Ni-Sepharose (Amersham) for overnight at 4°C with agitation. The His-tagged reporter protein was purified by Ni-NTA agarose chromatography as described by the resin manufacturer. The purified protein was dialysed against thrombin cleavage buffer (20 mM Tris-Cl pH 8.4, 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, from Novagen) and digested with thrombin (Novagen) at 26°C for 16 h.

### Mass spectrometry analysis

Multiple Reaction Monitoring (MRM) experiments were carried out on a hybrid quadrupole/linear ion-trap mass spectrometer (4000 QTrap; Applied Biosystems/MDS Sciex) using electrospray source and sample flow injection (FIA, Ultimate 3000; Dionex). The mass spectrometer was operated in triple quadrupole mode, with Q1 set to the specific precursor *m/z* value and Q3 set to the specific *m/z* value corresponding to a specific fragment of that peptide. The collision energy was tuned to optimize the intensity of the fragment ions of interest. In the MRM mode, a series of single reactions (precursor/fragment ion transitions) were measured sequentially and the cycle (2 s) was looped throughout the entire time of the FIA. MRM transitions were determined from the MS/MS spectra of the existing peptides (Supplementary Figures 1–3). Other parameters were tuned for best performance (Stahl-Zeng *et al.*, 2007).

### Sporulation assays

To determine sporulation efficiency, integrated cells were grown on sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose) for up to 12 days. At each time point, appropriate culture dilutions were prepared and both cells and tetrads were counted using a Neubauer chamber. For spore viability studies, aliquots of 2- to 4-day sporulation cultures were treated with

zymolyase for 10 min at room temperature. Digested asci were spread on thin plates and dissected with a MSM System Series 300 micromanipulator (Singer). After 4 and 7 days of incubation, plates were photographed in a GelDoc1000 (Bio-Rad) and colonies were counted. Spore viability was assessed in YPD (1% yeast extract, 2% peptone, 2% glucose) with geneticin (200 mg/l).

### Mating assays

Haploid control and ambiguous cells were obtained by sporulation as described above and their mating type was assessed by PCR as described (Huxley *et al.*, 1990). Cells with opposite mating type and distinct auxotrophic markers (His<sup>+</sup> or Ura<sup>+</sup>) were crossed. To do this, exponentially growing cells were counted in a Neubauer chamber and the volumes corresponding to 10<sup>6</sup> cells of each culture were mixed. Serial dilutions of each mating reaction were spotted in MM-His-Ura<sup>+</sup> geneticin, and after 3–4 days incubation at 30°C, plates were photographed as above.

### DNA content analysis

DNA content of cells was determined using flow cytometry, and fluorescence imaging of DAPI-stained cells was carried out with an Axio Imager.Z1 microscope (Zeiss), as described (Fortuna *et al.*, 2000).

### Transcriptome analysis

DNA microarray analyses were performed with six independent cultures for each strain and hybridized against the reference in dye-swap (three control strain cultures labelled Cy5 and three labelled Cy3), in a total of six microarrays for each mutant strain. RNA isolation, labelling and hybridization were carried out as described (van de Peppel *et al.*, 2003). Slides were scanned using the Agilent G2565AA DNA microarray scanner and raw data were extracted with ImageGene 4.0 (Biodiscovery). After print-tip Lowess normalization (Yang and Speed, 2002), data visualization and analysis were performed using GeneSpring (Silicon Genetics) and SAM (Tusher *et al.*, 2001). Comparison of GeneSpring data ( $P < 0.05$  by Student's *t*-test, fold change  $> 1.6$ ) and SAM analysis ( $\Delta = 2.15$ ; false discovery rate = 0.001) resulted in a common set of 170 significant genes, from which 81 were selected based on an average fold change of more than 1.6 in both mutant strains.

### Proteome analysis

Proteins were *in vivo* labelled with [<sup>35</sup>S]methionine and extracted and separated by 2D-PAGE as described (Boucherie *et al.*, 1995; Boucherie and Monribot, 2005). Radioactive protein spots were detected using a PhosphorImager (MolecularDynamics) and data analysis was performed with the ImageMaster software (Amersham Pharmacia Biotech).

### Proteasome activity assay

Proteasome activity was determined essentially as described, using the fluorogenic peptide succinyl-leucine-leucine-valine-tyrosine-MCA (s-LLVY-MCA) as substrate (Demasi *et al.*, 2003). Exponentially growing cells were harvested by centrifugation and disrupted with glass beads on a MiniBeadBeater (Biospec Products), in lysis buffer (10 mM K-Hepes, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>). Activity was determined for 100  $\mu$ g of protein extract in assay buffer (10 mM Tris-Cl, pH 8, 20 mM KCl 1 M, 5 mM MgCl<sub>2</sub>) with 5  $\mu$ M of s-LLVY-MCA. After a 60-min incubation at 37°C, fluorescence emission was measured at 435 nm (excitation at 365 nm) with a Perkin Elmer Luminescence Spectrometer (LS 50B).

### Trehalose and glycogen accumulation

Trehalose and glycogen were quantified as described (Parrou and Francois, 1997). Exponentially growing or heat-shocked cells were harvested by centrifugation, lysed in 0.25 M Na<sub>2</sub>CO<sub>3</sub> at 95°C for 4 h and incubated overnight either with trehalase (Sigma) or amylo-glucosidase (MERCK). Glucose present in the supernatant, from trehalose or glycogen breakdown, was determined using the glucose oxidase/oxidase kit (Sigma).

### Stress plate assays

Integrated cells were sporulated as described above, and spore viability was assessed in YPD, MM (0.67% yeast nitrogen base, 2% glucose, 0.2% drop-out mix containing all amino acids), SD (0.67% yeast nitrogen base without amino acids and ammonium sulphate,

2% glucose, 100 µg/ml of the required amino acids), MM + CdCl<sub>2</sub> (100 µM) or MM + H<sub>2</sub>O<sub>2</sub> (1.5 mM).

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

### Accession numbers

MIAME compliant protocols and datasets in MAGE-ML are accessible from ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) with the following accession numbers: E-TABM-196.

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