A Genetic Code Alteration Is a Phenotype Diversity Generator in the Human Pathogen Candida albicans

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Background. The discovery of genetic code alterations and expansions in both prokaryotes and eukaryotes abolished the hypothesis of a frozen and universal genetic code and exposed unanticipated flexibility in codon and amino acid assignments. It is now clear that codon identity alterations involve sense and non-sense codons and can occur in organisms with complex genomes and proteomes. However, the biological functions, the molecular mechanisms of evolution and the diversity of genetic code alterations remain largely unknown. In various species of the genus Candida, the leucine CUG codon is decoded as serine by a unique serine tRNA that contains a leucine 5’-CAG-3’ anticodon (tRNA\textsubscript{CAG}\textsuperscript{Ser}). We are using this codon identity redefinition as a model system to elucidate the evolution of genetic code alterations.

Methodology/Principal Findings. We have reconstructed the early stages of the Candida genetic code alteration by engineering tRNAs that partially reverted the identity of serine CUG codons back to their standard leucine meaning. Such genetic code manipulation had profound cellular consequences as it exposed important morphological variation, altered gene expression, re-arranged the karyotype, increased cell-cell adhesion and secretion of hydrolytic enzymes. Conclusion/Significance. Our study provides the first experimental evidence for an important role of genetic code alterations as generators of phenotypic diversity of high selective potential and supports the hypothesis that they speed up evolution of new phenotypes.

INTRODUCTION

A number of exceptions to the standard genetic code have been discovered in prokaryotic and eukaryotic organisms, involving nonsense-to-sense and sense-to-sense codon identity changes [1,2]. Twenty five of such alterations have been recorded in mitochondrial genetic codes of metazoans, fungi, red algae, green plants, alveolates, stramenopiles, haptophytes and euglenozoans [3]. The most remarkable alterations involve metazoan arginine AGA and AGG (AGR) codons, which changed their identity to serine at the base of the phylogenetic tree, and later on to translation-stop in vertebrates and to glycine in urochordates [4,5].

In bacteria and eukaryotic cytoplasmic systems, 18 genetic code alterations have also been recorded, but unlike in mitochondria, they involve, with one exception, nonsense-to-sense codon identity changes or codon unassignments (codons that vanished from genomes). For example, in Micrococcus spp., Masploasma spp. and Pseudomonas rhizovarhodni the AGA/AUA, CGG and UGA codons are unassigned, respectively. In Bacillus subtilis UGA codons are used to terminate mRNA translation (stop codons) and to insert tryptophan, creating readthrough proteins [6]. In various species of ciliates, 1 or 2 stop codons changed their identity to either glutamine (UAA and UAG), glutamate (UAU) or cysteine (UGA). Interestingly, these genetic code alterations apparently minimize nonsense errors arising from re-assembly of the ciliates fragmented genome [7–10].

Those genetic code alterations show that certain codons, namely codons that start with uridine (UNN) or adenosine (ANN), in particular stop (UAA, UAG or UGA), arginine (AGR), serine (AGY) and isoleucine (AUU) codons are more prone to identity changes than others. The only exception to this first codon base rule occurs in yeast mitochondria where cytosine starting codons (CNN), namely leucine CUN codons, changed their identity to threonine. Also, the leucine CUG codon altered its identity to serine in the cytoplasm of various Candida and Debaryomyces species [11–13]. These findings suggest that the strength of the interaction of the first codon-anticodon base pair plays an important role in the evolution of genetic code alterations. Indeed, the change of identity of UAA and UAG stop codons to glutamine in various ciliates involved first base misreading by glutamine tRNAs, which decode glutamine CAA and CAG codons [8,14]. But, it also indicates that other forces beyond codon-anticodon interaction play important roles in codon identity redefinition.

The unexpected flexibility of the genetic code described above is further highlighted by insertion of selenocystein (21st amino acid) in the active sites of prokaryotic and eukaryotic selenoproteins and insertion of pyrolysine (22nd amino acid) in the active site of monomethylamine methyltransferase of Methanosaeraa barkeri [15,16]. These expansions involving reprogramming of UGA and UAG stop codons, respectively, highlight the potential of genetic code alterations/expansions to generate functional innovation. This hypothesis is supported by recent artificial expansion of the genetic code through synthetic biology methodologies. Indeed, 49 non-natural amino acids have already been incorporated into E. coli, yeast and mammalian cells [17–20], to produce novel proteins of biotechnological and biomedical interest. This dramatic demonstration of genetic code flexibility also unveiled an extraordinary capacity of complex organisms to tolerate partial codon identity redefinition [21].
We are using C. albicans as a model system to elucidate the evolution of genetic code alterations. In this case, a unique serine tRNA (tRNACAG{superscript{Ser}}) decodes leucine CUG codons as serine [22–24]. However, the tRNACAG{superscript{Ser}} is recognized by both leucyl- and seryl-tRNA synthetases (LeuRS and SerRS) and it is aminoacylated in vitro with both serine (97%) and leucine (3%) [25]. This unusual dual aminoacylation of the tRNACAG has been preserved to the present day [26,27], raising the intriguing possibility that it may play a role in C. albicans biology. It also provides strong support for the hypothesis that codon identity redefinition is driven through codon decoding ambiguity [28,29].

In here, we have reconstructed the early stages of the C. albicans genetic code alteration to shed new light into those questions. This partial reversion of CUG identity from serine back to leucine triggered morphogenesis, phenotypic switching, and up-regulated expression of genes involved in cell adhesion and hyphal development and increased secretion of proteinas and phospholipases. Important karyotype alterations were also observed. The overall data suggest that C. albicans CUG ambiguity is an important phenotypic diversity generator and highlight important and yet overlooked functional roles for genetic code alterations.

RESULTS
Reverting CUG identity from serine back to leucine

The CUG identity alteration from leucine to serine was initiated 272 ± 25 My ago through a mutant serine tRNA (tRNACAG{superscript{Ser}}), containing a 3'-CAG-3' anticodon, which could decode CUG codons (see introduction) [26,22]. Initially, this unique tRNACAG{superscript{Ser}} competed with endogenous tRNA{superscript{Leu}}, which decoded CUG codons as leucine [30], creating a new situation where both leucines and serines were inserted at CUG positions on a genome wide scale (Figure 1). For reasons not yet fully understood, the wild type tRNA{superscript{Leu}} disappeared from the Candida ancestor genome leaving CUG decoding exclusively to the mutant tRNACAG{superscript{Ser}}. Disappearance of the tRNA{superscript{Leu}} should have abolished the ambiguous status of CUG codons, however the tRNACAG{superscript{Ser}} is recognized by both LeuRS and SerRS (see above), creating a serine tRNA that exists in 2 distinct forms, namely ser-tRNACAG{superscript{Ser}} (charged with serine) and leu-tRNACAG{superscript{Ser}} (charged with leucine). This ambiguous tRNA still exists in C. albicans [25].

We have re-created in C. albicans (CAI-4 strain) the high ambiguity status of CUG codons that existed 272 ± 25 My ago in the Candida ancestor (Figure 1). For this, we have expressed Saccharomyces cerevisiae wild type and mutant tRNAs, which decode CUG codons as leucine, in C. albicans (Figure 2A–D). These tRNAs{superscript{Leu}} competed with the novel tRNACAG{superscript{Ser}} for CUG codons at the ribosome A-site, but were not lethal. We have hypothesized that such genetic manipulation would increase CUG ambiguity and could uncover phenotypes associated to the residual ambiguity (3%) [25] of CUG codons in C. albicans.

Three S. cerevisiae tRNA{superscript{Leu}} genes, namely a wild type tRNA{superscript{Leu}}, an S. cerevisiae LeuRS and two mutant tRNACAG{superscript{Ser}} (containing U32 or G32 in the anticodon-loop), plus a control tRNAAGA{superscript{Ser}} (Figure 2B–D) were successfully expressed in C. albicans CAI-4 cells, as shown by Northern blot analysis of tRNAs fractionated by acidic-PAGE, which separates charged from uncharged tRNAs [31] (Figure 3A). This is in line with previous experiments that showed that the identity elements of C. albicans and S. cerevisiae LeuRS and SerRS are identical [32,33]. Transformation efficiency of plasmids carrying the S. cerevisiae tRNA{superscript{Leu}} genes was lower than that of control plasmids (Figure 3B) containing no tRNA gene or containing a cognate serine decoder tRNA (pUA16) (Figure 2D). In other words, the tRNA{superscript{Leu}} were slightly toxic to C. albicans, which supported the hypothesis that they were fully functional and could incorporate leucine at CUG positions. Remarkably, clones that survived transformation showed no decrease in growth rate (Figure 3C), suggesting that they adapted to increased CUG ambiguity.

Ambiguous CUG decoding generated important phenotypic diversity

Interestingly, expression of those S. cerevisiae tRNA{superscript{Leu}} genes in C. albicans triggered morphogenesis in both solid and liquid media (Figure 4). The pUA15 clones displayed extensive morphological variation (Figure 4B–C) and produced highly heterogeneous cell populations containing elongated-ovoid cells, pseudohypa and true hypha (not shown). Some pUA15 clones produced hypha that occupied sectors or entire colonies. Notably, morphological events that gave rise to these phenotypes happened spontaneously without external inducing factors. As expected, control pUA12 and pUA16 clones had homogeneous morphology and formed smooth-white colonies similar to those of untransformed C. albicans CAI4 (Figure 4A). Similar results were obtained with clones pUA13 and pUA14 (data not shown). Apart from morphogenesis, CUG ambiguity also induced phenotypic switching, which is a C. albicans phenotype characterized by reversible induction of opaque or myceliated sectors in white smooth colonies [34]. High frequency of phenotypic switching (63–88%) was obtained for all clones expressing S. cerevisiae leucine tRNAs (pUA13–15), but not for control clones (pUA12 and pUA16) (Figure 4D).

CUG ambiguity also increased cell adhesion in liquid and solid media (Figure 5A), and once more, this phenotype was exacerbated in pUA15 clones, as they displayed strong flocculation in liquid media (Figure 5A). Interestingly, more than 50% of the genes involved in adhesion contain CUG codons. For example, the ALS gene family which encodes cell-surface glycoproteins that mediate
adhesion to host surfaces [35], contain various CUG codons (3CUGs-ALS2, ALS3, ALS8; 4 CUGs-ALS4; 5CUGs-ALS1; 11CUGs-ALS6; 12CUGs-ALS9; 18CUGs-ALS7). It is not yet clear whether the change of serine (polar) for leucine (hydrophobic) at CUG positions in the Als proteins is responsible for the flocculation and exacerbated agar adhesion observed in pUA15 clones. But, the strong adhesion phenotypes resulting from expression of ALA1, EAP1 and members of the C. albicans ALS gene family, namely ALS1 and ALS5 in S. cerevisiae, support the hypothesis that the replacement of serines with leucines at CUG positions increases adhesion [36–41].

Finally, expression of S. cerevisiae tRNA^{Leu} (pUA15) in C. albicans increased production of extracellular hydrolases, namely secreted aspartic proteinases (SAP) and phospholipases, as determined on agar plates supplemented with BSA and egg yolk, respectively (Figure 5B,C). Hydrolysis of these substrates lead to formation of an halo of precipitated peptides around the colonies, indicative of SAP and phospholipase production [42]. Since adhesion, SAPs and phospholipases are important virulence attributes [43–45], those phenotypes are relevant to C. albicans pathogenesis and indicate, for the first time, that codon ambiguity and the Candida genetic code alteration may play a role in infection. It will be most interesting to put this hypothesis to experimental test as a positive result would clearly show that the negative impact of codon decoding ambiguity could be overcome by high selective potential of novel phenotypic diversity.

**Gene expression alterations**

In order to elucidate how CUG ambiguity generated phenotypic diversity we have carried out gene expression profiling of C. albicans cells expressing the S. cerevisiae tRNA^{Leu} (pUA15 clones), using DNA microarrays. However, the diversity of phenotypes and permanent switching between phenotypes in culture (Figure 4) created cell variability and culture instability that prevented us from obtaining meaningful mRNA expression profiles (data not shown). To overcome this limitation, we have tried to stabilize some of the phenotypes on solid media, but, once again, this turned out to be very difficult to achieve due to very high switching between morphological forms. As the mRNA profiles represented average values of a variety of phenotypes, reproducibility was low and most genes failed to pass our microarray statistical filters. Despite this, we were able to detect 4 genes whose expression was consistently altered in the microarray data sets and were relevant to the pUA15 phenotypes, namely the hypha-specific G1 cyclin-related protein HGC1 (2.64 fold), the hypha-specific gene HWP1 (41.76 fold), the white-opaque switch regulator WOR1 (7 fold) and the transcription factor MCM1 regulator of hyphal growth (-1.84 fold) (Figure 6). Expression of these genes was confirmed by Real Time PCR, as described in materials and methods.

The WOR1 gene (7 fold up-regulated) is a master regulator of white-opaque switching and its expression induced the white-opaque phase transition [46,47]. This provides a likely explanation for the high switching between morphological forms observed in the pUA15 clones.
for the strong induction of white-opaque switching in pUA15 clones, whose cells switch at high frequency. The \textit{HWP1} gene (41,76±9,96 up-regulated) encodes a hyphal-specific cell wall mannoprotein, which is a substrate for mammalian transglutaminases and plays a crucial role in adhesion of \textit{C. albicans} to epithelial cells [48]. Interestingly, \textit{HWP1} expression was correlated with \textit{MCM1} repression (-1.84 fold), confirming previous results that showed that expression of one of these genes represses expression of the other [49]. \textit{MCM1} plays an important role in cell morphology and its expression is auto-regulated by a feedback control mechanism. Since both low and high Mcm1p levels lead to hyphal formation, it may act as a recruiting regulatory factor for morphogenesis in \textit{C. albicans}. Depletion of Mcm1p induced transcription of \textit{HWP1}, however no Mcm1p binding site was found in the \textit{HWP1} promotor and it is not yet clear how the former activates transcription of the later. Finally, the \textit{HGC1} gene was also up-regulated (2.64 fold). This gene is crucial for hyphal formation by promoting apical bud elongation and it is strongly induced during morphogenesis. It is not required for expression of hypha-specific genes (HSGs), like \textit{HWP1}, but is positively regulated by the cAMP/PKA pathways and repressed by Tup1 and Nrg1 morphogenesis repressors [50].

**Increased CUG ambiguity increased \textit{C. albicans} ploidy**

Up-regulation of the \textit{WOR1} gene and the high percentage of opaque cells (mating competent) observed in pUA15 clones prompted the question of whether CUG ambiguity would induce mating in \textit{C. albicans}. Indeed, pUA15 opaque cells formed conjugation tubes and mating figures which were readily observed by optical microscopy (Figure 7A). Furthermore, flow cytometry analysis of pUA15 clones identified sub-populations of 4N, 6N and 8N cells (Figure 7B), supporting the hypothesis that ambiguous \textit{C. albicans} mated at high frequency. Since \textit{C. albicans} is a diploid organism with a sexual life cycle [51], and considering that its mating locus (MTL) is heterozygotic in mating incompetent white cells (MTLa/\textit{a}) and homozygotic in mating competent opaque cells (MTLa/\textit{a} or MTLa/\textit{a}) [52], the latter were isolated from pUA15 clones and were analysed for MTL homozygoty. All clones analysed were MTLa/\textit{a}, suggesting that expression of tRNALeu \textsubscript{(pUA15)} induced biased MTLa/\textit{a} homozygoty (Figure 7C), creating an excess of MTLa/\textit{a} over MTLa/\textit{a} cells. These results were in good agreement with up-regulation of the \textit{WOR1} gene as its expression is controlled by the MTLa1-/\textit{a}2 heterodimer, which, in turn, controls white-opaque transition and mating competence [46,47]. That is, switching from white-to-opaque phase required conversion of heterozygotic MTL to homozygotic MTL inducing mating competence [52]. Therefore, it is likely that MTL homozygoty induced by tRNALeu derepressed the \textit{WOR1} gene, which, in turn, promoted the white-to-opaque transition and mating.

The presence of cells with high ploidy (6N and 8N) in pUA15 opaque cultures (Figure 7B), prompted us to monitor ploidy variability. Most clones showed increased ploidy ranging from 4N to 8N (data not shown), however very large cells with remarkably high ploidy (>64N) were also observed (Figure 8A). In general, ploidy variability between clones was higher than previously described [53,54] and raised the question of whether those high chromosome numbers could return to 2N over time. Since wild

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**Figure 3. Expression of \textit{S. cerevisiae} tRNA\textsubscript{Leu} in \textit{C. albicans}.** A) Aminoacylation \textit{in vivo} in \textit{C. albicans} of \textit{S. cerevisiae} tRNA\textsubscript{UAG/CAG}\textsubscript{Leu} and tRNA\textsubscript{AGA}\textsubscript{Ser} was monitored by Acidic Page and Northern Blot analysis. For this, total tRNAs were extracted under acidic conditions from pUA13, pUA14, pUA15, and pUA16 clones and fractionated on an acidic polyacrylamide gel, as described in materials and methods. These gels separated deacylated (-AA) from aminoacylated tRNAs (+AA), which were detected using a tDNA\textsubscript{Leu/Ser} probe labeled with [32P]. B) Transformation efficiencies of plasmids encoding tRNALeu, which decoded the \textit{C. albicans} serine codons as leucine, was significantly lower than that of control plasmids (pUA12 and pUA16), indicating that the leucine tRNAs were slightly toxic. C) However, clones that survived the transformation procedure adapted readily to CUG ambiguity and showed growth rates similar to control clones (pUA12).

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type C. albicans undergoes a process of chromosome loss after mating, which decreases its ploidy from 4N back to 2N [55–57], we hypothesized that high ploidy in pUA15 clones could also be reduced. To test this hypothesis, clones with very high ploidy (large cells) were successively re-plated on fresh agar plates and their ploidy was monitored over time by flow cytometry. Indeed, ploidy reverted to 2N or 4N after several passages on fresh agar, confirming the above hypothesis (Figure 8B).

The above results also prompted us to check whether transformation of C. albicans with the pUA15 plasmid destabilized its karyotype. The C. albicans karyotype is characterized by frequent chromosome rearrangements, in particular of the chromosome R, which contains rDNA cistrons [58,59]. We wondered whether the tRNAlue would affect rRNA metabolism and protein synthesis. Indeed, various rearrangements of the R-chromosome were readily observed (Figure 8C). In particular, the size of R-chromosomes increased in some clones, decreased in others and these rearrangements affected most cells (Figure 8C). It will now be most interesting to investigate whether CUG ambiguity affects ribosome assembly and the rate of protein synthesis.

**DISCUSSION**

The identity of CUG codons is variable in the genus Candida. Indeed, C. glabrata decodes CUGs as leucine, C. cylindracea changed their identity completely to serine and the other Candida species decode them ambiguously [13,25,60,61]. These differences in CUG decoding arose from differences in the structure of the tRNA_{CAG}^Ser, which is the only cognate tRNA for CUG codons in Candida. Indeed, the various tRNA_{CAG}^{Ser} have identity determinants for both SerRS and LeuRS [1]. For example, the C. albicans tRNA_{CAG}^{Ser} contains identity elements for the LeuRS, namely m1G37 and the middle base of the anticodon (A35), but the discriminator base at position 73 (G73) is specific for SerRS and not for LeuRS (A73 discriminator) [62]. This base is critical for the recognition of tRNAs by aminoacyl-tRNA synthetases (aaRSs) and one would expect that the LeuRS would not recognize tRNAs with G73. Therefore, charging the tRNA_{CAG}^{Ser} with leucine [25] suggests that the Candida LeuRS may have evolved a unique mode of recognition of its cognate tRNA_{Lue}. Interestingly, the presence of a unique guanosine in the turn of the anticodon-loop (G-turn) of the tRNA_{CAG}^{Ser} (G33), a conserved position occupied by U33 (U-turn), reduced leucylation efficiency of the tRNA_{CAG}^{Ser} [25]. That is, recognition of the ancestral tRNA_{CAG}^{Ser} by the LeuRS was efficient and G33 acted as a leucine identity anti-determinant. The reason for G33 selection is not yet clear, but one possibility is that it may have decreased the toxicity of the mutant tRNA_{CAG}^{Ser} during the early stages of CUG identity alteration [27,63].

The dual recognition of the tRNA_{CAG}^{Ser} by the LeuRS and SerRS indicates that there are two forms of the tRNA_{CAG}^{Ser} in the cytoplasm of C. albicans, namely Ser-tRNA_{CAG}^{Ser} and Leu-tRNA_{CAG}^{Ser}, which are charged with serine and leucine, respectively. These 2 tRNAs generate ambiguity at CUG codons since they compete with each other for CUGs at the ribosome A-site. Interestingly, such CUG ambiguity was not constant over the 272±25My of evolution of the genetic code alteration (see introduction) [30]. It was high during the early stages of CUG identity change (when the tRNA_{CAG}^{Ser} gene appeared), and
decreased gradually due to elimination of the tRNA<sup>Leu</sup> gene from the genome of the Candida ancestor [30]. Reconstruction of the high level of CUG ambiguity, which existed during the early stages of CUG identity alteration, provided the first insight on how the genetic chaos created at the onset of CUG identity change may have generated phenotypic diversity of evolutionary and adaptive relevance. In extant C. albicans, morphological variation alters cell surface antigens and it is likely that this pathogen uses its sophisticated capacity to generate morphological variation as a strategy to escape the immune system. Furthermore, secreted proteinases and phospholipases are important C. albicans virulence attributes and their increased activity in pUA15 clones may also be relevant to virulence. It will now be interesting to engineer stable high level mistranslation in C. albicans and test the virulence of the recombinant strains in mice models.

The high phenotypic diversity of pUA13, pUA14 and pUA15 clones and constant transition between phenotypes prevented us from carrying out a detailed study of the impact of CUG ambiguity on gene expression. However, the strong up-regulation of the hyphal specific gene (HWP1) and of the master regulator of the white-opaque transition (WOR1), may explain, at least in part, some of the phenotypes observed. As a hyphal specific gene (HSG), high HWP1 expression, induced by CUG ambiguity, is in agreement with spontaneous morphogenesis events that generate filamentous cell populations. Furthermore, HWP1 up-regulation may also increase adhesion since Hwp1p is a glycosylphosphatidylinositol cell wall adhesin (GPI-CWP) and mediates attachment of C. albicans cells to human endothelial and epithelial cells [44]. The observed adhesion phenotype (Figure 5A) may have also resulted from the combined up-regulation of HWP1 and WOR1 genes, since the later, previously known as EAP2 (enhanced adhesion to polystyrene), mediates C. albicans and S. cerevisiae adhesion to polystyrene and epithelial cells [41].

Apart from its putative role in adhesion, up-regulation of WOR1 may also explain the white-opaque phenotype since Wor1p is a transcriptional regulator of white-opaque switching [46,47]. Indeed, Wor1p is present in very low amounts in white cells and accumulates in opaque cells. WOR1 is repressed by the heterodimer MTL α1/α2 and is activated by Wor1p itself when cells become homozygous MTL αα/αα or MTL αα/αα. Increased accumulation of Wor1p triggered white-opaque switching and repressed its own transcription by a feedback regulatory mechanism [46,47]. The up-regulation of the HGC1 gene (Figure 6), which is a hypha-specific gene encoding a G1 cyclin-related protein, that plays a role in hyphal morphogenesis, was also significant. Since it is transcriptionally regulated by hyphal-inducing rather than cell cycle signals [50], its up-regulation in ambiguous cells supports the hypothesis that it functions independently of other cell cycle cyclins.

CUG ambiguity also generated karyotype alterations and formation of polyploids and aneuploids. Ploidy-shift has been associated with chromosomal rearrangements [53,54,64] which also generates morphological variation [65–67], antifungal resistance [68], adaptation to alternative carbon sources [69,70], or even with homozigosity of chromosome-V [71]. This suggests that part of the phenotypic diversity observed in pUA13, pUA14 and pUA15 clones may have resulted from karyotype alterations, or from a combination of up-regulation of the genes described above.
Genetic code alterations pose important new biological questions whose answers remain elusive. It is now clear that a number of them evolved through codon decoding ambiguity, required significant structural change of protein synthesis machineries and reprogrammed codon usage [1,5,30]. However, codon decoding ambiguity is toxic, decreases fitness and may ultimately lead to cell death, as is the case in multicellular organisms [19,74]. For these reasons, evolution of genetic code alterations through codon ambiguity is most puzzling. This study unveiled possible ways of overcoming the negative impact of codon ambiguity by high selective potential generated through generation of phenotypic diversity. The molecular mechanism used to generate such phenotypic diversity remains to be elucidated. However, the adaptive potential of the unveiled phenotypes strongly suggests that CUG ambiguity may have been preserved in *Candida* spp. as a novel generator phenotypic diversity. We have previously shown that codon ambiguity in *S. cerevisiae* creates a competitive edge under stress by inducing the general stress response and pre-adapting cells to sudden environmental changes [75]. Therefore, the toxicity of codon ambiguity is not an impediment to codon identity redefinition, supporting the hypothesis that codon misreading plays a critical role in the evolution of genetic code alterations and genetic code expansion.

Plasmids

A multi-cloning site was inserted [NruI/EcoRV] in the low-copy *C. albicans* vector pRM1 [76]. The resulting vector was named pUA12. For heterologous expression of the *S. cerevisiae* tRNAs genes in CAI4, a genomic DNA fragment containing *S. cerevisiae* tRNA gene (700 bp), previously amplified by PCR, was cloned into the multi cloning site of pUA12 plasmid. *S. cerevisiae* Leu-tRNAGAG and a 300 bp flanking region, upstream and downstream of the gene, was amplified with the following set of primers: 5'-GGCGACTGTCCAGACTTAGTAAAGCT-3' (tRNAGAG); and reverse primer 5'-ATGCATAAAAACAAAA-3'. These primers introduced a mutation in the *tRNA* gene, which controls cell morphology through the recruitment of other morphogenesis regulatory factors.

Conclusions

Genetic code alterations pose important new biological questions whose answers remain elusive. It is now clear that a number of them evolved through codon decoding ambiguity, required significant structural change of protein synthesis machineries and reprogrammed codon usage [1,5,30]. However, codon decoding ambiguity is toxic, decreases fitness and may ultimately lead to cell death, as is the case in multicellular organisms [19,74]. For these reasons, evolution of genetic code alterations through codon ambiguity is most puzzling. This study unveiled possible ways of overcoming the negative impact of codon ambiguity by high selective potential generated through generation of phenotypic diversity. The molecular mechanism used to generate such phenotypic diversity remains to be elucidated. However, the adaptive potential of the unveiled phenotypes strongly suggests that CUG ambiguity may have been preserved in *Candida* spp. as a novel generator phenotypic diversity. We have previously shown that codon ambiguity in *S. cerevisiae* creates a competitive edge under stress by inducing the general stress response and pre-adapting cells to sudden environmental changes [75]. Therefore, the toxicity of codon ambiguity is not an impediment to codon identity redefinition, supporting the hypothesis that codon misreading plays a critical role in the evolution of genetic code alterations and genetic code expansion.
CCGCTCGAGCGGGATGCACAATCGTTGTCTGTAATGA-3′ and 5′-GCTATGGGCCCAAGCACAAA TGGTTATGA-CAATTG ATG-3′. The pUA16 control plasmid was constructed by inserting a XhoI/AvaIII genomic DNA fragment (600 bp), containing *S. cerevisiae* Ser-tRNAAGA gene amplified by PCR with the following pair of primers 5′-CCGC TCGAGCGGGAGGATTCCTATATCCTTGAGGAG-3′ and 5′-GGCTCGATGCATGCCAGGAA-GAAATACACTGC-3′, into the multicloning site. All DNA amplifications were carried out using a Mastercycle gradient (Eppendorf) and standard PCR protocols.

**Plasmid transformation**

Transformation of *E. coli* was carried out as described by [77] CAI4 transformation was performed by the spheroplast method as described in the Manual for Preparation and Transformation of *Pichia pastoris* Spheroplasts (version A, Invitrogen).

**Northern Blot analysis**

Acidic Northern Blot analysis was performed as described by Santoso et al. [96]. For total tRNA extractions, 250 ml cultures grown overnight in YEPD or MM-uri medium were harvested at an OD<sub>600</sub> of 0.7–0.9 and the pellets were frozen at −70°C overnight. Cells were resuspended in 5 ml lysis buffer (0.3M sodium acetate, pH 4.5, 10 mM EDTA), 1 vol. phenol equilibrated with sodium citrate pH 4.5 and baked glass beads. Cell suspension was vigorously shaken ≥30 seconds and incubated on ice for periods longer than 30 seconds, this procedure was repeated 8 times [78]. The aqueous phase containing RNAs was separated from the phenolic phase by centrifugation at 3200×g for 20 min at 4°C and then transferred to a new Falcon tube and re-extracted with fresh phenol. Aqueous phases containing RNAs were harvested by centrifugation at 3200×g for 20 min at 4°C and applied to a 20 ml DEAE-cellulose column equilibrated with 0.1 M sodium acetate pH 4.5. tRNAs were eluted with 0.1 M sodium acetate/1 M sodium chloride and precipitated with 2.5 vol. ethanol, resuspended in 10 mM sodium acetate pH 5.0/1 mM EDTA, and stored at −20°C. The deacylated tRNAs were obtained by incubation at 37°C for 1 h in 1 M Tris pH 8, 1 mM EDTA buffer [26].

tRNAs were fractionated at 4°C in 7.5% agarose gel of 8 M urea (30 cm long, 0.8 mm thick), buffered with 0.1 M sodium acetate pH 5.0. The 7.5% acidic gels were run at 300 V until bromophenol blue dye reached the bottom [31]. Fractionated tRNAs were transferred to nitrocellulose membranes (Hybond N, Amersham). Membranes were pre-hybridized for 6 h in a Hybridization oven at 50°C in 50% formamide, 5×SSC, 1% SDS, 0.04% Ficoll, 0.04% polyvinylpyrrolidone and 250 μg/ml sheared salmon sperm DNA [79]. Hybridization was performed overnight in the above buffer using probes labelled with [α-<sup>32</sup>P]dCTP by PCR, using standard protocols [80], except that the amount of dCTP was reduced from 100 to 50 mM and 5 mmol (30 μCi) 6000 Ci/mmolv [α-<sup>32</sup>P]dCTP was added to the reaction mixture. In order to decrease the background level of free radioactivity, 50 PCR cycles were performed to decrease the amount of non-incorporated [α-<sup>32</sup>P]dATP. Membranes were washed at low stringency in 1×SSC, 0.5% SDS at 50°C or at high stringency in 0.1×SSC,
0.5% SDS at 65°C for 1 h. The membranes were exposed overnight with intensifying screens and developed using a Molecular Imager FX (Biorad).

Switching frequencies and phenotypic characterization

*C. albicans* grown overnight at 30°C in MM-uri were serially diluted to 1000 cells per ml. Approximately 50 cells were plated onto fresh agar plates and then allowed to grow at 30°C for 7 days in a humidified incubator to prevent drying of the agar surface. Sected colonies displaying atypical colonies were scored and the data was analyzed for statistical significance using ANOVA. Colonies were photographed using a Stemi 2000-C dissecting microscope equipped with AxioCam HRc camera and Axio Vision Software from Zeiss. Cells were photographed using a Zeiss MC80 Axioplan 2 light microscope.

Real Time RT-PCR

Total RNA was prepared from *C. albicans* using hot acid phenol [81]. First-strand cDNA synthesis was carried out using the Superscript II RT kit from Invitrogen and its quantification was carried out in an Applied Biosystems 7500 Real Time PCR system using the SYBR Green I dye quantification assay (Power SYBR Green PCR master Mix). Primer concentrations were tested (0.2 to 0.4 μM) to ensure the lowest threshold cycle (Ct) and the highest signal magnitude against the target template and to ensure non-specific product formation, resulting from primer dimmerization. After amplification, reactions were checked for presence of non-specific products through dissociation curve analysis. Each gene was quantified using 9 replicas of both control (pUA12) and ambiguous (pUA15) clones and a mean value was calculated. Outliers were rejected using critical values of Dixon’s “Q” parameter at 95% confidence level [82].

Determination of extra cellular hydrolytic activity

*C. albicans* strains were screened for the production of extra cellular phospholipase and secreted aspartic proteinase activity by growing cells on MM-uri agar supplemented with 10% egg yolk (Merck) and 10% bovine albumin (Sigma), respectively. A 3×2 μl suspension of 10^7 cells/ml in PBS was plated on the surface of the agar medium and left to dry at room temperature. The culture

Figure 8. Ambiguous CUG decoding induced karyotype rearrangements and ploidy-shift. A) In ambiguous cell lines (pUA15) polyplody was predominant and very high ploidy was often detected (~32N). Aneuploidy was also observed (6N). B) However, after plating cells several consecutive times on fresh agar chromosome numbers were reduced indicating that most cells returned to low ploidy (2N or 4N). Ploidy reduction after mating normally occurs by chromosome loss in *C. albicans* and it is likely that such mechanism also played a role in ploidy reduction in pUA15 transformed cells. C) CUG ambiguity also promoted extensive rearrangements of the R-chromosome (highlighted in white circles). Chromosomes were separated by PFGE on 0.6% agarose gels under the following conditions: 120–300 s for 24 h at 80 V, then 420–900 s for 48 h at 80 V. The numbers 1–7 and R identify *C. albicans* chromosomes.

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Karyotype analysis was performed using Pulsed-Field Gel Electrophoresis (PFGE) [85].

**Genome analysis**

DNA content of *C. albicans* cells was determined using FACS analysis [84]. Karyotype analysis was performed using Pulsed-Field Gel Electrophoresis (PFGE) [85].

**MTL analysis**

PCR analysis of the MTL configuration was carried out in pUA12 and pUA15 cells, through amplification of *db*/*p* and *MTLa1* genes, respectively, using the following pairs of primers: 5′-GGTTGCAATGAGGCTGATAC 3′ and 5′-ACATGTGTGC-GCCCAACTCC 3′; 5′-TTGAAAGCTGAGGCGAGG 3′ and 5′-ATGAATCCCCCTTCTCCATTAGG 3′.

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**Author Contributions**

Conceived and designed the experiments: MS. Performed the experiments: GM RR IM SM DM. Analyzed the data: MS GM IM LC. Contributed reagents/materials/analysis tools: MS. Wrote the paper: MS IM.
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