LETTER

doi:10.1038/nature10165

Converting nonsense codons into sense codons by targeted pseudouridylation

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All three translation termination codons, or nonsense codons, contain a uridine residue at the first position of the codon[1–3]. Here, we demonstrate that pseudouridylation (conversion of uridine into pseudouridine (Ψ), ref. 4) of nonsense codons suppresses translation termination both *in vitro* and *in vivo*. *In vivo* targeting of nonsense codons is accomplished by the expression of an H/ACA RNA capable of directing the isomerization of uridine to Ψ within the nonsense codon. Thus, targeted pseudouridylation represents a novel approach for promoting nonsense suppression *in vivo*. Remarkably, we also show that pseudouridylated nonsense codons code for amino acids with similar properties. Specifically, ΨAA and ΨAG code for serine and threonine, whereas ΨGA codes for tyrosine and phenylalanine, thus suggesting a new mode of decoding. Our results also suggest that RNA modification, as a naturally occurring mechanism, may offer a new way to expand the genetic code.

Ψ, the C5-glycoside isomer of uridine, has many structural and biochemical differences from uridine[5] (Supplementary Fig. 1). Thus, it is possible that replacement of the uridine within a nonsense codon with Ψ may affect translation termination. To investigate the possible effect of Ψ on translation termination, we developed an *in vitro* nonsense suppression assay (Fig. 1a). Briefly, we synthesized an artificial messenger RNA that encoded a 6× histidine (6His) tag at the amino terminus and a Flag tag at the carboxy terminus. In between the 6His tag and Flag tag, a pseudouridylated nonsense codon (ΨAA) was inserted (Fig. 1a). In addition, two control transcripts were created with the same sequence except that the Ψ of the nonsense codon was either changed to uridine (U), thus forming an authentic nonsense codon (UAA), or substituted with a cytidine (C), thus eliminating the nonsense codon (CAA) (Fig. 1a). Anti-6His immunoblot analysis indicated that all three RNAs were equally translated in rabbit reticulocyte lysate (Fig. 1b, top panel). Remarkably, however, according to the anti-Flag blot, the presence of a Ψ within the termination codon resulted in robust nonsense suppression (Fig. 1b, lower panel). Specifically, the ΨAA-containing transcript produced a strong Flag signal which is almost comparable to that produced by the CAA-containing transcript (Fig. 1b). In contrast, only a background level of Flag was produced when the UAA-containing transcript was used (Fig. 1b). Our results thus indicate that presence of Ψ in a termination codon effectively suppresses translation termination *in vitro*.

The *in vitro* results prompted us to investigate whether the pseudouridylation of a termination codon would elicit nonsense suppression *in vivo*. Taking advantage of the *CUP1* reporter system[6], we introduced a premature termination codon (PTC) at the second codon of the *CUP1* gene, thus creating a new *CUP1* reporter gene (termed *cup1-PTC*). Cup1p is a copper chelating protein that mediates resistance to copper sulphate ($CuSO_4$)[7]. Thus, upon transformation of the *cup1-PTC* plasmid (pcup1-PTC) into a *Saccharomyces cerevisiae cup1-Δ* strain, one should be able to measure nonsense suppression by plating the cells on selective medium containing $CuSO_4$ (Fig. 2A).

To direct site-specific Ψ formation *in vivo*, we took advantage of the H/ACA ribonucleoprotein (RNP) family. H/ACA RNPs are primarily responsible for the post-transcriptional isomerization of uridine to Ψ within RNA (Supplementary Fig. 2)[8,9]. To target the PTC within *cup1-PTC* we derived an H/ACA RNA from *SNR81*, a naturally occurring yeast H/ACA RNA. The newly derived H/ACA RNA, *snR81-1C*, contained a guide sequence capable of targeting the PTC within *cup1-PTC*. In addition, we also constructed a control H/ACA RNA, *snR81-Random*, which contained a random guide sequence.

To ensure that *cup1-PTC* was pseudouridylated in response to expression of *snR81-1C*, we measured Ψ formation within the PTC both *in vitro* and *in vivo*. To analyse Ψ formation *in vitro*, we monitored, by thin layer chromatography (TLC), Ψ formation on a 39-nucleotide fragment of RNA corresponding to the region of *cup1-PTC* containing the PTC (Fig. 2B). Incubation of the transcript in extracts prepared from cells expressing *snR81-1C* resulted in the formation of Ψ (Fig. 2B, lane 5), whereas extracts containing an empty vector or *snR81-Random* did not result in the formation of Ψ (lanes 3 and 4). These results indicate that *snR81-1C* is capable of directing pseudouridylation within the RNA fragment, most probably at the target site, that is, the uridine of the PTC.

To determine the pseudouridylation status of *cup1-PTC in vivo*, we analysed the PTC of cellularly derived *cup1-PTC* mRNA using a site-specific and quantitative pseudouridylation assay, namely site-specific cleavage and radiolabelling followed by nuclease digestion and

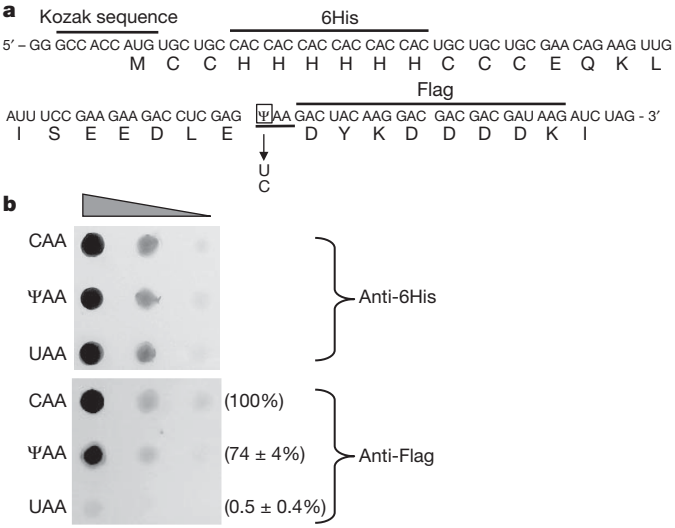

Figure 1 | Pseudouridylation of a termination codon promotes nonsense suppression *in vitro*. **a**, Nucleotide sequence of the *in vitro* transcription product and its translated sequence are shown. Positions of the Kozak sequence, as well as epitopes (6His and Flag) within the nucleotide and protein sequences are labelled. The pseudouridylated nonsense codon is indicated. Changes of Ψ to U and Ψ to C are also indicated. **b**, Anti-6His and anti-Flag immunoblot analysis of the *in vitro* translation lysate following translation of an RNA lacking a termination codon (CAA), an RNA containing a pseudouridylated termination codon (ΨAA), or an RNA containing an authentic termination codon (UAA). Relative efficiency of read-through (anti-Flag/anti-6His) was calculated and indicated in parentheses (the control, CAA, is set to 100%). Error is given as the standard deviation of three independent experiments.

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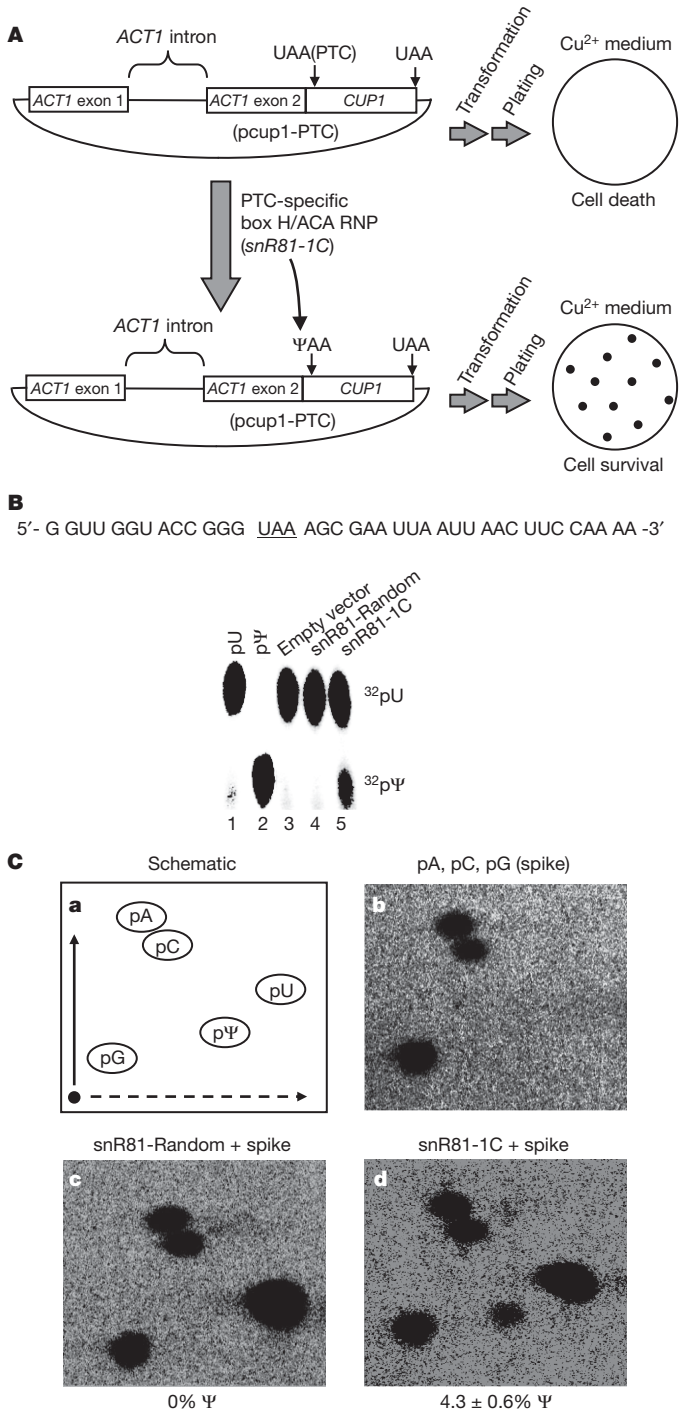

Figure 2 | Quantification of *cup1-PTC* pseudouridylation. **A**, Schematic of the *in vivo* nonsense suppression assay. **B**, *In vitro* pseudouridylation assay by thin layer chromatography. 5′ ^{32}P-radiolabelled uridylate (pU) and pseudouridylate (pΨ) markers were run in parallel. The substrate—[α^{32}P]UTP uniformly labelled RNA fragment—is shown. **C**, Quantification of *cup1-PTC* pseudouridylation *in vivo*. The percentage of pseudouridylation was calculated (pΨ/(pΨ + pU)). **a**, Schematic; **b**, Spike, **c**, snR81-Random; **d**, snR81-1C. Adenosine 5′-monophosphate (pA), cytidine 5′-monophosphate (pC), guanosine 5′-monophosphate (pG), uridine 5′-monophosphate (pU), and pseudouridine 5′-monophosphate (pΨ), are indicated. •, origin. Error is given as the standard deviation of three independent experiments.

two-dimensional TLC (2D-TLC)[10]. To help locate the uridine and Ψ spots on the TLC plate, we spiked each reaction with ^{32}P-radiolabelled adenosine 5′-monophosphate (pA), cytidine 5′-monophosphate (pC) and guanosine 5′-monophosphate (pG) (Fig. 2C, panel b). Consistent with the results of our *in vitro* analysis (Fig. 2B), only *CUP1* mRNA isolated from cells expressing *snR81-1C* produced a spot corresponding to Ψ (Fig. 2C, compare panel **c** with panel **d**). Quantification indicated that approximately 5% of the *cup1-PTC* transcript was pseudouridylated. Thus, our *in vivo* pseudouridylation results would predict that, upon expression of *snR81-1C*, a functional Cup1p (although in small amount) would be translated from the *cup1-PTC* mRNA.

Figure 3a shows the results of the *in vivo* nonsense suppression assay (see Fig. 2a for illustration). As expected, when transformed with wild-type pCUP1, *cup1Δ* cells grew healthily on media containing 0 mM or 0.02 mM $CuSO_4$ (top row). However, when transformed with pcup1-PTC, only cells co-transformed with psnR81-1C were able to survive on medium containing 0.02 mM $CuSO_4$ (compare the middle row with the bottom row). Partial restoration of growth seems to be consistent with the previous quantitative analysis demonstrating a low level of pseudouridylation (~5%) at the PTC (Fig. 2c).

Because nonsense suppression could be achieved both at the level of translation (termination suppression) and the level of mRNA decay where PTC-containing mRNA is usually the target of NMD (nonsense-mediated decay), it remained possible that the suppression we observed in Fig. 3a was a result of NMD suppression rather than suppression of translation termination. To test this possibility, we measured the levels of *CUP1* mRNA using northern blot analysis (Fig. 3b). As expected, steady-state levels of *cup1-PTC* mRNA dropped significantly when compared with the level of wild-type *CUP1* mRNA (compare lanes 6 and 7 with lane 5). However, expression of the PTC-specific guide RNA (*snR81-1C*) had no effect on steady-state levels of *cup1-PTC* mRNA; nearly identical levels of *cup1-PTC* mRNA were detected in cells transformed with either psnR81-1C or with empty vector (compare lane 6 with lane 7). These results indicated that the observed suppression was a result of nonsense codon suppression rather than a result of suppressing NMD. To completely eliminate the potential complications of NMD, we deleted *UPF1* (also known as *NAM7*), a gene required for NMD[11], and then repeated the nonsense suppression assay and northern blotting. As expected, deletion of *UPF1* resulted in the stabilization of *cup1-PTC* (Fig. 3d). Consistent with previously published results[12,13], we also observed a small degree of nonsense suppression as evidenced by a low (but above background) level of growth on medium containing a low concentration (0.013 mM) of $CuSO_4$ (Supplementary Fig. 3, compare row 5 with row 2). However, no growth was observed when $CuSO_4$ concentration was raised to 0.02 mM (Fig. 3c, middle row). Apparently, the nonsense suppression phenotype conferred by deletion of *UPF1* is not sufficient to promote growth on 0.02 mM $CuSO_4$. To assess nonsense suppression induced by PTC pseudouridylation, we plated cells on media containing either 0.02 mM or 0.013 mM $CuSO_4$ (Fig. 3c and Supplementary Fig. 3). Under both conditions, expression of *snR81-1C* provided a growth advantage; the level of growth rescue is similar to that observed when *UPF1* was intact (compare Fig. 3c with Fig. 3a, and Supplementary Fig. 3). These results further support the notion that expression of *snR81-1C* or pseudouridylation of the PTC resulted in suppression of translation termination rather than suppression of NMD. Given that the control, where *snR81-Random* was similarly expressed (Fig. 3d), showed no suppression (Fig. 3c), our results also indicate that the observed suppression of translation termination is guide-RNA-specific.

To determine further whether Ψ-mediated nonsense suppression can be generalized as well as which amino acids are incorporated at Ψ-substituted nonsense codons, we took advantage of a plasmid containing a C-terminally tagged *TRM4* gene (also known as *NCL1*), pTRM4-WT (Fig. 4a). Through site-directed mutagenesis the codon for phenylalanine at position 602 (F602) of the *TRM4* gene was changed to a nonsense codon (TAA, TAG or TGA), creating three variants of the plasmid (pTRM4-F602X(TAA), pTRM4-F602X(TAG), and pTRM4-F602X(TGA)) (Fig. 4a).

Figure 4b shows the western blot analysis of extracts prepared from wild-type cells expressing wild-type *TRM4* (nonsense-free) or

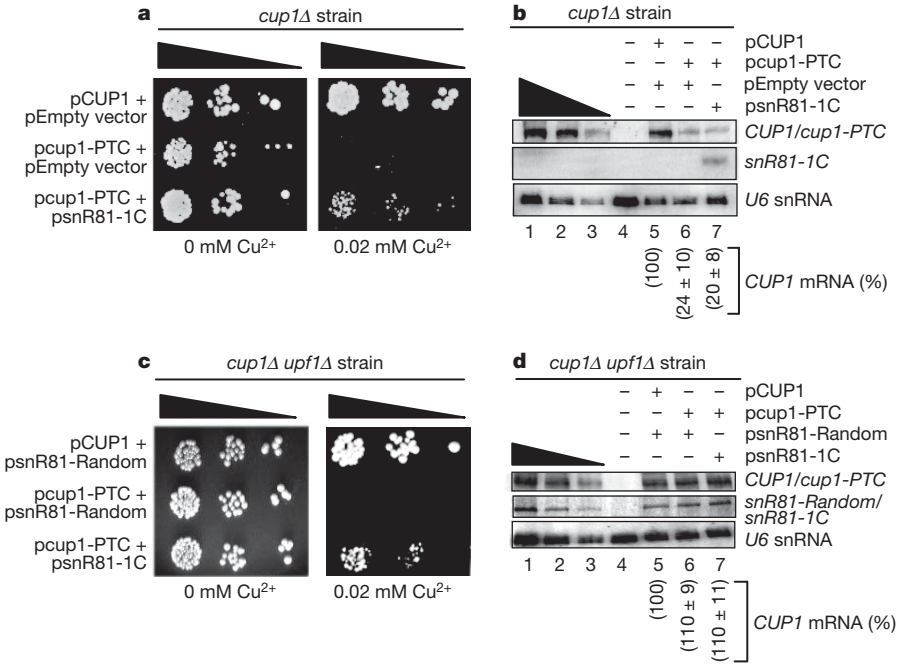

Figure 3 | Expression of an H/ACA RNA targeting the PTC of *cup-PTC* for pseudouridylation promotes nonsense suppression. **a**, pCUP1 or pcup1-PTC along with either an empty vector or psnR81-1C were transformed into a *cup1Δ* strain. Cell growth was assessed on solid synthetic medium (−Ura −Leu) containing either 0 mM or 0.02 mM $CuSO_4$, as indicated. **b**, Northern blot analysis of RNA extracted from cells described in **a**. Normalized levels of *CUP1* mRNA (lane 5) and *cup1-PTC* mRNA (lanes 6 and 7) are indicated in parentheses under each lane. Error is given as the standard deviation of three independent experiments. **c**, *cup1Δ upf1Δ* strain was transformed with either pCUP1 or pcup1-PTC along with either psnR81-Random or psnR81-1C. Cell growth was assessed on solid synthetic medium (−His −Ura −Leu) with or without $CuSO_4$, as indicated. **d**, Northern blot analysis of RNA extracted from cells described in **c**. Normalized levels of *CUP1* mRNA (lane 5) and *cup1-PTC* mRNA (lanes 6 and 7) are indicated in parentheses under each lane. Error is given as the standard deviation of three independent experiments.

TRM4-F602X(TAA). When cells were transformed with the wild-type *TRM4* plasmid, a strong protein A-tag signal was detected (Fig. 4b, lane 4). However, when cells were transformed with pTRM4-F602X(TAA), a protein A signal was only detected when co-transformed with a *TRM4-F602X(TAA)*-specific guide RNA, indicating nonsense suppression (Fig. 4b, compare lane 5 with lane 6).

Next, we carried out large-scale immunoprecipitations to purify full-length Trm4p produced as a consequence of Ψ-mediated nonsense suppression. Figure 4c shows an example [pTRM4-F602X(TAA)] of such experiments. The bands corresponding to full-length Trm4p produced as a consequence of Ψ-mediated nonsense suppression were excised and sequenced by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Fig. 4c and Supplementary Figs 4–7). Remarkably, LC-MS/MS analysis indicated that pseudouridylated UAA and UAG (ΨAA and ΨAG) both directed the incorporation of either serine or threonine (Fig. 4d and Supplementary Figs 4–6). Taking into account that the third base of a codon is usually non-specific (the wobble base), it makes sense that both ΨAA and ΨAG code for the same amino acids. With respect to targeted pseudouridylation of UGA (ΨGA), it directed the incorporation of tyrosine and phenylalanine (Fig. 4d and Supplementary Fig. 7). As all three termination codons directed the incorporation of two amino acids, we quantified their frequency of incorporation (Supplementary Fig. 8). Interestingly, although ΨAA and ΨAG both code for serine and threonine, serine is predominantly incorporated at ΨAG, whereas serine and threonine are incorporated at a roughly similar frequency for ΨAA. Furthermore, ΨGA primarily directs the incorporation of tyrosine.

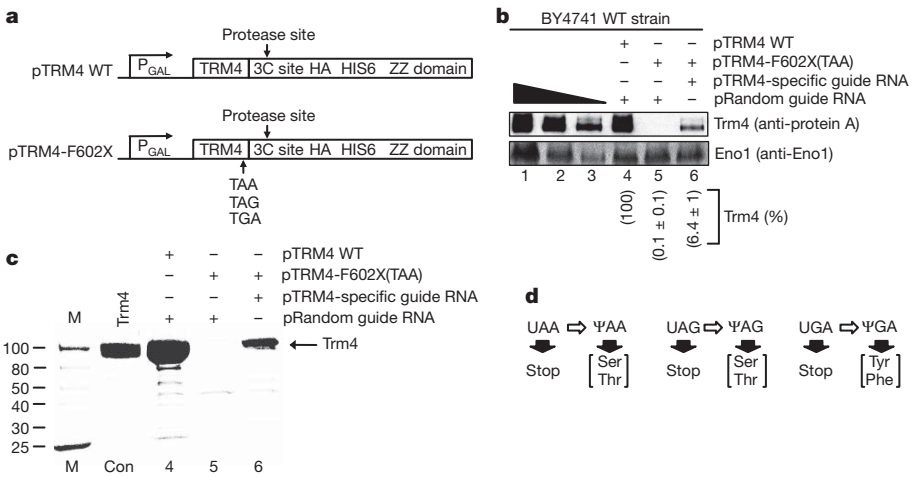

Figure 4 | Generalization of Ψ-mediated nonsense suppression and determination of amino acids coded for by pseudouridylated nonsense codons. **a**, Schematic representation of the constructs used for protein purification (also see text). **b**, Western blot analysis was carried out using extracts prepared from wild-type cells transformed with either pTRM4 wild type (WT) and a plasmid containing a random guide RNA gene (pRandom guide RNA) (lane 4), pTRM4-F602X(TAA) and pRandom guide RNA (lane 5), or pTRM4-F602X(TAA) and a plasmid containing a guide RNA gene that targets the nonsense codon (UAA 602) of TRM4-F602X(TAA) (lane 6). Enolase (Eno1) was probed as a loading control. The normalized levels of Trm4p are indicated in parentheses under each lane. Error is represented as the standard deviation from three independent experiments. **c**, Cell cultures described in **b** were scaled up, and Trm4 proteins were purified and analysed on a SDS–PAGE gel (stained with Coomassie blue); lanes correspond to those in **b**. In the control lane (Con), a known amount (6 μg) of purified Trm4p was loaded. M, molecular weight marker. **d**, Identification of amino acids incorporated at Ψ-containing termination codons (also see Supplementary Figs 4–8).

Interestingly, however, given that the anticodons of the transfer RNA^{Ser} and tRNA^{Thr} families do not look alike (Supplementary Fig. 9), our experimental data raise an important question: how is the same pseudouridylated nonsense codon (for example, ΨAA or ΨAG) recognized by the different families of tRNA? Although it is possible that the presence of Ψ in mRNA–tRNA duplexes acts to stabilize interactions between the mRNA and near- or non-cognate tRNAs[14,15], an alternative explanation is that the presence of Ψ within the A-site may disorder the local RNA (or ribosome) structure, somehow allowing for the binding or accommodation of near- or non-cognate tRNAs, possibly through altering the hydration state of the nonsense codon[16]. It is also possible that unique RNA modifications in the anti-codon loop of tRNA^{Ser}, tRNA^{Thr}, tRNA^{Phe} or tRNA^{Tyr} contribute to the recognition of pseudouridylated nonsense codons, thus allowing them to be decoded. In fact, modifications within the anticodon loop of tRNA have previously been demonstrated to impact recoding[17]. Perhaps more interestingly, it has not escaped our attention that the amino acids incorporated at each termination codon are biochemically and structurally similar. Specifically, serine and threonine, which are coded for by ΨAA and ΨAG, are both hydroxylated short-chain amino acids. Likewise, tyrosine and phenylalanine, which are coded for by ΨGA, both contain an aromatic ring. Although the decoding centre is ~75 Å away from the peptidyl transferase centre[18], whether there is a role for the amino acid in the decoding of Ψ-containing termination codons is an interesting idea that requires further analysis. If true, such a mechanism would represent a completely new mode of decoding. It is interesting to note that frameshifting at sense codons also shows a strong preference for using tRNA^{Ser} and tRNA^{Thr} (ref. 19). Although detailed mechanisms are still unclear, the similarities in using similar polar amino acids (serine and threonine) in frameshifting and in decoding of pseudouridylated nonsense codons certainly deserve further attention.

Our data demonstrate that artificial H/ACA guide RNAs are able to direct the pseudouridylation of nonsense codons of mRNA, thus leading to nonsense suppression. It should be noted that artificial guide RNAs may have an unintended target(s), thus raising concerns about substrate specificity. We did, however, realize this concern when designing sense-to-nonsense mutations and their corresponding guide RNAs, and purposely avoided the sites and their guide sequences that could target other endogenous mRNAs. In fact, BLAST search against the yeast genome did not generate any other potential targets that appear to be suitable substrates for our artificial H/ACA RNAs. Thus, it is unlikely that the observed effects are due to the nonspecific effect of modifications of unintended off-targets.

Our RNA-guided modification strategy is of significant clinical interest, given the current estimates that approximately 33% of genetic diseases can be attributed to the presence of a PTC[20]. On the other hand, because the artificial guide RNAs are derived from naturally occurring H/ACA RNAs (only the short guide sequence is changed), we predict that the nonsense codons of some mRNAs are naturally pseudouridylated by endogenous H/ACA RNAs as long as the guide sequence matches the target. Indeed, using computational algorithms to predict nonsense codons that may be natural targets of the endogenous H/ACA RNP machinery yields a number of potential candidates (Supplementary Fig. 10). In addition, our lab has recently demonstrated that an exact match between the H/ACA RNA guide sequence and the target sequence is not necessary for efficient modification under certain conditions. In fact, the mismatches are required for inducible pseudouridylation in response to cell stress[21]. Thus, there are probably a large number of pseudouridylation targets in mRNAs. Whereas some of these potential targets are nonsense codons, a majority of them are expected to be sense codons. Given our surprising discovery that pseudouridylation of nonsense codons converts them into sense codons, it is not impossible that pseudouridylation of sense codons will alter their decoding, making mRNA pseudouridylation a novel mechanism of RNA editing. If this is true, the genetic code would expand considerably. We predict that targeted pseudouridylation of mRNA is a yet-to-be appreciated mechanism of generating protein diversity.

METHODS SUMMARY

Relevant properties of strains and growth conditions are described in the text. Strain construction and additional growth conditions are described in the Methods. Standard procedures were used for all protein and RNA analyses and are described in the Methods. Mass spectrometry was performed at the University of Rochester Medical Center Proteomics Core and is described in the Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 26 January; accepted 28 April 2011.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank F. Hagen and the Proteomics Core at the University of Rochester for performing the mass spectrometry analysis. We also thank E. Phizicky and B. Grayhack for the wild-type TRM4 construct, C. Guthrie for the *cup1Δ* yeast strain, D. Mcpheeters for the wild-type CUP1 construct, and M. Dumont for the anti-Eno1p antibody. Lastly, we would like to thank members of the Yu laboratory, especially X. Zhao, for helpful discussions.

Author Contributions J.K. and Y.-T.Y. designed and interpreted the experiments. Mass spectrometry was performed at the Proteomics Core at the University of Rochester Medical Center. J.K. performed all other experiments.

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METHODS

Yeast strains, transformation and growth assay. The *cup1-Δ* yeast strain was kindly provided by C. Guthrie[6]. The *UPF1* locus was deleted from a *cup1-Δ* strain using a standard protocol as described previously[22]. For the analysis of $CuSO_4$ resistance the appropriate plasmids were transformed into either *cup1-Δ* or *cup1-Δ upf1-Δ* yeast strains as previously described[22], except that after heat shock cells were precipitated and resuspended in 100 µl of water rather than YPD (yeast peptone dextrose). Single colonies were selected and grown to saturation in SGal (synthetic galactose) drop-out media, cells were diluted to an $OD_{600} = 0.001$ and then a series of fivefold dilutions were spotted on to SGal drop-out media, with or without $CuSO_4$. Growth phenotypes were assessed after cells were grown for 3–5 days at 30 °C.

Plasmids. The pCUP1 plasmid was a gift from D. Mcpheeters, and pTRM4 WT was a gift from E. Phizicky and B. Grayhack. pcup1-PTC and pTRM4 F602X variants were created by site-directed mutagenesis using Pfu polymerase (Stratagene) and the appropriate oligonucleotides and plasmids. Novel H/ACA RNA genes were constructed by PCR using four overlapping oligonucleotide primers and were either cloned into 2 µm *URA3* or 2 µm *LEU2* vector (both gifts from E. Phizicky) as BamHI/HindIII fragments[23].

***In vitro* transcription and translation.** To generate mRNA transcripts for *in vitro* translation, DNA templates were synthesized through PCR using two overlapping DNA oligonucleotides. The double-stranded DNA templates thus synthesized contained either a TAA nonsense codon or a CAA codon in the middle, flanked by a 6His-coding sequence near the 5′ end and a Flag-coding sequence at the 3′ end (Fig. 1b). For efficient *in vitro* translation, the templates also contained a Kozak sequence immediately upstream of the 6His coding sequence (Fig. 1b). In addition, a T7 promoter sequence was included at the 5′ terminus. Following *in vitro* T7 transcription[24,25], UAA- or CAA-containing mRNA transcripts were synthesized (see Fig. 1b). To create a similar mRNA, with the uridine of the nonsense codon (or the cytidine of the CAA codon) changed to Ψ, a two-piece splint ligation was employed[24]. The 5′ RNA was *in-vitro*-synthesized through T7 transcription, ending with CUC at its 3′ terminus (see Fig. 1b), and the 3′ piece (5′-GAGΨAAGACUACAAGGACGACGACGACAAGAUCUAG-3′) (see Fig. 1a) was chemically synthesized (Thermo Scientific). The 5′ and 3′ halves were ligated using a bridging oligonucleotide and T4 DNA ligase[24]. *In-vitro*-synthesized RNAs were gel-purified before being used in the *in vitro* translation reactions. *In vitro* translation reactions were carried out in 30 µl reactions of Red Nova Lysate as described by the supplier (Novagen). PCR of two overlapping oligodeoxynucleotides was also used to generate the template for *in vitro* transcription of the substrate used in the *in vitro* pseudouridylation assay (Fig. 2C).

Northern blot analysis. Total RNA was isolated from yeast using TRIzol essentially as described by the supplier (Invitrogen), except that cells were vortexed with acid-sterilized glass beads for 5 min. For northern blot analysis, 6 µg of total RNA was separated on 8% polyacrylamide–7 M urea gels and electrotransferred at 4 °C to Amersham Hybond-N^+ membranes in 0.5× TBE buffer for 16 h at 15 V. Hybridizations were preformed essentially as described[23].

Protein purification and immunoblot analysis. For the analysis of Trm4p protein sequence, BY4741 was transformed with the appropriate plasmids as described before[22]. Yeast whole-cell extracts and IgG Sepharose chromatography was preformed as previously described[26]. For analysis of *in vitro* translation products, membranes were probed with either a monoclonal Flag antibody (M2; Sigma-Aldrich) or monoclonal His-probe (H-3; Santa Cruz Biotechnology). Goat anti-mouse IgG (H+L)–alkaline phosphatase (AP) conjugate (Bio-Rad) was then used as a secondary antibody. Proteins were visualized using 1-Step NBT/BCIP (Pierce). For the analysis of Trm4p, yeast crude extracts were separated on 4–15% Tris-HCl Ready gels (BioRad). Proteins were then transferred to 0.1 µm nitrocellulose membranes (Whatman) and probed with either monoclonal Protein A (Sigma-Aldrich) or anti-Eno1p (a gift from M. Dumont). Goat anti-Mouse IgG (H+L)–AP conjugate (Bio-Rad) was used as a secondary antibody. Proteins were visualized using 1-Step NBT/BCIP (Pierce).

Pseudouridylation assays. *In vitro* pseudouridylation assays were performed using yeast whole-cell extract. Cells were grown to mid log phase and pelleted. Pellets were resuspended in 200 µl of extraction buffer containing 20 mM HEPES at pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 25% glycerol. Sterile acid-washed glass beads (400 µl) were added to the cell suspension, and cells were subsequently homogenized through vigorous vortexing (5 × 30 s) at 4 °C. Following a 5-min centrifugation (14,000*g*, 4 °C), the supernatant was recovered, and used for the pseudouridylation assay. The substrate RNA was prepared by *in vitro* transcription in the presence of [α-P^{32}]UTP. The substrate was gel purified and incubated in the extracts for 2 h. The radiolabelled substrate was recovered and digested with nuclease P1 and analysed by one-dimensional-TLC as previously described[10]. Pseudouridylation of cellularly derived *cup1-PTC* RNA was analysed as previously described[10], except that modifications were analysed by 2D-TLC[27].

Mass spectrometry. Mass spectrometry was preformed at the University of Rochester Proteomics Center. Coomassie-stained gel bands corresponding to full-length Trm4p were subjected to in gel trypsin digestion. An 80-min LC-MS/MS run was performed in-line with a Finnigan LTQ Ion Trap mass spectrometer (Thermo Scientific), using a flow rate of 250 µl min^{-1}. The data collected from the LTQ runs was searched using MASCOT (Matrix Science), initially against the full *Saccharomyces* database, second against a custom database which included the wild type Trm4p sequence, as well as a Trm4p sequence with an "X" in the amino acid position that corresponds to the stop codon. Peptides identified by Mascot with an ion score of 15 or greater were inspected further for MS/MS fragmentation patterns that map through most of the peptide sequence, especially on and through the mutant amino acid position. Peptides with Expect values greater than 0.05 were not accepted. To allow for relative quantification, we repeated the LC-MS/MS experiments using dynamic inclusion for only the peptides of interest. Total spectral counts obtained by dynamic inclusion therefore represent the relative abundances of each respective peptide.

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