

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' ³²P-radiolabelled uridylyate (pU) and pseudouridylyate (pΨ) markers were run in parallel. The substrate—[α-³²P]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 ³²P-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 pseudouridylyated. 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 CuSO₄ (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 CuSO₄ (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).

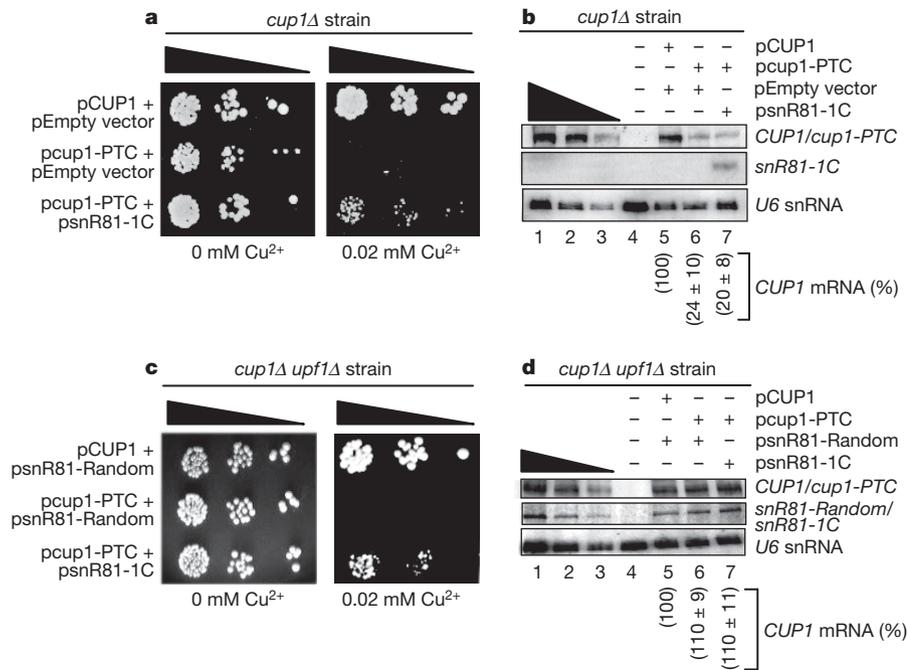
Because nonsense suppression could be achieved both at the level of translation (termination suppression) and the level of mRNA decay where PTC-containing mRNA is usually the target of NMD (nonsense-mediated decay), it remained possible that the suppression we observed in Fig. 3a was a result of NMD 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 NMD¹¹, 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 CuSO₄ (Supplementary Fig. 3, compare row 5 with row 2). However, no growth was observed when CuSO₄ 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 CuSO₄. To assess nonsense suppression induced by PTC pseudouridylation, we plated cells on media containing either 0.02 mM or 0.013 mM CuSO₄ (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 *cup1*-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 (Ψ AA and Ψ AG) 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 Ψ AA and Ψ AG code for the same amino acids. With respect to targeted pseudouridylation of UGA (Ψ GA), 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 Ψ AA and Ψ AG both code for serine and threonine, serine is predominantly incorporated at Ψ AG, whereas serine and threonine are incorporated at a roughly similar frequency for Ψ AA. Furthermore, Ψ GA primarily directs the incorporation of tyrosine.

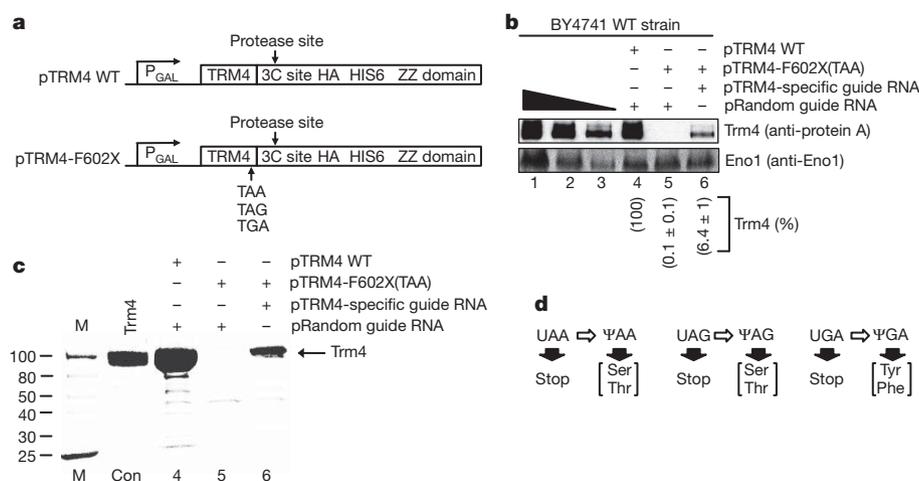


Figure 4 | Generalization of Ψ -mediated nonsense suppression and determination of amino acids coded for by pseudouridylated nonsense codons. **a**, Schematic representation of the constructs used for protein purification (also see text). **b**, Western blot analysis was carried out using extracts prepared 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 μ g) 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 codon¹⁶. 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 recoding¹⁷. 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 centre¹⁸, 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 PTC²⁰. 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 stress²¹. 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.

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

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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. Guthrie⁶. The *UPF1* locus was deleted from a *cup1-Δ* strain using a standard protocol as described previously²². For the analysis of CuSO₄ resistance the appropriate plasmids were transformed into either *cup1-Δ* or *cup1-Δ upf1-Δ* yeast strains as previously described²², 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 OD₆₀₀ = 0.001 and then a series of fivefold dilutions were spotted on to SGal drop-out media, with or without CuSO₄. 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 fragments²³.

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 employed²⁴. 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 ligase²⁴. *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 performed essentially as described²³.

Protein purification and immunoblot analysis. For the analysis of Trm4p protein sequence, BY4741 was transformed with the appropriate plasmids as described before²². Yeast whole-cell extracts and IgG Sepharose chromatography was performed as previously described²⁶. 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 MgCl₂, 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 (14,000g, 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 -³²P]UTP. The substrate was gel purified and incubated in the extracts for 2 h. The radio-labelled substrate was recovered and digested with nuclease P1 and analysed by one-dimensional-TLC as previously described¹⁰. Pseudouridylation of cellularly derived *cup1-PTC* RNA was analysed as previously described¹⁰, except that modifications were analysed by 2D-TLC²⁷.

Mass spectrometry. Mass spectrometry was performed 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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