Self-protective responses to norvaline-induced stress in a leucyl-tRNA synthetase editing-deficient yeast strain

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ABSTRACT

The editing function of aminoacyl-tRNA synthetases (aaRSs) is indispensible for formation of the correct aminoacyl-tRNAs. Editing deficiency may lead to growth inhibition and the pathogenesis of various diseases. Herein, we confirmed that norvaline (Nva) but not isoleucine or valine is the major threat to the editing function of Saccharomyces cerevisiae leucyl-tRNA synthetase (ScLeuRS), both in vitro and in vivo. Nva could be misincorporated into the proteome of the LeuRS editing-deficient yeast strain (D419A/ScΔleuS), potentially resulting in dysfunctional protein folding and growth delay. Furthermore, the exploration of the Nva-induced intracellular stress response mechanism in D419A/ScΔleuS revealed that Hsp70 chaperones were markedly up-regulated in response to the potential protein misfolding. Additionally, proline (Pro), glutamate (Glu) and glutamine (Gln), which may accumulate due to the conversion of Nva, collectively contributed to the reduction of reactive oxygen species (ROS) levels in Nva-treated D419A/ScΔleuS cells. In conclusion, our study highlights the significance of the editing function of LeuRS and provides clues for understanding the intracellular stress protective mechanisms that are triggered in aaRS editing-deficient organisms.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRSs) are responsible for charging their cognate tRNAs with the corresponding amino acids to generate aminoacyl-tRNAs for protein biosynthesis (1). For most aaRSs, the aminoacylation reaction occurs in two steps: amino acid activation and tRNA aminoacylation. In more detail, aaRSs use adenosine triphosphate (ATP) to activate amino acid by generating aminoacyl-adenylate (aa-AMP) intermediates, and the aminoacyl moiety is then transferred to the tRNA to form aminoacyl-tRNA (aa-tRNA) (1,2). Since many amino acids and some of their metabolic intermediates share similar structures, there is the potential for a specific aaRS to mischarge non-cognate amino acids; and to counteract this and ensure translation fidelity, many aaRSs use a ‘double-sieve’ mechanism to eliminate non-cognate amino acids (3,4). The first sieve involves the catalytic active site, which can discriminate different amino acids. For many closely related or slightly smaller amino acids, some aaRSs have evolved a distinct editing active site that acts as a second sieve to hydrolyze misactivated or mischarged amino acids (3,5).

Based on the sequence and architecture of the catalytic domain, aaRSs are divided into two classes (6,7). Leucyl-tRNA synthetase (LeuRS), as well as isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS), belongs to class I aaRSs that share a common Rossmann fold and two conserved HIGH and KMSK motifs in their catalytic domains (6,8–11). Besides the cognate Leu, LeuRS can also activate norvaline (Nva), isoleucine (Ile) and valine (Val) (12–15). The non-proteinogenic Nva is a metabolic side product of leucine (Leu) biosynthesis and it differs from Leu only by a single methyl group, posing a prime threat to the fidelity of leucylation (16). Under microaerobic conditions, Nva can accumulate in Escherichia coli and be misincorporated into the proteome to the extent that ~10% of Leu residues are substituted with Nva (17,18). Nva can also be misincorporated into antibacterial peptides produced by Bacillus subtilis and recombinant human haemoglobin produced in E. coli cells under certain conditions (19,20). To prevent non-cognate amino acids from being misincorporated into newly synthesized proteins, LeuRS performs both pre-transfer (hydrolysis of aa-AMP) and post-transfer (hydrolysis of aa-tRNA) editing to...
ensure translation accuracy (5,21–24). For *Saccharomyces cerevisiae* LeuRS (ScLeuRS), post-transfer editing is an important proofreading mechanism that preserves yeast cell survival in the presence of surplus Nva *in vivo* (25). The connective peptide 1 (CP1), which is flexibly inserted into the catalytic domain, performs post-transfer editing (12,22,26–28). In particular, a conserved Asp within the CP1 domain of *LeuRS* (EcLeuRS-Asp354, *Natrialba magadhi* LeuRS1-Asp35, ScLeuRS-Asp319, and *Homo sapiens* LeuRS-Asp390) is responsible for the post-transfer editing activity, and mutation of this Asp to Ala could completely abolish this activity (24,25,29,30).

A deficiency in aaRS editing activity may lead to proteomic instability, cell growth inhibition and severe disruption of physiological function (31–34). For example, editing deficiency of alanyl-tRNA synthetase induces neurodegeneration and cardiac proteinopathy in mice (31,32). In aging bacteria with an editing-defective IleRS, the SOS response mechanism was activated and the DNA mutation rate was dramatically increased (33). A recent study found that Nva but not Ile was the biggest threat to the fidelity of LeuRS editing in *E. coli*, emphasizing the importance of LeuRS editing in ensuring the survival of bacteria in environments with fluctuating oxygen levels (16). However, the detailed stress protective mechanism induced by non-cognate substrates in editing-deficient organisms is little understood. Herein, we utilized a LeuRS post-transfer editing-deficient yeast strain (D419A/ScΔleuS) to address this question.

The heat shock response is the most universal stress adaptation mechanism in organisms, involving the upregulation of heat shock proteins (Hsp) that facilitate refolding or degradation of misfolded proteins and prevent their aggregation (35). In *Saccharomyces cerevisiae*, there are eight Hsp70 family members, of which six are localized in the cytoplasm or nucleus (SSA1, SSA2, SSA3, SSA4, SSB1 and SSB2) and two (KAR2 and SSC) are localized in organelles (35). KAR2, also known as Bip, is the sole Hsp70 chaperone in the endoplasmic reticulum (ER), whose upregulation can relieve ER stress (35,36). Besides, there are two Hsp90 homologs (Hsc82 and Hsp82) in *S. cerevisiae* that are responsible for quality control of mature proteins and macromolecular assemblies (35).

In addition to the heat shock response, organisms could accumulate specific small molecules to counteract stress. Proline (Pro) is a well-known multifunctional stress protector which inhibits protein aggregation and scavenges intracellular reactive oxygen species (ROS) (37–39). Pro content increases under certain stress conditions in plants (40), and yeasts that accumulate Pro are more tolerant to stress (37). Pro can be synthesized from glutamate (Glu) stepwise by increases under certain stress conditions in plants (40), and vice versa can be converted to glutamine (Gln), and yeast that accumulate Pro are more tolerant to stress (37). Glu can be converted to Pro by ornithine transaminase (CAR2) (42,43). Pro can be converted back to Glu in mitochondria by Pro dehydrogenase (PUT1) and P5C dehydrogenase (PUT2) (44,45). Glu can be converted to glutamine (Gln), and *vice versa*. The riboflavin transporter (MCH5) is a plasma membrane-bound protein that transports riboflavin into the cytosol and produces flavin adenine dinucleotide (FAD) which serves as the cofactor for PUT1 (46). Importantly, Pro, Glu and Gln are all *bona fide* ROS scavengers. Pro can quench OH and O₂⁻ radicals due to its low ionization potential (38). Gln can be used to generate Glu and maintain an appropriate intracellular Glu pool, and then Glu can be used directly for the synthesis of glutathione, which is a major redox buffering compound (47). In addition, Glu and Gln can be converted to α-ketoglutarate (α-KG), which then enter the TCA cycle to supply NADPH and ATP-producing pathways (48).

In this study, we confirmed that Nva is the major editing target of ScLeuRS and can significantly inhibit the growth of the *ScΔleuS* strain harboring the editing-deficient *ScLeuRS*-D419A (D419A/ScΔleuS). Nva can be misincorporated into the proteome of D419A/ScΔleuS, which may cause protein instability and the upregulation of heat shock proteins. To reduce the cytotoxicity of Nva, D419A/ScΔleuS cells may initiate protective responses to convert some Nva to non-toxic metabolites, such as Pro, Glu and Gln. The accumulation of these antioxidative amino acids can lead to the reduction of intracellular ROS levels. In conclusion, our results provide a better understanding of the intracellular protective mechanisms in response to aaRS editing deficiency induced stress and provided insights into aaRS editing deficiency associated diseases.

**MATERIALS AND METHODS**

**Materials**

L-Leu, L-Nva, BioUltra L-Ile, BioUltra L-Val, dithiothreitol (DTT), ATP, CTP, GTP, UTP, 5'-GMP, tetradsodium pyrophosphate, inorganic pyrophosphate, Tris–HCl, MgCl₂, NaCl and activated charcoal were purchased from Sigma-Aldrich (St Louis, MO, USA). Pyrophosphatase (PPiase) was obtained from Roche Applied Science (Switzerland). [³²P]Leu, [³²P]tetradsodium pyrophosphate and [α-³²P]ATP were obtained from PerkinElmer Inc. (Waltham, MA, USA). Nuclease S1 was obtained from Thermo Scientific (Pittsburgh, PA, USA). Ni²⁺-NTA Superflow resin was purchased from TianGen (China). Oligonucleotide primers were synthesized by Biosune Bioscience (China). Competent *E. coli* Top10 and Rosetta 2 (DE3) cells were prepared in our laboratory.
Gene cloning, expression and protein purification

Recombinant plasmids pET28a-ScLeuRS and pET28a-ScLeuRS-D419A were constructed in our laboratory (25) and transformed into E. coli Rosetta 2 (DE3) cells for protein expression. Purified proteins were dialysed into buffer A (20 mM Tris–HCl pH 7.5, 10 mM MgCl2, 2 mM DTT, 4 mM ATP, 10–1000 nM Leu, 0.3–500 nM Nva, 5–100 mM Ile or 3–150 mM Val). Reactions were started by the addition of 50–100 nM ScLeuRS after incubation at 30°C for 2–3 min. Aliquots (9 μl) at specific time points were added to 200 μl of quenching solution (2% activated charcoal, 3.5% HClO4 and 5% acetic acid). A total of 3.5 μl crude aminoacylated tRNAleu was prepared according to our previously reported method (49). The deacylation reaction was performed in 60 mM Tris–HCl (pH 7.5), 10 mM MgCl2 and 100–500 nM ScLeuRS or ScLeuRS-D419A at 30°C. Reactions were started by the addition of 3.5 μM crude aminoacylated tRNAleu. Samples were treated in the same way as described above for the aminoacylation reaction.

Construction of WT/ScΔleuS and D419A/ScΔleuS strains

The yeast knockout strain ScΔleuS that contains the rescue plasmid p416GPD-ScLeuRS was constructed in our lab previously (51). The gene encoding ScLeuRS was subcloned from pET28a-ScLeuRS-6 × His and inserted into the PstI and XhoI restriction enzyme sites of p425TEF to generate p425TEF-ScLeuRS-6 × His. p425TEF-ScLeuRS-D419A-6 × His was obtained by site-directed mutagenesis using p425TEF-ScLeuRS-6 × His as a template. The ScΔleuS strain was cultured in yeast extract peptone dextrose (YPD) medium overnight at 30°C, and plasmids were respectively transformed into ScΔleuS using the Yeastmaker Yeast Transformation System 2 according to the manufacturer’s protocol. Transformants were cultured on SD/Leu/-5-FOA plates to discard the rescue plasmid, and surviving transformants were picked and named WT/ScΔleuS and D419A/ScΔleuS, respectively (Figure 2A).

Yeast total RNA extraction and transcriptional profile analysis

Yeast cells (WT/ScΔleuS and D419A/ScΔleuS) were cultured in 50 ml SD/Leu− medium for 12 h to A600 = 0.6. Each strain was then supplemented with or without 0.5 mM Nva and cultured for 12 h. Total RNA from WT/ScΔleuS and D419A/ScΔleuS was extracted using the hot phenol method according to previous reports (52). Yeast cells were suspended in 400 μl AE buffer (50 mM NaAc pH5.2, 10 mM ethylenediaminetetraacetic acid (EDTA)). A total of 40 μl 10% sodium dodecyl sulphate and 400 μl acidic saturated phenol (pH 4.5) were then added and vortexed for 5–10 s. Samples were incubated at 65°C for 10 min to ensure sufficient lysis and vortexed for 5–10 s per min. Samples were then incubated on ice for 5 min, centrifuged at 15 000 × g for 10 min at 4°C and supernatants were extracted with an equivalent volume of phenol (pH 4.5) and chloroform/isoamylol (24:1) in order. Samples were then mixed with 1 ml ethanol and 40 μl 3 M NaAc (pH 5.2) for precipitation. Precipitated total RNA was centrifuged, dried and resolved in 50 μl ddH2O. After quality verification, total RNA was sent to Beijing Genomics Institute for transcriptional profile analysis.
cDNA synthesis and qRT-PCR
cDNA was synthesized using 0.5 μg yeast total RNA and PrimeScript RT Master Mix according to the manufacturer’s protocol. qRT-PCR was performed using SYBR Green Real-time PCR Master Mix on an ABI 7500 Fast Real-time PCR System (ThermoFisher Scientific). cDNA from WT/ScΔleuS cultured in normal medium was used as a control. Data were normalized against the reference gene ACT1 (53). Primers used for qRT-PCR are listed in Supplementary Table S1.

Protein tags insertion and western blot detection
We inserted the gene sequences encoding 3 × HA tag at the 3’ terminus of the PUT2, Pro1, Pro3, CARI and CAR2 genes, and the sequences encoding 13 × Myc tag at the 3’ terminus of the MCH5 and PUT1 genes, in both D419A/ScΔleuS and WT/ScΔleuS cells by PCR-mediated homologous recombination (54). Correct integration was confirmed by PCR. After treatment with or without 0.5 mM Nva for 12 h, the yeast cells were harvested and resuspended in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF and 1 mM DTT, mixed with a few glass beads, vortexed vigorously for 30 min, then centrifuged at 10 000 × g for 15 min, 4°C. The supernatant was collected and proteins were detected by western blot. Anti-HA antibody was obtained from Sigma (H3663), anti-Myc antibody was from Proteintech (66004), anti-beta Actin antibody was from Abcam.

Detection of Nva misincorporated into proteins
Yeast cells (WT/ScΔleuS and D419A/ScΔleuS) were cultured in 50 ml SD/Leu− medium for 12 h to A600 = 0.6. Yeast cells were then supplemented with or without 0.5 mM Nva and cultured continuing for 12 h. Samples were collected at 2000 × g for 5 min, cells were washed twice with 0.9% NaCl, suspended in buffer B (20 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF), mixed with a few glass beads, vortexed vigorously for 1 h. After centrifugation at 12 000 × g for 15 min, supernatants were collected, washed with 100 mM potassium phosphate buffer. Samples were treated with 58.4 μM 2′,7′-dichlorofluorescein diacetate (H2DCFDA) for 2 h in the dark. After propidium iodide staining, the fluorescence intensity was immediately quantified by flow cytometry using a BD Accuri C6 flow cytometer (BD Biosciences). A minimum of 30 000 yeast cells were analyzed in each sample.

RESULTS
Nva can be efficiently misactivated by ScLeuRS
To explore the editing properties of ScLeuRS, ATP-PPI exchange experiments were initially performed to measure the amino acid activation activity. The results showed that among the non-cognate amino acids, only Nva could be strongly misactivated by ScLeuRS with a discrimination factor of 1/118 (Table 1). This is much higher than the error rate in protein biosynthesis (1/3000) (55), indicating that Nva represents a major threat to the fidelity of ScLeuRS aminoacylation in vitro. In contrast, Ile and Val could be well discriminated by ScLeuRS with discrimination factors of 1/11 700 and 1/5900, respectively, both of which are significantly lower than 1/3000 (Table 1).

ScLeuRS-D419A catalyses sufficient Nva-tRNALeu formation
The high amino acid activation activity of ScLeuRS for Nva suggests that ScLeuRS must possess a robust editing activity to guarantee the accuracy of aminoacylation. The aminoacylation reaction showed that ScLeuRS was barely able to misaminoacylate CatRNALeu with Nva and Ile (Table 2). However, in the presence of CatRNALeu, the aminoacylation catalytic efficiency of ScLeuRS-D419A, which has lost the post-transfer editing function, for Nva (0.92 s⁻¹·mM⁻¹) and Ile (0.038 s⁻¹·mM⁻¹) was 1/67 and 1/1631 of that for the cognate Leu (62 s⁻¹·mM⁻¹), respectively (Table 2), suggesting that the post-transfer editing pathway plays an important role in preventing Nva and Ile from being mischarged (error rate in protein biosynthesis
Table 1. Amino acid activation kinetics of ScLeuRS with various amino acids

<table>
<thead>
<tr>
<th>Amino acid structure</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$M$^{-1}$)</th>
<th>Discrimination Factor (DF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>37.6 ± 4.1</td>
<td>102.4 ± 14.7</td>
<td>2723</td>
<td>1</td>
</tr>
<tr>
<td>Nva</td>
<td>3100 ± 300</td>
<td>71.6 ± 8.0</td>
<td>23.1</td>
<td>1/118</td>
</tr>
<tr>
<td>Ile$^a$</td>
<td>19400 ± 1900</td>
<td>4.5 ± 0.6</td>
<td>0.232</td>
<td>1/11700</td>
</tr>
<tr>
<td>Val$^a$</td>
<td>72500 ± 7700</td>
<td>33.7 ± 1.8</td>
<td>0.465</td>
<td>1/5900</td>
</tr>
</tbody>
</table>

The results are presented as the mean ± SD (n = 3).

*aBioUltra > 99.5%.

*bDF corresponds to the relative ratio of $k_{cat}/K_m$ for non-cognate amino acids compared with that for Leu.

Table 2. Aminoacylation kinetics of ScLeuRS (WT) and ScLeuRS-D419A (D419A)

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>ScLeuRS</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$M$^{-1}$)</th>
<th>Relative $k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>WT</td>
<td>3.3 ± 0.1</td>
<td>0.28 ± 0.04</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>D419A</td>
<td>2.6 ± 0.3</td>
<td>0.16 ± 0.03</td>
<td>62</td>
<td>73</td>
</tr>
<tr>
<td>Nva</td>
<td>WT</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>D419A</td>
<td>306 ± 28</td>
<td>0.28 ± 0.03</td>
<td>0.92</td>
<td>1.08</td>
</tr>
<tr>
<td>Ile$^a$</td>
<td>WT</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>D419A</td>
<td>1600 ± 100</td>
<td>0.060 ± 0.002</td>
<td>0.038</td>
<td>0.0447</td>
</tr>
<tr>
<td>Val$^a$</td>
<td>WT</td>
<td>4020 ± 100</td>
<td>0.11 ± 0.02</td>
<td>0.027</td>
<td>0.0318</td>
</tr>
<tr>
<td></td>
<td>D419A</td>
<td>5120 ± 130</td>
<td>0.083 ± 0.017</td>
<td>0.016</td>
<td>0.0188</td>
</tr>
</tbody>
</table>

The results are presented as the mean ± SD (n = 3).

*aBioUltra > 99.5%.

nd, not determined.

is 1/3000) (55). Once the post-transfer editing function of ScLeuRS was disrupted, Nva-tRNA$^{Leu}$ and Ile-tRNA$^{Leu}$ was likely to form (Supplementary Figure S1). Val did not need to be edited because it is only poorly aminoacylated by both ScLeuRS (0.027 s$^{-1}$M$^{-1}$) and ScLeuRS-D419A (0.016 s$^{-1}$M$^{-1}$; Table 2 and Supplementary Figure S1).

**ScLeuRS employs distinguishing post-transfer editing activities for different mischarged products**

To further study the post-transfer editing activity of ScLeuRS, deacylation reactions were performed using pre-prepared Nva-CatRNA$^{Leu}$, Ile-CatRNA$^{Leu}$ and Val-CatRNA$^{Leu}$. The results showed that ScLeuRS, but not ScLeuRS-D419A, could efficiently deacylate Nva-CatRNA$^{Leu}$ and Ile-CatRNA$^{Leu}$ (Figure 1A–D). In contrast, both ScLeuRS and ScLeuRS-D419A showed minimal deacylation activity for Val-CatRNA$^{Leu}$ (Figure 1E and F). The $k_{obs}$ values for deacylation of Nva-CatRNA$^{Leu}$ and Ile-CatRNA$^{Leu}$ were 0.51 and 0.54 s$^{-1}$, respectively, which was ~12-fold higher than that for Val-CatRNA$^{Leu}$ (0.041 s$^{-1}$; Table 3). These results further suggest that the post-transfer editing function of ScLeuRS is effective for preventing Nva and Ile from attaching to tRNA$^{Leu}$, consistent with a previous report on EcLeuRS (16).
Figure 1. ScLeuRS efficiently deacylates Nva-CatRNA\textsuperscript{Leu} and Ile-CatRNA\textsuperscript{Leu}, but not Val-CatRNA\textsuperscript{Leu}. (A, C and E) Representative images showing the deacylation activities of 100 nM ScLeuRS and ScLeuRS-D419A with 3.5 μM Nva-CatRNA\textsuperscript{Leu} (A), Ile-CatRNA\textsuperscript{Leu} (C) and Val-CatRNA\textsuperscript{Leu} (E) at pH 7.5 based on TLC assays. (B, D and F) Quantification of the deacylation activities of 100 nM ScLeuRS (■) and ScLeuRS-D419A (□) with 3.5 μM Nva-CatRNA\textsuperscript{Leu} (B), Ile-CatRNA\textsuperscript{Leu} (D) and Val-CatRNA\textsuperscript{Leu} (F) in (A), (C) and (E), respectively. Values are the mean ± SD (n = 3).

Table 3. Deacylation $k_{\text{obs}}$ values for ScLeuRS with misacylated CatRNA\textsuperscript{Leu}

<table>
<thead>
<tr>
<th>aa-CatRNA\textsuperscript{Leu}</th>
<th>$k_{\text{obs}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nva-CatRNA\textsuperscript{Leu}</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>Ile-CatRNA\textsuperscript{Leu}</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>Val-CatRNA\textsuperscript{Leu}</td>
<td>0.041 ± 0.004</td>
</tr>
</tbody>
</table>

The results are presented as the mean ± SD (n = 3).

Studies on EcLeuRS revealed that the side chain of Thr\textsuperscript{252} limits the size of the ‘editing pocket’ to prevent the deacylation of the correct product Leu-EctRNA\textsuperscript{Leu} (14,56,57). Given the minimal deacylation activity of ScLeuRS for Val-CatRNA\textsuperscript{Leu}, we postulated that Val might also be excluded by the conserved Thr\textsuperscript{319} (corresponding to Thr\textsuperscript{252} in EcLeuRS) to prevent the hydrolysis of the wrong Val-CatRNA\textsuperscript{Leu} product. Indeed, ScLeuRS-T319A was capable of hydrolyzing both Leu-CatRNA\textsuperscript{Leu} and Val-CatRNA\textsuperscript{Leu}, which verified our hypothesis (Supplementary Figure S2). Therefore, the catalytic site may assume all responsibility for discriminating Val. This distinction in ScLeuRS editing functions might be due to the long-term co-evolution between its aminoacylation and editing activities.

Nva inhibits the growth of D419A/ScΔleuS

Given that Ile and Val could be effectively excluded from the catalytic centre, Nva was deduced to be the biggest threat to the editing function of ScLeuRS in vitro. To study the in vivo effect of Nva on LeuRS editing-defective yeast, we constructed two strains: WT/ScΔleuS (containing ScLeuRS) and D419A/ScΔleuS (containing ScLeuRS-
Figure 2. Nva inhibits the growth of LeuRS editing-defective yeast. (A) Schematic representation of the construction of yeast strains containing Sc-LeuRS (WT/ScΔleuS) and Sc-LeuRS-D419A (D419A/ScΔleuS). (B and C) Representative growth curves of WT/ScΔleuS (B) and D419A/ScΔleuS (C) in the presence of 0.5 mM Ile (□), Leu (▲), Nva (▽), Val (⃝) or the absence of any additional amino acids (●) (initial A600 = 0.1). Values are the mean ± SD (n = 3). (D) Growth of yeast cells on SD/Leu− solid media under various concentration of Nva, Ile or Val.

D419A; Figure 2A). Both yeast strains were cultured in SD/Leu− medium supplemented with or without 0.5 mM Nva/Ile/Val/Leu (initial A600 = 0.1). In the absence or presence of various amino acids, WT/ScΔleuS grew at a similar rate (Figure 2B). However, D419A/ScΔleuS grew noticeably slower in the presence of 0.5 mM Nva (Figure 2C). To further confirm the inhibitory effect of Nva on the growth of D419A/ScΔleuS, yeast was spotted on solid SD/Leu− plates supplemented with varying concentration of Nva (0, 0.2, 0.5 or 1 mM). The growth rate of D419A/ScΔleuS was not affected in the presence of 0.2 mM Nva, but was inhibited more and more obviously with increasing Nva concentration (Figure 2D). This dose-dependent phenotype indicates that Nva is only deleterious for D419A/ScΔleuS cells when its amount exceeds a threshold. In sharp contrast, Ile/Val did not affect the growth of D419A/ScΔleuS, even at a concentration of 20 mM (Figure 2D). This is consistent with the in vitro biochemical data that showed Ile and Val were well discriminated by ScLeuRS-D419A. Besides, it should be noted that excess Ile or Val may be used preferentially by their cognate aaRSs to reduce the possibility of misrecognition by ScLeuRS-D419A.

Nva can be misincorporated into the proteome of D419A/ScΔleuS

ScLeuRS-D419A has lost post-transfer editing capac-
ity for Nva, potentially resulting in the accumulation of Nva-tRNA^Leu in D419A/ScΔleuS under Nva stress conditions. Therefore, we predicted that under such conditions, some Leu residues may be replaced by Nva in the proteome of D419A/ScΔleuS, which could jeopardize the translation accuracy. To verify our hypothesis, WT/ScΔleuS and D419A/ScΔleuS were grown in SD/Leu^- medium supplemented without or with Nva (WT/ScΔleuS, WT/ScΔleuS+Nva, D419A/ScΔleuS and D419A/ScΔleuS+Nva). Total proteins were acid hydrolyzed to their constituent amino acids and analyzed by high performance liquid chromatography (HPLC) as described above (see ‘Materials and Methods’ section). A new, small peak corresponding to the Nva standard was observed in the chromatogram of D419A/ScΔleuS+Nva (Figure 3), but not in the chromatograms of D419A/ScΔleuS (Figure 3), WT/ScΔleuS and WT/ScΔleuS+Nva (Supplementary Figure S3). This result further indicates that Nva could be misincorporated into the proteome of D419A/ScΔleuS by substituting for some Leu residues.

Heat shock proteins are upregulated in Nva-treated D419A/ScΔleuS

Given that Nva is smaller and less hydrophobic than Leu, replacement of Leu by Nva may affect the correct folding of proteins, and the accumulation of misfolded proteins may pose a severe threat to physiological processes and ultimate survival. We therefore proceeded to explore the stress responses in D419A/ScΔleuS to Nva stress. By comparing the transcriptional profile of Nva-treated WT/ScΔleuS and D419A/ScΔleuS, we found that Hsp70 family proteins KAR2, SSA1 and SSA2, and Hsp90 member Hsp82 were markedly upregulated in Nva-treated D419A/ScΔleuS (Figure 4A), which was further verified by qRT-PCR (Figure 4B–D). The protein level of Hsp70 was significantly upregulated in Nva-treated D419A/ScΔleuS, while that of Hsp90 showed slight downregulation (Figure 4E). From this it could be deduced that significant accumulation of misfolded proteins in the cells leads to upregulation of these Hsp70 chaperones to alleviate stress in the Nva-treated D419A/ScΔleuS cells, reflecting their ability to facilitate the refolding or degradation of misfolded proteins (35). In contrast, Ile treatment did not induce the upregulation of Hsp70 and Hsp90 in D419A/ScΔleuS, even at a concentration of 20 mM (data not shown), consistent with its inability to affect cell growth.

Genes related to Pro catabolism are upregulated at the transcription level

Interestingly, most pathways identified by transcriptional profiling were related to cell metabolism (Supplementary Figure S4), suggesting Nva stress may have a global effect on metabolism. In particular, MCH5, PUT1 and PUT2, all of which are involved in Pro catabolism, were significantly upregulated in Nva-treated D419A/ScΔleuS (Figures 4A and 5A), which was further confirmed by qRT-PCR (Figure 5B–D). However, the expression of these genes was unaffected in Ile-treated D419A/ScΔleuS (data not shown), suggesting upregulation of Pro catabolism-related genes may occur in response to Nva misincorporation-induced stress.

We also measured the expression levels of MCH5, PUT1 and PUT2 by the insertion of HA or Myc protein tags (see ‘Materials and Methods’ section). Unexpectedly, the expression of MCH5, PUT1 and PUT2 were all downregulated in Nva-treated D419A/ScΔleuS but not in WT/ScΔleuS (Figure 5E). To interpret the possible reason for the inconsistency between transcriptional and translational levels of these genes, we further measured the expression of other genes, such as the Pro metabolism-related...
proteins (Pro1, Pro3, CAR1 and CAR2), and LeuRS by the insertion of protein tags. The results showed that the expression of these proteins were also downregulated in D419A/ScΔleuS after Nva treatment (Supplementary Figure S5). The global decrease in protein amount may result from compromise of translation efficiency under stress and/or the instability of proteins due to Nva misincorporation (58).

Pro, Glu, Gln and Orn accumulate in Nva-treated D419A/ScΔleuS

The transcription of MCH5, PUT1 and PUT2 genes are all activated by Pro utilization trans-activator (PUT3) in the presence of sufficient Pro, suggesting that Pro content may increase in Nva-treated D419A/ScΔleuS. It is also of interest to examine the variation of other free amino acids in D419A/ScΔleuS after Nva treatment. The data showed that Nva-treated D419A/ScΔleuS cells indeed had higher Pro content than Nva-untreated D419A/ScΔleuS cells (t-test; Figure 6A), indicating the transcription of MCH5, PUT1 and PUT2 was activated by PUT3 due to the accumulation of Pro. Moreover, Glu, Gln and Orn, all of which are involved in Pro metabolism (Figure 5A), were also found to accumulate in Nva-treated D419A/ScΔleuS compared with those in Nva-untreated D419A/ScΔleuS cells in a dose-dependent manner (t-test; Figure 6B–D and Supplementary Table S2). According to the metabolic pathway, we deduced that there may exist metabolic flow from Orn to Pro and then to Glu and Gln in Nva-treated D419A/ScΔleuS cells, leading to the accumulation of Pro, Glu and Gln (Figure 5A). The fact that the medium lacks Pro, Glu and Gln excludes the possibility of their uptake from the medium.

Surprisingly, in the presence of exogenous Nva, less Nva accumulated in the cytoplasm of D419A/ScΔleuS than in WT/ScΔleuS (t-test; Figure 6E), presumably because WT/ScΔleuS possesses an editing proficient LeuRS and could tolerate a higher concentration of intracellular Nva. Besides, Nva may be converted to some non-toxic products in D419A/ScΔleuS to reduce its cytotoxicity. Given that Nva is an inhibitor of CAR1 and only lacks an amino group compared with Orn (59), it can be deduced that Nva may be converted to Orn or other amino acids in D419A/ScΔleuS (Figure 6A), causing the accumulation of Orn.
Figure 5. Pro metabolism-related genes are upregulated at the transcriptional level but downregulated at the translational level in Nva-treated D419A/ScΔleuS. (A) Schematic overview of Pro metabolism in Nva-treated D419A/ScΔleuS. Amino acids subject to accumulation are shown in red front. Genes subject to over-transcription are shown in red italic front, while proteins subject to downregulation are shown in blue front. Red arrows represent the metabolism direction we suggested, while the red dashed arrow represents the potential conversion. Red hollow arrows represent the regulation of gene transcription. MCH5, riboflavin transporter; PUT1, Pro oxidase; PUT2, P5C dehydrogenase; PUT3, Pro utilization trans-activator. Pro1, γ-glutamyl kinase; Pro2, γ-glutamyl phosphate reductase; Pro3, P5C reductase; CAR1, arginase; CAR2, ornithine aminotransferase. Arg, arginine; Orn, ornithine; Pro, proline; P5C, 1′-pyrroline-5-carboxylate; GSA, glutamate-γ-semialdehyde; Glu, glutamic acid; Gln, glutamine; Nva, norvaline. (B–D) qRT-PCR analysis of mRNA levels of MCH5 (B), PUT1 (C) and PUT2 (D) in WT/ScΔleuS (black columns) and D419A/ScΔleuS (gray columns) treated with 0.5 mM Ile/Leu/Nva for 12 h (initial A600 = 0.6). Data are normalized against ACT1 and presented as the mean ± SD (n = 3). Asterisks indicate significant differences between different treatments (**P < 0.02; *P < 0.05; two-tailed Student’s t-test). (E) Both D419A/ScΔleuS and WT/ScΔleuS yeast cells were treated with or without 0.5 mM Nva for 12 h (initial A600 = 0.6). Left panel, protein levels of MCH5–13 × Myc (90 kDa), PUT1–13 × Myc (85 kDa) and PUT2–3 × HA (70 kDa) were determined by immunoblotting. Right panel, quantification of bands in left panel by normalization against Actin. Black columns represent the amount of protein in Nva-untreated D419A/ScΔleuS, which is designated as 100%. Gray columns represent the amount of protein in Nva-treated D419A/ScΔleuS. Error bars represent the mean ± SD (n ≥ 2).
Figure 6. Measurement of free amino acids in Nva-treated D419A/ScΔleuS (A–E) Intracellular Pro (A), Glu (B), Gln (C), Orn (D) and Nva (E) content (µmol/g yeast dry weight) of WT/ScΔleuS (black columns) and D419A/ScΔleuS (gray columns) treated with Nva (0, 0.5, 1 or 2 mM) for 12 h (initial A600 = 0.6). Data are presented as the mean ± SD (n = 3). Asterisks represent significant differences between different treatments (**P < 0.02; *P < 0.05; two-tailed Student’s t-test).

In contrast, the amount of other amino acids either decreased (Arg, glycine, histidine, lysine and threonine) or showed no obvious change (Ile, Leu, Val, phenylalanine, serine and tryptophan) in Nva-treated D419A/ScΔleuS cells compared with that in Nva-untreated D419A/ScΔleuS cells (t-test; Supplementary Table S2), suggesting that Nva stress may affect the metabolism of some other amino acids in LeuRS editing-defective yeast.

Pro, Glu and Gln contribute to lower intracellular ROS levels in Nva-treated D419A/ScΔleuS

As redox homeostasis is indispensable for protein folding (60) and Pro, Glu and Gln are all well documented as antioxidants (37,38,47), we wondered whether the accumulation of these amino acids could decrease ROS levels in Nva-treated D419A/ScΔleuS. WT/ScΔleuS and D419A/ScΔleuS yeast strains were cultured in the absence or presence of Nva, and ROS levels were monitored by flow cytometry using H2DCFDA as a ROS indicator. As expected, ROS levels in Nva-treated D419A/ScΔleuS were significantly decreased compared with untreated yeast (Figure 7A and B), consistent with the accumulation of Pro, Glu and Gln (Figure 6A–C). In contrast, Nva did not affect ROS levels in WT/ScΔleuS (Figure 7C and D), consistent with the unchanged amount of Pro, Glu and Gln (Figure 6A–C).

To further confirm the important role of intracellular Pro, Glu and Gln in scavenging ROS in Nva-treated D419A/ScΔleuS cells, exogenous Pro, Glu or Gln was added to the medium and ROS levels were monitored. The results showed that the addition of Glu (10 mM), Gln (1 mM) or Pro (10 mM) was enough to lower intracellular ROS levels (Figure 7E and F), further suggesting that accumulation of Pro, Glu and Gln contribute to scavenging of intracellular ROS.

Only Leu, but not Pro, Glu or Gln can strikingly ameliorate Nva stress

As described above, Pro, Glu and Gln accumulated in Nva-treated D419A/ScΔleuS cells, which contribute to lowering ROS levels. In addition to their synthesis in vivo, these three amino acids could also be assimilated from the medium. We therefore supplemented Nva-containing minimal medium with these amino acids to identify their function in Nva-treated D419A/ScΔleuS. However, exogenous addition of Pro, Glu or Gln only slightly rescued the Nva-induced growth delay of D419A/ScΔleuS (Figure 8A), indicating that their accumulation may not be a major approach for D419A/ScΔleuS cells to ameliorate Nva stress.

Additionally, Leu was more effective at relieving the Nva-induced growth delay (Figure 8B), presumably by out-competing Nva. Generally, amino acid analogs enter cells through the same transporter as their corresponding amino acids, but they have a lower affinity for the transporter, resulting in a relatively low transport rate (61). Moreover, cognate amino acids are activated and aminoacylated more efficiently than their analogs, which reduces the possibility of misincorporation when both cognate amino acids and their analogs are present. This is consistent with the observation that an increase in the Leu:Nva ratio decreases Nva misincorporation into haemoglobin (20).
Figure 7. ROS generation in different yeast strains. (A) Intracellular ROS levels in D419A/ScΔleuS treated with Nva (0, 0.2, 0.5 or 1 mM). Approximately 30,000 cells were counted. Data were processed using Flowjo 2.0 software. The graph shows the ROS intensity in live cells. (C) Intracellular ROS levels in WT/ScΔleuS treated with Nva (0, 0.2, 0.5 or 1 mM). (E) Intracellular ROS levels in D419A/ScΔleuS treated with 10 mM Glu, 1 mM Gln and 10 mM Pro. (B, D and F) Quantification of the relative ROS levels in (A, C and E), respectively. Data are the mean ± SD (n = 3). n.s., no significant differences between Nva-treated and non-treated WT/ScΔleuS. Asterisks indicate significant differences between treated and non-treated D419A/ScΔleuS (**P < 0.02; *P < 0.05; two-tailed Student’s t-test).
**DISCUSSION**

Nva is the biggest threat for LeuRS among Leu analogs

Translation is a highly complex process with multiple checkpoints that cooperate to ensure fidelity. AaRSs are key players in this process and their ‘double-sieve’ mechanism prevents misincorporation of non-cognate amino acids into growing polypeptide chains. Previously, both Nva and Ile were thought to be targets for LeuRS editing. However, it was recently shown that the editing function of EcLeuRS is only required to prevent Nva misincorporation, not misincorporation of Ile (16). Nva is a by-product of Leu metabolism and can accumulate in *E. coli* cells under anaerobic conditions (17). Nva can replace about 10% of Leu residues in the proteome of LeuRS editing-defective *E. coli*, resulting in reduced cell viability (16,18). In the present study, we used the simple eukaryote, *S. cerevisiae*, to reveal that Nva is also the sole threat for eukaryotic LeuRS both in vitro and in vivo. Once the post-transfer editing function of ScLeuRS was disrupted, some Nva could be misincorporated into the proteome of yeast. Therefore, Nva should be the biggest threat for both prokaryotic and eukaryotic LeuRSs among the various Leu analogs.

Editing deficiency of aaRS inhibits cell growth and could induce diseases

Non-cognate amino acids will be misincorporated into proteins when the editing function of one specific aaRS is deficient, impairing the overall translation fidelity and inducing protein misfolding. Misfolded proteins are extremely deleterious to cells, which may be due to their dysfunction or even toxic gain-of-function. Besides, misfolded proteins are prone to nucleate other proteins to form harmful aggregates. Many studies have shown that the editing deficiency of aaRS can impact many physiological functions including growth (16), neurodegeneration (31), SOS response (33) and apoptosis (34). However, the severity of the cellular responses appears to correlate with the degree of mistranslation. For example, in our study, D419A/*ScΔleuS* cells could withstand low concentration (0.2 mM), but not higher concentrations (0.5 and 1 mM) of Nva, suggesting that disease or growth inhibition will only occur when the level of misfolded proteins exceed a threshold.

Heat shock proteins are upregulated to deal with misfolded proteins

Proteins must be correctly folded in order to carry out their biological function, thus all cells possess an extensive proteostasis network to cope with misfolded proteins. The upregulation of heat shock proteins by the cell facilitates the refolding or degradation of misfolded proteins, and prevents protein aggregation (35). Indeed, Hsp70 chaperones were upregulated in Nva-treated D419A/*ScΔleuS*, suggesting the accumulation of misfolded proteins in editing-deficient cells. In particular, the level of the ER chaperone KAR2 (Bip) was strikingly upregulated. KAR2 is also upregulated in AlaRS editing-defective Purkinje cells (31). The ER is an important organelle for protein folding and quality surveillance. The accumulation of misfolded proteins within the ER could disrupt the balance between the unfolded protein load and the ER protein surveillance system, which is known as ER stress. Cells may initiate the unfolded protein responses to relieve ER stress (60).

A new protective response to decreased editing

The lower level of Nva in Nva-treated D419A/*ScΔleuS* cells compared with that in Nva-treated WT/*ScΔleuS* cells suggests that Nva may be excluded from the cells or converted into other metabolites, such as α-ketovaleric acid, which could be further converted to other amino acids (17,62). Due to a difference of only an amino group, we assumed that a portion of intracellular Nva may be converted into Orn in Nva-treated D419A/*ScΔleuS* and Orn could be further converted into Pro, Glu and Gln. By converting Nva into other non-toxic amino acids, cells could reduce the toxicity of Nva.

Pro, Glu and Gln were indeed found to accumulate in Nva-treated D419A/*ScΔleuS*. Previous studies demonstrated that Pro is a multifunctional molecule (37–39). It can inhibit protein aggregation through interactions with misfolded proteins via the hydrophobic backbone and side-chain (39). Given that misfolded proteins accumulate in...
Nva-treated D419A/ScΔleuS cells, the concomitant accumulation of Pro may help prevent protein aggregation. It is noteworthy that, Pro, Glu and Gln are all bona fide antioxidants (37,38,47). In our study, we found that both the endogenous accumulation and exogenous addition of Pro, Glu and Gln contributed to lowering ROS generation. Although Pro, Glu and Gln have been studied previously as individual antioxidants, their synergistic action and metabolic modulation in reducing ROS levels has not been described before. However, the exogenous supplementation of Pro, Glu or Gln only slightly relieves the Nva-induced growth inhibition, suggesting that accumulation of antioxidant amino acids (Pro, Glu and Gln) and reduction of ROS may not be major approaches for D419A/ScΔleuS cells to relieve Nva stress but are the products of the metabolic detoxification process.

In conclusion, we explored the intracellular protective mechanism in a LeuRS post-transfer editing-deficient S. cerevisiae strain and identified a novel protective pathway to deal with non-cognate amino acids in editing-deficient cells. We propose that the non-cognate amino acid itself has the potential to participate in the protective pathway to reduce its cytotoxicity by conversion to non-toxic amino acids. Our results provide insights into the protective responses in aaRS editing-deficient cells and may assist the development of therapies for editing-deficient diseases.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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