Context-specific action of macrolide antibiotics on the eukaryotic ribosome

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Macrolide antibiotics bind in the nascent peptide exit tunnel of the bacterial ribosome and prevent polymerization of specific amino acid sequences, selectively inhibiting translation of a subset of proteins. Because preventing translation of individual proteins could be beneficial for the treatment of human diseases, we asked whether macrolides, if bound to the eukaryotic ribosome, would retain their context- and protein-specific action. By introducing a single mutation in rRNA, we rendered yeast Saccharomyces cerevisiae cells sensitive to macrolides. Cryo-EM structural analysis showed that the macrolide telithromycin binds in the tunnel of the engineered eukaryotic ribosome. Genome-wide analysis of cellular translation and biochemical studies demonstrated that the drug inhibits eukaryotic translation by preferentially stalling ribosomes at distinct sequence motifs. Context-specific action markedly depends on the macrolide structure. Eliminating macrolide-arrest motifs from a protein renders its translation macrolide-tolerant. Our data illuminate the prospects of adapting macrolides for protein-selective translation inhibition in eukaryotic cells.
Many human diseases result from expression of unwanted proteins\textsuperscript{1–3}. While the most common therapies for such diseases are based on blocking the functions of the undesirable proteins, this approach mitigates their harmful effect but does not eliminate the culprit. Inhibiting the production of a malicious protein could be a better strategy than targeting its activity. To achieve this goal, significant efforts have been invested in developing mRNA-targeting approaches for selective destruction of specific mRNAs or blocking their translation\textsuperscript{1}. However, very limited research has been dedicated towards finding molecules, that could curb the production of specific proteins by acting upon the ribosome. The lack of interest for undertaking such task could be justified by the traditional notion that ribosome-targeting compounds indiscriminately prevent ribosomes from synthesizing all proteins. Therefore, the discovery of PF846, a small molecule that binds to eukaryotic ribosomes and selectively inhibits translation of only a subset of polypeptides, including the therapeutically-significant protein PCSK9 involved in cholesterol homeostasis, came as a big and welcomed surprise\textsuperscript{5,6}. Illuminating biochemical and structural studies have shown that PF846 binds in the nascent peptide exit tunnel (NPET) of the large ribosomal subunit and interferes with translation of several specific nascent polypeptides, that assume an idiosyncratic conformation in the tunnel\textsuperscript{6,7}. However, because many details of the mechanism of PF846 action remain unknown, predicting the proteins whose synthesis would be inhibited by this compound would be a difficult task.

Even though the concept of selective inhibition of eukaryotic translation by ribosome-targeting small molecules emerged only recently, it has been long recognized for antibiotics that act upon the bacterial ribosome. Over four decades ago it was found that inducible resistance to macrolide antibiotics is regulated by programmed translation arrest relying on the ability of the drugs to stop ribosomes at specific mRNA codons, while allowing for unpinned translation through the preceding ones\textsuperscript{8,9}. More recent studies have shown that in fact many drugs that target the bacterial ribosome act in a context-specific manner, causing translation arrest at specific sites in mRNA, where the nascent peptide sequence, tRNA nature or mRNA structure are conducive to the antibiotic action (reviewed in the ref. \textsuperscript{10}). Yet, the ribosome-targeting antibiotics whose context-selective action is best understood are the macrolides\textsuperscript{11}.

All macrolide antibiotics, from the prototype of this class, erythromycin (ERY), to those of later generations, e.g., telithromycin (TEL) or solithromycin (SOL)\textsuperscript{12} (Fig. 1), bind in the NPET of the bacterial ribosome, close to the peptidyl transferase center (PTC)\textsuperscript{13–16}. They establish interactions with several RNA residues, including the adenosine at position 2058 of the 23S rRNA (Escherichia coli numbering) which, while conserved in bacteria, is replaced with guanine in eukaryotes (G2400 in the yeast Saccharomyces cerevisiae 23S rRNA) (Supplementary Fig. 1a, b), and is thought to be the key determinant of the bacterial selectivity of macrolide action\textsuperscript{14}. Because the bulky macrolide molecule narrows the lumen of the NPET, these antibiotics were initially thought to completely block the passage of any nascent polypeptide and to only allow the synthesis of peptides a few amino acids long\textsuperscript{17,18}. Subsequent studies have shown, however, that the growing protein chain can be threaded through the macrolide-obstructed NPET, and that macrolides selectively abolish production of specific proteins by preventing the ribosome from polymerizing specific amino acid sequences\textsuperscript{11,19–22}. Remarkably, the context-specificity of the macrolide action is not based on the inability of some nascent polypeptides to bypass the antibiotic in the NPET. Instead, NPET-bound macrolides interfere with peptide bond formation when the bacterial ribosome attempts to synthesize specific amino acid motifs\textsuperscript{23–27}. In addition, the spectrum of the macrolide arrest motifs, and hence the range of the inhibited proteins, is defined by the chemical structure of the drug bound in the NPET of the bacterial ribosome\textsuperscript{11,19,22,28}.

Context-specificity and protein-selectivity of macrolide action in bacteria make this class of antibiotics a promising platform for developing selective inhibitors of eukaryotic translation. The prospect of such an approach hinges, however, on the macrolides retaining their context-selectivity and protein-selectivity of inhibition of protein synthesis in a eukaryotic cell. Unfortunately, little of what has been learned about macrolide action in bacteria could be extrapolated to eukaryotic translation, because the structures and functional properties of NPETs in bacterial and eukaryotic cytoplasmic ribosomes are substantially different\textsuperscript{29–33} (Supplementary Fig. 1b, c). Furthermore, whether macrolides can actually bind in the eukaryotic ribosomal NPET remains unclear, especially considering that mutation of the key discriminating nucleotide G2400 to A, failed to render yeast ribosomes or cells sensitive to ERY\textsuperscript{34}.

**Fig. 1 Macrolide antibiotics.** Chemical structures of the main macrolide antibiotics used in this study. The numbering of the macrolactone atoms is shown on the ERY structure. C5-desosamine sugars and alkyl-aryl side chains of the extended macrolides are indicated by black and red rectangles, respectively.
Here, we used biochemical and structural analyses to explore whether macrolide antibiotics retain their inhibitory activity and context-specificity of action when bound to the eukaryotic (yeast) ribosome. We show that engineered yeast cytoplasmic ribosomes with the G2400A mutation are capable of binding macrolides, which contain an extended side chain and that the binding mode of such compounds in the NPET is analogous to that on the bacterial ribosome. We further show that macrolide binding to the 80S ribosome inhibits translation in vivo and in vitro. Genome-wide Ribo-seq studies revealed that in eukaryotic cells, macrolides interfere with protein synthesis in a context-specific way, with some of the prevalent arrest motifs overlapping with those in bacteria, while others being specific for the eukaryotic 80S ribosome. We found that minor changes in the polypeptide-encoding sequence can drastically alter the sensitivity of protein translation towards a particular macrolide. We also show that by altering the structure of the macrolide antibiotics bound to the eukaryotic ribosome, it is possible to modulate their effect upon synthesis of individual proteins.

Results

A single rRNA mutation sensitizes yeast cells and cytoplasmic 80s ribosomes towards extended macrolide antibiotics. A previous work had shown that the 25S rRNA G2400A mutation (A2058 in E. coli; through the rest of the text, the E. coli numbering is shown in parentheses) in the macrolide binding site, which replaced the eukaryote-specific guanine with bacteria-specific adenine, did not render yeast 80S ribosomes sensitive to ERY34. We reasoned that newer and more active macrolides such as TEL or SOL (Fig. 1), with extended side chains that establish additional interactions in the NPET of the bacterial ribosome15,35,36, could perhaps bind to the G2400A mutant yeast ribosomes. Therefore, we engineered the G2400A mutation de novo in the S. cerevisiae strain NOY891, where the rRNA-encoding RDN locus on chromosome XII is deleted and rRNA is expressed from a plasmid37,38 (see “Methods” section). Primer extension analysis confirmed that the cytoplasmic 80S ribosomes in the resulting strain contained exclusively the mutant G2400A 25S rRNA (Supplementary Fig. 2a). In agreement with the previous report34, the engineered G2400A mutant yeast remained resistant to ERY and the closely related azithromycin (AZI). However, TEL and SOL completely abolished the growth of the mutant at concentrations below 200 µg/ml (TEL) or below 50 µg/ml (SOL) (Fig. 2a and Supplementary Table 1). The growth arrest was likely caused by inhibition of protein synthesis because exposure of the mutant yeast cells to SOL resulted in a rapid decline of incorporation of [1-35S]-methionine into newly synthesized polypeptides (Fig. 2b). Thus, the G2400A mutation in 25S rRNA of the large subunit of the eukaryotic cytoplasmic ribosome sensitized yeast to the extended macrolide antibiotics. We used the engineered mutant to explore the mode of binding of this class of inhibitors to the 80S ribosome, and the effects of macrolides upon translation in the eukaryotic cell.

Extended macrolides bind in the exit tunnel of the mutant yeast ribosome. To verify that extended macrolides inhibit protein synthesis in the mutant yeast cells by acting upon ribosomes, we carried out binding studies using radiolabeled [14C]-SOL. While wild-type (wt) ribosomes were essentially impervious to binding of SOL, ribosomes with the G2400A mutation showed a significantly increased affinity to the drug, allowing binding with an apparent dissociation constant in the sub-micromolar range (K_d = 0.26 µM ± 0.09) (Fig. 2c). To further analyze whether binding of SOL and TEL takes place in the NPET of the mutant eukaryotic ribosome, we analyzed drug-rRNA interactions by chemical RNA probing. In bacteria, NPET-bound macrolides shield A2058 and A2059 in the 23S rRNA from modification by dimethylsulfate (DMS)39–41. In mutant (but not wt) yeast ribosomes, SOL and TEL protected the two equivalent rRNA residues, A2400 and A2401, from DMS modification (Fig. 2d and Supplementary Fig. 2b) revealing binding of these antibiotics to the NPET of the 80S ribosome. ERY and AZI that lacked the alkylaryl side chain present in the extended macrolides afforded only marginal protection of these residues (Supplementary Fig. 2c), corroborating a very weak binding of these antibiotics, which therefore, were excluded from the subsequent experiments.

While revealing the general location of the macrolide binding site in the mutant yeast ribosome, the results of rRNA probing are unable to reveal the atomic interactions and the exact orientation

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**Fig. 2 Macrolide antibiotics bind to the mutant yeast ribosome and inhibit protein synthesis and cell growth.** a SOL arrests growth of the G2400A mutant S. cerevisiae cells. Plots show the growth of mutant cells in the absence of the drug (filled black circles) or after addition of 8× MIC of SOL (open gray circles). The inset shows that wt yeast is resistant to SOL. The graphs represent results of two independent experiments with individual data points indicated. b Residual protein synthesis in wt (squares) and G2400A mutant (circles) yeast cells exposed to SOL. Translation was assessed by measuring incorporation of [1-35S]-methionine into polypeptides after exposure of cells for 10 min to different concentrations of SOL. [1-35S]-methionine incorporation in the untreated mutant cells was set to 100%. The data points of two independent experiments are indicated by squares (wt) and circles (G2400A mutant). c Equilibrium binding of [14C]-SOL to wt (squares) or G2400A mutant (circles) ribosomes. The data are presented as mean values of three independent experiments; error bars show standard deviation. d SOL and TEL protect A2400 and A2401 of the 25S rRNA from chemical modification. The gel shows the primer extension analysis of the 25S rRNA extracted from the untreated or dimethyl sulfate (DMS)-modified G2400A 80S ribosomes incubated without or with the antibiotics. The position of the cDNA bands corresponding to the nucleotide residues A2400 and A2401 are indicated by the arrows. The chemical modification of wt ribosomes in the presence of SOL and TEL and that of mutant ribosomes in the presence of ERY and azithromycin are shown in Supplementary Fig. 2b, c, respectively. The uncropped gel can be found in the Source data file. The result is a typical representative of two independent experiments.
of the drugs in the 80S ribosome NPET, which can be dramatically affected by the distinct architectures of ribosomes from different species (Supplementary Fig. 1). To understand the precise binding mode of the extended macrolides in the yeast G2400A mutant ribosome, we determined the cryo-EM structure of the mutant G2400A ribosome in complex with TEL with an average resolution of 3.1 Å (Supplementary Fig. 3). The resolution was further improved with focused refinement on the large 60S subunit (Supplementary Fig. 3), leading to a final reconstruction of the yeast 60S-TEL complex (Fig. 3a) with an average resolution of 2.9 Å and extending to 2.5 Å within the core of the particle (Supplementary Fig. 4a–d and Supplementary Table 2). The density for TEL observed within the NPET of the 60S subunit was well-resolved, enabling an unambiguous placement of the 14-membered macrolactone ring, C5-desosamine sugar and the C10–C11 alkyl-aryl side chain (Fig. 3b and Supplementary Fig. 4e). TEL binds within the NPET of the yeast large ribosomal subunit with desosamine sugar extending towards the PTC and placed adjacent to 25S rRNA nucleotides A2400(2058) and A2401(2059) (Fig. 3c), consistent with the DMS protection
Macrolides elicit context-specific ribosome stalling in yeast cells. Having demonstrated that macrolides can interfere with translation by binding in the NPET of the mutant eukaryotic ribosome, we wanted to know whether, similar to their action in bacteria, they preferentially arrest the ribosome at specific sites in yeast cells.

We applied pLogo analysis to 749 sites associated with the sites of most prominent macrolide-induced translation stalling, mapping the ribosome footprints onto the RNAs in the TEL-treated yeast cells compared to the control. The most common motif (24% of the strong arrest sites) is represented by the sequence Arg/Lys-X-Arg/Lys (where X, which could be any amino acid, corresponds to the penultimate residue of the nascent chain). The second most prevalent motif, found in 15% of the strongest arrest sites in yeast, is characterized by the presence of proline as the penultimate amino acid (Fig. 4c). This pattern matches the one commonly found in TEL-arrest sites in bacteria, called the “+X+” motif, because both Arg and Lys side chains carry a positive charge. The second most prevalent motif, found in 15% of the strongest arrest sites in yeast, is characterized by the presence of proline as the penultimate amino acid (Fig. 4c).

Macrolides elicit context-specific ribosome stalling in yeast cells. Having demonstrated that macrolides can interfere with translation by binding in the NPET of the mutant eukaryotic ribosome, we wanted to know whether, similar to their action in bacteria, they preferentially arrest the ribosome at specific sequence motifs. To address this question in an unbiased way, we analyzed re-distribution of ribosomes on the mRNAs in the TEL-exposed S. cerevisiae cells by ribosome profiling (Ribo-seq). In order to minimize secondary stress-related effects, the exponentially growing G2400A yeast cells were exposed for only a brief time (10 min) to a high concentration of TEL (1.5 mg/mL equivalent to 8x MIC), that result in ~90% inhibition of protein synthesis (Supplementary Fig. 8a, b). Under these conditions, the polysome profile of the treated cells virtually showed no changes in comparison with the untreated control (Supplementary Fig. 8c). The Ribo-seq data were highly reproducible between the biological replicates, when gene scores (the relative densities of ribosome footprints mapped to a gene) or pause scores (the relative codon occupancies) were compared (Supplementary Fig. 8d–f). However, comparison of TEL datasets relative to the controls showed a number of sites with a notably increased pause score in the antibiotic-treated samples, reflecting a redistribution of ribosomes within mRNAs in response to the exposure to the drug (Supplementary Fig. 8g–i).

Noteworthy, although the PDK sequence is one of the preferred sites of TEL-induced arrest in bacteria, the general PDX sequence is not particularly problematic for the TEL-bound bacterial ribosome. While the variable X amino acid within the +X+ and PDX motifs might play a secondary role, its identity apparently modulates the efficiency of stalling (Supplementary Fig. 9a, b). Importantly, not every +X+ or PDX sequence, even when containing a favorable X amino acid within the motif, was associated with increased ribosome occupancy in the TEL-treated yeast cells (Supplementary Fig. 9c). Therefore, the general signal of macrolide-induced stalling likely includes additional elements.
**Fig. 4 Macrolides arrest translating ribosomes at specific sequences.** 

a Examples of TEL-induced translation arrest within *S. cerevisiae* genes. Comparison of ribosome footprint densities in untreated G2400A yeast cells (“no drug”, downwards extending black plots) or cells treated for 10 min with 1.5 mg/mL (8× MIC) of TEL (upwards-extending orange plots). The arrows indicate prominent footprint density peaks; the amino acid in the P site of the stalled ribosome is shown in bold.

b The correlation of pause score ratios in TEL-treated and untreated cells for 46,445 sites within the actively expressed genes between two independent experiments. The red rectangle includes the 749 sites with ≥2.5-fold enrichment of ribosomal density in TEL samples in both experiments, which were used for the pLogo analysis shown in c–e. The green rectangle marks the 1809 sites of the least efficient TEL action (pause scores reduced by ≥2-fold in the TEL-treated samples). c pLogo analysis of the sequences of the nine C-terminal residues of the nascent protein (positions 0 to −8) and the A-site amino acid (position +1) at the sites of strongest TEL action. d pLogo analysis of the sites of the strongest TEL action conforming to the +X+ arrest motif.

e pLogo analysis of the strongest arrest sites where proline (P) is present in the penultimate position of the nascent chain. f, g Bottom panels: Metagene analyses of ribosome density around the +X+ (f) and PDX (g) arrest motifs in TEL-treated (orange plots) and untreated (black plots) cells. Top panels show the ratio between the TEL and control metagene plots. The P-site codons of the +X+ and PDX motifs (highlighted by gray shadowing) are assigned as position 0. Note that the large metagene peak upstream from the highlighted peak in the PDX plot results from TEL-independent ribosome stalling at Pro codons..

h Relative occurrence of the different prevalent motifs among the 749 sites of strongest TEL-induced arrest. Left pie-chart shows all 749 sites, middle chart represents the subset of sites conforming to the +X+ motif and the chart on the right illustrates the subset of sites corresponding to the PXX motif.
besides the identified short motifs. No well-defined sequence motifs were detected for the remaining 61% of the strongest sites of TEL-induced arrest that do not conform to the +X+ or PDX motifs (Fig. 4h), except possibly for an increased incidence of the presence of Lys and Arg in the P-site of the TEL-stalled ribosome (Supplementary Fig. 9d).

We also analyzed the mRNA sites, where translation was least sensitive to inhibition by TEL. Due to drug-induced redistribution of ribosomes on mRNAs, such codons are characterized by diminished pause scores in the TEL-treated cells relative to the control (Fig. 4b, lower left quadrant). The yeast ribosome is least susceptible to TEL inhibition when the nascent chain contains Asn at the C-terminus, or when Glu is the incoming amino acid (Supplementary Fig. 9e, f). However, we did not find these two residues to be associated in a single sequence context. Rather, their effect on reducing TEL inhibition appeared to be independent from each other (Supplementary Fig. 9f). Neither a C-terminal Asn within the +X+ stalling motif, nor a Glu residue in the PDX arrest sequence are able to counteract the TEL action (Supplementary Fig. 9e, f). However, we did not find these two residues to be associated in a single sequence context. Rather, their effect on reducing TEL inhibition appeared to be independent from each other (Supplementary Fig. 9f). Neither a C-terminal Asn within the +X+ stalling motif, nor a Glu residue in the PDX arrest sequence are able to counteract the TEL action (Supplementary Fig. 9e, f). However, we did not find these two residues to be associated in a single sequence context. Rather, their effect on reducing TEL inhibition appeared to be independent from each other (Supplementary Fig. 9f). Neither a C-terminal Asn within the +X+ stalling motif, nor a Glu residue in the PDX arrest sequence are able to counteract the TEL action (Supplementary Fig. 9e, f). However, we did not find these two residues to be associated in a single sequence context. Rather, their effect on reducing TEL inhibition appeared to be independent from each other (Supplementary Fig. 9f). Neither a C-terminal Asn within the +X+ stalling motif, nor a Glu residue in the PDX arrest sequence are able to counteract the TEL action (Supplementary Fig. 9e, f). However, we did not find these two residues to be associated in a single sequence context. Rather, their effect on reducing TEL inhibition appeared to be independent from each other (Supplementary Fig. 9f). Neither a C-terminal Asn within the +X+ stalling motif, nor a Glu residue in the PDX arrest sequence are able to counteract the TEL action (Supplementary Fig. 9e, f). However, we did not find these two residues to be associated in a single sequence context. Rather, their effect on reducing TEL inhibition appeared to be independent from each other (Supplementary Fig. 9f). Neither a C-terminal Asn within the +X+ stalling motif, nor a Glu residue in the PDX arrest sequence are able to counteract the TEL action (Supplementary Fig. 9e, f). However, we did not find these two residues to be associated in a single sequence context. Rather, their effect on reducing TEL inhibition appeared to be independent from each other (Supplementary Fig. 9f).

Context-specific inhibition of translation depends on the structure of the macrolide antibiotic. We asked whether variation in the structure of the drug bound in the NPET of the yeast ribosome would affect specificity of translation inhibition. Several representative extended macrolides differing in the structure of the macrolactone ring and side chains were selected for these experiments (Fig. 6a). RNA chemical probing showed that in contrast to ERY or AZI, all these extended macrolides efficiently bind to the mutant yeast ribosome, and protect the A2400 and A2401 residues (A2058/A2059) from DMS modification (Fig. 6b and Supplementary Fig. 2c). Tylosin (TYL) and spiramycin (SPI), that carry a C5 disaccharide side chain (boxed in Fig. 6a), additionally protected A2404 (A2062) (Fig. 6b). Such protection, caused by a direct interaction of the drugs’ C5 side chain with this nucleotide, which has been noted previously in archaeal and bacterial ribosomes51,52, is indicative of a similar binding mode of these drugs in the eukaryotic ribosome.

We then examined the effects of all these antibiotics on the in vitro synthesis of three different polypeptides (the Gfp reporter and the two yeast proteins Slt2 and Zeo1), that we used in the previous experiments (Fig. 5). Strikingly, different drugs affected protein synthesis in a very distinct fashion. The C3-cladinose-containing compounds RU69874 and CEM103 efficiently blocked the expression of all three proteins, including TEL-resistant Zeo1 (Fig. 6c–e). Because no accumulation of truncated peptides was observed, these inhibitors possibly block protein synthesis at very early stages of cell-free translation. TEL, SOL, cethromycin (CET), and RU3004, all of which carry the C3-keto group instead of cladinose, had only a small effect on expression of Zeo1 (Fig. 6e), but readily inhibited translation of Gfp and Slt2 (Fig. 6c, d). As we had observed with TEL (Fig. 5d, f, g), SOL, CET, and RU3004 caused accumulation of specific truncated polypeptide products due to translation arrest at the identified macrolide arrest motifs within the Slt2-coding and Gfp-coding sequences. The remaining two drugs, 16-membered ring TYL and SPI with C5 disaccharide side chains barely affected expression of the tested proteins in the cell-free system (Fig. 6c–e) in spite of the robust binding of these compounds to the G2400A ribosome (Fig. 6b). The efficient synthesis of full-length polypeptides potentially reflected the ability of the yeast ribosomes to polymerize the problematic motifs

Extent of inhibition of protein expression by TEL depends on the polypeptide sequence. Although Ribo-seq analysis showed that TEL-bound yeast ribosomes stall at distinct sites during translation of a gene, the redistribution of ribosome footprints on mRNA does not directly reveal how the yield of the encoded protein is affected. To explore whether the context specificity of macrolide action can be manifested as sequence-selective inhibition of protein expression, we studied the effects of TEL on the synthesis of individual proteins in a cell-free system driven by the G2400A yeast ribosomes.

Cell-free expression of the Zeo1 protein, encoded by an ORF where the ribosome footprints pattern remained unchanged in the TEL treated cells, was barely affected even by high concentrations of the macrolide (Fig. 5a, c). By contrast, expression of the Slt2 polypeptide was highly sensitive to TEL and instead of the full-size protein, a shorter (~11 kDa) product accumulated (Fig. 5b, d). Appearance of the truncated Slt2 fragment possibly results from translation arrest at the PDG98 sequence of the SLT2 ORF (Fig. 5b, d) that conforms to the PDX stalling motif.

To directly test whether the protein sequence impacts the extent of inhibition of its translation by TEL, we followed its expression in the presence of TEL, the 12 kDa peptide no longer appeared (Fig. 5g, compare lanes 2 and 4), confirming that the wt KYKTR109 sequence was the site of the drug-induced translation arrest. Similarly, inactivation of the PDX motif by mutating the Pro196 codon within the PDG197N sequence to the Ala codon (P196A mutation) abolished the appearance of the 22 kDa product (Fig. 5g, compare lanes 2 and 6). The double K107A/P196A mutant that lacked both of the aforementioned macrolide arrest sites in GFP, prevented accumulation of both the 12 and 22 kDa truncated polypeptides and rendered translation of the full-size protein highly resistant to inhibition by TEL (Fig. 5g, compare lanes 2 and 8). In an independent assay, where Gfp expression was monitored by following its fluorescence, even at concentrations of TEL as high as 100 µM, translation of the full-size protein carrying the K107A/ P196A mutations remained at ~75% of the control, whereas production of wt Gfp was reduced to a lesser than 20% level (Fig. 5b). These results demonstrate that inhibition of protein production by binding of a macrolide to a eukaryotic ribosome critically depends on the sequence of the translated polypeptide, and revealed macrolides as potential protein-selective inhibitors of eukaryotic translation.
Fig. 5 TEL selectively inhibits in vitro translation of proteins with specific sequence motifs. a, b Ribosome footprint density in the ZEO1 (a) and SLT2 (b) genes in yeast cells treated (orange plots) or not (black plots) for 10 min with 8× MIC of TEL. PDX and +X+ motifs present in the SLT2 gene are indicated by arrows. Increased ribosome occupancy at early codons of SLT2 (orange asterisk) occurs at sites not conforming to the +X+ or PDX motifs. c, d SDS-gel analysis of the [35S]-radiolabeled proteins generated by in vitro translation of the ZEO1 (c) or SLT2 (d) templates by the G2400A yeast ribosome in the absence of the drug or in the presence of 100 µM TEL. Arrowheads indicate full-size proteins. The truncated Slt2 polypeptide, likely a product of TEL-mediated translation arrest at the PD34G motif, is indicated by an arrow and asterisk. e Locations of +X+ and PDX motifs in the GFP reporter gene. The molecular weights (kDa) of the proteins whose translation would be terminated at the corresponding sites are indicated. f Top: SDS-gel analysis of the [35S]-labeled products of in vitro translation of the GFP template in the absence or presence of increasing concentration of TEL. The band of the full-size GFP is indicated. Truncated GFP polypeptides resulting from TEL-induced ribosome arrest are marked with arrows and asterisks (colored as shown in e). Bottom: quantification of the relative radioactivity associated with the full-size and truncated GFP polypeptides in the SDS gel above. g SDS-gel analysis of the in vitro translation products of the wild-type (WT) GFP gene or its mutant variants in the absence or presence of 100 µM TEL. Translation products are labeled as in f. h Expression of wt GFP (GFPwt) or its K107A/P196A mutant (GFPmut) in the yeast cell-free translation system in the presence of increasing concentrations of TEL. Accumulation of functional GFP was followed by its fluorescence. The activity of GFPmut in the “no-drug” samples was set at 100%. The curves represent average of two independent experiments with individual data points indicated by filled (GFPwt) or open (GFPmut) circles. The uncropped gel can be found in the Source data file. The results shown in e, c, d, f, and g are typical representatives of at least two independent experiments.

Discussion

In this study, we demonstrated that binding of macrolide antibiotics to the eukaryotic cytoplasmic ribosome creates a hurdle for translation. Importantly, neither in bacteria, nor in yeast, the nascent chain residues critical for the drug-induced ribosome stalling are juxtaposed with the drug molecule in the NPET. Instead, they are located at the PTC for the drug-induced ribosome stalling to be terminated at the corresponding sites.

Interestingly, both macrolide arrest motifs, +X+ and PDX, were found previously at the sites of ribosome pausing in yeast cells depleted of the elongation factor eIF5A, which helps the ribosome in polymerizing problematic combinations of amino acids. It is possible, therefore, that binding of TEL to the yeast ribosome aggravates the burden of polymerizing intrinsically-difficult sequences. Noteworthy, however, in the untreated cells the ribosome often pauses at proline codons, with yeast ribosome aggravates the burden of polymerizing problematic combinations of amino acids. In contrast, in the TEL-bound yeast ribosome, the presence of proline in the penultimate position of the nascent protein creates a hurdle for translation. Importantly, in eukaryotes, just like in bacteria, TEL appears to act as an inhibitor of peptide bond formation between specific donor and acceptor substrates rather than as a discriminating gateway for the growing protein chain.

The main arrest motif in yeast, +X+, is identical to the primary motif of macrolide-induced stalling in bacteria. Although prolines are also found in some of the macrolide arrest motifs in bacteria, they are most commonly placed in the P or A sites of the stalled ribosome. In contrast, in the TEL-bound yeast ribosome, the presence of proline in the penultimate position of the nascent protein creates a hurdle for translation. Importantly, both macrolide arrest motifs, +X+ and PDX, were found previously at the sites of ribosome pausing in yeast cells depleted of the elongation factor eIF5A, which helps the ribosome in polymerizing problematic combinations of amino acids. It is possible, therefore, that binding of TEL to the yeast ribosome aggravates the burden of polymerizing intrinsically-difficult sequences. Noteworthy, however, in the untreated cells the ribosome often pauses at proline codons, with the nascent chain ending with proline (Fig. 4g). TEL additionally pauses translation at the following codon, when a proline residue is present in the penultimate position of the nascent chain (Fig. 4g). The pLogo plots also showed a somewhat increased presence of methionine at position –8 relative to the proline residue.
site of TEL-induced arrest (Fig. 4c–e and Supplementary Fig. 9d). However, this effect may simply reflect the fact that in the ten amino acid window that we used in our analysis, the initiator Met will be always found at this position when the window is placed at the beginning of the ORFs.

The macrolide arrest motifs identified by our bioinformatics analysis likely represent only a part of the signal required for the drug-induced ribosome stalling. Indeed, the Riboseq data revealed that the TEL-bound ribosome stalls only at a fraction of the sequences matching the identified arrest motifs. This conclusion was additionally reinforced by our in vitro translation experiments, where only some of the +X+ or PDX sequences caused accumulation of the corresponding truncated polypeptides (Fig. 5f, g). These observations argue that other factors, operating at the level of the polypeptide chain, mRNA or RNA trap might be also at play. In particular, more remote segments of the nascent protein, within or even outside of the NPET, could suppress or stimulate the arrest imposed by the tunnel-bound antibiotic, similar to the influence of the distal nascent chain context on ribosomes pausing during polymerization of polyproline sequences56,57 or of the native stalling peptides58,59.

The effect of macrolides on eukaryotic translation critically depends on the structure of the NPET-bound antibiotic (Fig. 6). The ketolides (macrolides with a C3-keto group) exhibit strong context dependence of the translation arrest (Fig. 6a–e). Similar compounds but with a C3 cladinose inhibit protein synthesis but do not yield any truncated protein products, likely because they interfere with the very early stages of translation (Fig. 5c–e). Strikingly, the 16-member macrolactone ring containing drugs TYL and SPI, in which a disaccharide moiety replaces the C5 desosamine present in other tested macrolides, had only limited effect on the yield of the three tested proteins (Fig. 6a–c). One possibility is that the advancing N-terminus of the growing polypeptide could displace TYL or SPI from their binding site, as has been suggested for the mode of action of short macrolide resistance peptides in bacteria60–62. Alternatively, these antibiotics could be much more selective compared to ketolides and the three proteins used in our in vitro experiments simply lacked the required arrest motifs. Noteworthy, some reports alluded that TYL could induce premature stop codon readthrough in mammals63, suggesting a possible effect of the antibiotic on translation in the wt eukaryotic cell, although it remains unknown whether this activity was mediated by binding of the drug to the ribosome.

Besides macrolides, the only other known highly-selective protein synthesis inhibitor acting upon the eukaryotic ribosome is the compound PF846, which interferes with translation of a very narrow subset of proteins in mammalian cells5. The binding site of PF846 in the NPET partially overlaps with that of macrolides (Supplementary Fig. 10), but its specificity and mode of action are significantly different. In contrast to TEL, which arrests translation at distinct sites, PF846 slows down the progression of the ribosome over several consecutive mRNA codons at the site of arrest5–7. At each of these codons the ribosome operates with a different combination of donor and acceptor ligands and therefore, in contrast to TEL, PF846 specificity is less dependent on the nature of the PTC substrates, but rather on the unusual trajectory of the nascent chain in the NPET6,7. Consistently, while macrolides inhibit peptide bond formation24–27, PF846 interferes with ribosome translocation and with translation termination6,7. It is remarkable that in spite of these differences, PF846 and TEL achieve context-specific inhibition of translation by binding to overlapping sites in the NPET. Interestingly, it has been recently shown that the compound tetracyclomycin X binds to bacterial
and eukaryotic ribosomes in a cavity of the NPET located on the wall opposite to the macrolide binding site and appears to act, at least during bacterial translation, in a sequence-specific manner. Therefore, the PTCproximal NPET segment emerges as the best target for the inhibitors, whose action depends on the sequence context of the growing polypeptide.

Identifying compounds capable of selectively suppressing expression of unwanted proteins, especially those that are viewed as “undruggable targets”, is an attractive strategy for the development of new medicines. For example, upregulation of expression of ribosomal proteins uL6 (gene RPL9), eL15 (RPL15), and eL39 (RPL39) is associated with increased tumor growth and development of new medicines. For example, upregulation of expression of macrolides makes obtaining such compounds within a cytoplasmic ribosome. The recent progress in synthetic chemistry inspired compounds active against the unaltered eukaryotic eukaryote-like nature of their macrolide-binding site. These pharmaceuticals.

Authorized from Sigma-Aldrich or obtained from Aventis or Cempra bacteria growth were from Difco. Macrolide antibiotics used in the study were purchased from Sigma-Aldrich or obtained from Aventis or Cempra Pharmaceuticals.

In our study, we used several available macrolides which have been specifically selected or optimized by the pharmaceutical industry for their action upon the bacterial ribosome and the lack of the effects upon eukaryotic translation. Therefore, we were compelled to carry out our experiments with the yeast ribosome that was intentionally sensitized to macrolide action by introducing the G2400A substitution in the drug-binding site. This single-nucleotide mutation allowed for binding with considerable selectivity of several macrolide antibiotics with extended side chains. Noteworthy, some macrolides (e.g., TYL or SPI) are capable of binding to the NPET of the archaeal ribosomes, in spite of the eukaryote-like nature of their macrolide-binding site. These observations argue that a targeted drug optimization, especially if guided by high-resolution structural data, could yield macrolide-inspired compounds active against the unaltered eukaryotic cytoplastic ribosome. The recent progress in synthetic chemistry of macrolides makes obtaining such compounds within a realistic reach.

Methods

Reagents and radiochemicals. All chemicals were from Thermo Fisher Scientific or Sigma-Aldrich. Radiochemicals were from PerkinElmer ([35S]-methyl-thione and [32P]-y-ATP), American Radiolabeled Chemicals ([3H]-ERY) or Cephr Pharmaceuticals ([14C]-SOL). Premixed media and media components for yeast and bacteria growth were from Difco. Macrolide antibiotics used in the study were purchased from Sigma-Aldrich or obtained from Aventis or Cempra Pharmaceuticals.

Yeast strains and plasmids. S. cerevisiae strain NOY891 and plasmid pJD694 were kindly provided by Dr. Dinman (University of Maryland). S. cerevisiae NOY891 (MATa ade2-1 ura3-1 leu2-3 his3-11 trpl1 can1-100 rnl1AA-HIS3) carries the TRP1-selectable plasmid PNYO353, that contains the wild type RDN operon encoding 355 pre-rRNA under the control of GAL7 promoter. Plasmid pJD694 was a URA3 selectable marker and carries the 355 pre-rRNA operon under the control of the tetracycline (doxycycline) repressible TET promoter. The S. cerevisiae strain with the G2400A mutation in the 25S rRNA gene was prepared as follows. The G2400A mutation was introduced in plasmid pJD694 by overlap-extension PCR, and the mutant plasmid was transformed into the NOY891 strain. Transformants were selected by first plating cells on SC-Ura-Page media with 50 µM TEL for 15 h at 30 °C. The reactions were incubated for 1 h at 30 °C. Following incubation, ribosomes were captured using 0.5 mg/mL of TCA. Following collection of all samples, the content of the beaker with the disks was boiled for 5 min and the TCA wash procedure was carried out.

Inhibition of bulk protein synthesis in vivo. S. cerevisiae NOY891 G2400A mutant cells were grown exponentially at 30 °C in YPD medium supplemented with 100 µM KOAc, 10 mM NaOH, 2 mM DTT, 5 mM Mg(OAc)2, 1 M µCi of [3H]-SOL. Ribosomes were isolated as described previously and washed three times with 1 mL of ice-cold binding buffer and then resuspended in 30 µL of fresh SD medium supplemented with 40 µM/mL of each amino acid except methionine. Reactions were initiated by addition of 1 µL of 11 µL/µL of ([35S]-methione (specific activity 1175 Ci/mmol). After 5 min incubation at 30 °C, 25 µL of the tubes’ contents were spotted onto 25 mm Whatman 3MM paper disks. Disks were immediately immersed into a beaker containing 500 mL of 5% trichloroacetic acid (TCA). Following collection of all samples, the content of the beakers with the disks was boiled for 5 min and TCA was discarded. The TCA wash procedure was repeated one more time. Disks were rinsed with acetone, dried, placed in vials with 5 mL of scintillation cocktail, and the amount of radioactivity retained was determined by scintillation counting. The time course of TEL-induced inhibition of bulk protein synthesis was carried out following essentially the same protocol except that the cultures were incubated with the antibiotic for 0, 5, 10, and 40 min before metabolic labeling was carried out.

Purification of yeast 80S ribosomes. S. cerevisiae G2400A mutant 80S ribosomes were purified for the structural studies according to the published protocol. The ribosomes for the drug binding assays were purified using the same protocol except that the sucrose cushion (20% sucrose cushion in a buffer containing 20 mM Hepes-KOH, pH 7.5, 120 mM KCl, 8.3 mM MgCl2, 2 mM DTT, 0.3 mM EDTA). Specifically, ribosomes collected from the lysate by sequential differential precipitation with PEG 20 K (4% and then 9% w/v) were resuspended in 15 µL of buffer A (30 mM Hepes-KOH, pH 7.5, 150 mM KCl, 10 mM MgCl2, 8.5% mannnitol, 2 mM DTT, 0.5 mM EDTA), layered over a 15 µL sucrose cushion, and centrifuged for 16 h in a SW32Ti at 36,000 rpm (130,000×g) at 4 °C. The ribosome pellets were resuspended in a storage buffer containing 10 mM Hepes-KOH, pH 7.5, 50 mM KOAc, 10 mM NH4Cl, 2 mM DTT, 5 mM Mg(OAc)2, flash frozen in liquid nitrogen and stored at −80 °C.

Analysis of binding of macrolides to yeast ribosomes. Purified ribosomes were diluted to 80 nM (A260 nm = 4) and combined with varying concentrations of [35S]-SOL (specific activity 52 Ci/mmol) in 100 µL of binding buffer (80 mM Hepes-KOH, pH 7.5, 140 mM KCl, 1.5 mM DTT, 5 mM MgCl2). The reactions were incubated for 1 h at 30 °C. Following incubation, ribosomes were captured using 0.5 mg/mL diethyl amino ethyl (DEAE) magnetic beads (BioClone). Beads were rapidly washed three times with 1 mL of ice-cold binding buffer and then resuspended in 100 µL of 1% SDS as described. Ribosome-associated radioactivity was determined by scintillation counting. Data were analyzed using Prism software (GraphPad).

Analysis of binding of unlabeled drugs to ribosomes was performed by RNA chemical probing following the conventional procedure, but with minor modifications. Brieﬂy, ribosomes (final concentration 0.2 µM) and antibiotic (final concentration 100 µM) were incubated in 50 µL of binding buffer for 1 h at 30 °C. Two micromolars of dimethylsulfate (DMS) diluted 1/5 in ethanol were added to the reactions and incubation continued for additional 10 min at 30 °C. The reactions were quenched by addition of 50 µL of stop solution containing 0.6 M NaOAc and 1 M β-mercaptoethanol. Ribosomes were ethanol-precipitated and RNA was extracted. The extent of modifications of 25S rRNA residues in the macrolide binding site was assessed by primer extension using the 5′-[32P]-labeled primer 25S-2430 (Supplementary Table 3).

Cryo-EM and single-particle reconstruction of 60S-TEL complex. The 80S-TEL complex was generated by incubating purified ribosomes with 50 µM TEL for 15 min on ice in buffer 10 mM Hepes-KOH, pH 7.5, 30 mM KOAc, 10 mM NH4OAc, 2 mM DTT, 5 mM Mg(OAc)2. Four microliters of the reaction solution (absorbance at 260 nm = 0.4) was applied to pre-coated Quantifoil holey carbon supported grids (R033, 3 mm C, Cu 300 mesh, Q4669, C3-C18Cu30-01) and vitrified using a Vitrobot Mark IV (FEI). Data collection was performed on Titan Krios 300 kV equipped with a K2 direct detection camera (Gatan). Images of single ribosome particles were aligned using MotionCor2 and 329,333 particles were picked automatically using Gautomatch (https://www.mrc-lmb.cam.ac.uk/kzhang/). pick for analysis using Relion 3.0. For each particle, the resulting refinement map was used to generate a 3D map.

Defocus values were determined using Gctf software77 (https://www.mrc-lmb.cam.ac.uk/kzhang/). Images were processed with Relion 3.0. Picked particles were sorted by 2D classification and 242,959 ribosome-like particles were selected for initial 3D refinement using an S. cerevisiae 80S reference structure (PDB ID 65477) (Supplementary Fig. 3a). 3D classification yielded five classes with one class containing 80S ribosomes with only E-site tRNA (75,303 particles), three classes with
80S ribosomes and sub-stoichiometric E-site tRNA (combined 153,893 particles) and one lower resolution class with sub-stoichiometric E-site tRNA (13,763). (Supplementary Fig. 2b–d). Particles of different resolution classes 2–4 were 3D refined and resolution optimized by CTF refinement through Relion 3.0 resulting in an average resolution of 3.1 Å (unmasked) and 2.9 Å (masked) determined using the “gold-standard” criterion (FSC100). The models were manually adjusted and refined using COOT, while regions with poor density were not manually adjusted based on the initial model. The final model was refined using Phenix with structural restraints calculated by Phenix eLBOW. Model validation was carried out using Phenix and MolProbity Server (http://molprobity.biochem.duke.edu/). The map vs. model cross correlation at PSEC was calculated by Phenix (T.197, 41582) comprehensive cryo-EM validation for each map individually using the Phenix validation tool 82.

Molecular modeling of 60S-TEL complex. The molecular model of the 60S ribosomal subunit, containing ribosomal proteins and tRNA, were based on S. cerevisiae 80S ribosome (PDB ID 6Q8Y) and the molecular model of TEL was based on E. coli 70S-TEL (PDB ID 4Y75). Models were rigid body fitted into the electron density map using Chimera. The models were manually adjusted and refined using COOT, while regions with poor density were not manually adjusted based on the initial model. The final model was refined using Phenix with structural restraints calculated by Phenix eLBOW. Model validation was carried out using Phenix and MolProbity Server (http://molprobity.biochem.duke.edu/). The map vs. model cross correlation at PSEC was calculated by Phenix (T.197, 41582) comprehensive cryo-EM validation for each map individually using the final molecular model (Supplementary Fig. 4a, right). The statistics of the final model are presented in Supplementary Table 2.

Preparation of figures with Cryo-EM structures. Figures were generated using PyMOL (Schrödinger, LLC) and structural superpositions were generated by alignment to the bacterial or mammalian large ribosomal subunits. Isolated densities and density images were created using Chimera and visualized using ChimeraX.

The structures of the NPET surfaces (Supplementary Fig. 1c) were generated using the described algorithm with the following parameters: 60 Å cubic grid with adjacent grid points separated by 1 Å; 10 Å sphere radius for “filling up” the internal cavities of the ribosome, including NPET. The image was generated using PyMOL (Schrödinger, LLC).

Preparation of samples for Ribo-seq. Two independent experiments (carried out on different days and employing different library preparation protocols) were carried out for collecting the Ribo-seq data. S. cerevisiae G420A mutant cells were grown exponentially at 30 °C in two L 1-LS flasks containing 200 mL of YPD medium. When the culture density reached 0.6–0.8, the lysate was added to the final concentration of 1.5 mg/mL (8x MIC) to one of the L-LS flasks. Cultures were incubated with shaking for 10 min and cells were collected by rapid filtration through Express Plus Membrane filter (Millipore) as described. Cells were rapidly frozen in liquid nitrogen and lysed using Mixer Mill MM400 (Retsch) with 300 µL of lysis buffer (10 mM HEPES-KOH, pH 7.5, 50 mM KOAc, 10 mM NH4Cl, 2 mM DTT, 5 mM Mg(OAc)2) without addition of cycloheximide. The lysate was centrifuged at 20,000×g for 5 min and the supernatant (~1 mL) was desalted by gravity-flow gel-filtration through a 15 mL Sephadex G-25 column equilibrated in buffer containing 30 mM Heps-KOH, pH 7.6, 100 mM KCl, 2 mM DTT, 0.5 mM PMSF, and 2 mM Mg(OAc)2. The flow-through fractions with A260 were combined, flash-frozen in liquid nitrogen and lyzed using Mixer Mill MM400 (Retsch) as described above for ribosome profiling. The RNA footprints were then combined, flash-frozen in liquid nitrogen, and stored at −80 °C.

To use the lysate for in vitro transcription/translation assay, the endogenous mRNAs were removed by nuclease treatment. For that, 50 µL of the thawed lysate were supplemented with 1 mM CaCl2 and 3.5 units of micrococcus RNase (Thermo Fisher Scientific) and incubated for 10 min at room temperature. The reaction was quenched by addition of 2 mM EGTA and the lysate was immediately used for in vitro protein expression. Reactions were carried out in a total volume of 15 µL containing 7.5 µL of the nuclease-treated lysate supplemented with the following components (listed with their final concentrations): 22 mM Heps-KOH, pH 7.6, 5.5 mM magnesium glutamate, 120 mM potassium glutamate, 120 mM sodium glutamate, 1.7 mM DTT, 1.5 mM ATP, 1.5 mM GTP, 1.5 mM CTP, 1.5 mM UTP, 25 mM creatine phosphate, 0.26 mg/mL creatine kinase, 80 µM of each amino acid except methionine, 0.6 mM/µL of L-methionine (specific activity 1175 Ci/mmol) and 50 µg/mL of T7 RNA polymerase purified according to Durniak et al. The reactions were initiated by adding 0.5 µL of the RNA templates generated as described above. The reaction products were analyzed in 16 % SDS gels. Gels were fixed with 5% perchloric acid, stained with Coomassie blue, dried, and exposed overnight to a phosphorimager screen. Radioactivity was visualized in a Typhoon scanner (GE Healthcare). The intensity of the bands was quantified using ImageJ.

Preparation of DNA templates for in vitro transcription/translation. The DNA templates for the coupled transcription/translation reactions contained the untranslated region of tobacco mosaic virus RNA (also known as Ω leader) with the sequence of the analyzed gene, and ending with a poly-A tail. All the primers used for generation of the templates are listed in Supplementary Table 3. To generate the SLT2 and ZEO1 templates, the gene sequences were PCR-amplified from genomic DNA of S. cerevisiae strain NOY99 using the primers Ω-SLT2/SLT2-A30 and ZEO1-ZEO1/A30, respectively. One microliter of the PCR product was diluted 1:100 to then re-amplify it using the T7 + T7-Ω primers combined with reverse primer SLT2-A30, for the SLT2 template, or with ZEO1-A30 primer for the ZEO1 template. The GFP gene was PCR-amplified from the pl1-sfGFP plasmid, using primers Gfp-SfGFP and Gfp-sfGFP-reverse. The resulting PCR product was re-amplified using primers T7, T7-Ω, and sfGFP-A37. The mutant GFP templates were generated by introducing the desired mutations via cross-over PCR using the respective mutagenizing primers listed in Supplementary Table 3 in combination with the T7 and sfGFP-A37 primers. The PCR products were cloned into plasmid and the presence or absence of the desired mutations was verified by sequencing. The templates for transcription-translation reactions were generated by PCR from the corresponding plasmids using the primers T7 and sfGFP-A37.

Calculation of pause scores. Pause scores were generated by dividing the rpm associated with each nucleotide by the average rpm of the gene. TEL-induced pauses were calculated by dividing the pause scores obtained from TEL-treated cells by the pause scores in untreated cells. Nucleotides with at least 0.5 were included in the analysis. The change in pause scores resulting from TEL treatment was calculated by computing the average pause score per codon, and then dividing codon pause score in the TEL sample by the codon pause score in the untreated sample.

Gene scores were calculated by adding together the total rpm mapping to each coding sequence. Reads were shifted by 18 nt from their 3′ ends to map the P-site. Total number of reads for each gene were normalized by length of the gene (by dividing by the length in kilobases) to obtain rpmk values (gene scores).

Analysis of the amino acid context enrichment at the sites of TEL-induced ribosome stalling. The 10-amino acid long sequences associated with the sites of the most pronounced TEL-induced translation arrest were extracted. Each sequence included amino acids encoded by the eight codons preceding the codon positioned in the ribosomal P site, and by the P-site and A-site codons. The sequences associated with the sites showing ≥5-fold change in pause score in both TEL samples compared to the control were compared to the amino acid sequences associated with atheric codons (46,445 in the 8x MIC sample) included in the analysis using the plogo tool.

Preparation of yeast lysate for in vitro translation was prepared according to a procedure described by Hodgman et al. with minor modifications. The S. cerevisiae NOY99 G420A mutant cells were grown at 30 °C in 1 L of YPD medium supplemented with 100 µg/mL Amp. Upon reaching an absorbance of A600 ~0.7, cells were harvested by fast filtration, flash-frozen in liquid nitrogen and lysed using Mixer Mill MM400 (Retsch) as described above for ribosome profiling. After the steps were carried out at 4 °C, the lysate was clarified by centrifugation at 20,000×g for 5 min and the supernatant (~1 mL) was desalted by gravity-flow gel-filtration through a 15 mL Sephadex G-25 column equilibrated in buffer containing 30 mM Heps-KOH, pH 7.6, 100 mM KCl, 2 mM DTT, 0.5 mM PMSF, and 2 mM Mg(OAc)2. The flow-through fractions with A260 were combined, flash-frozen in liquid nitrogen and stored at −80 °C.

Computational processing of ribosome profiling data. Ribosome profiling data were processed following the described algorithms. Briefly, the fastq files were first trimmed to remove adapters, demultiplexed, and mapped to the genome of individual samples from pooled data using cutADAPT. To remove tRNA and rRNA reads, the files were then aligned to an index of noncoding RNAs with Bowtie (version 1.1.2) using the following parameters: -v 2 -y. The fastq files after noncoding RNA removal step were then aligned to coding regions and splice junctions using Bowtie2 (version 1.2.9) with the following parameters: -v 2 -a -m 1—best—strata (two mismatches allowed and multiple alignments suppressed), and using the R64-1-1-2488R reference genome assembly (SacCer3, Saccharomyces Genome Database Project). Only 25–34 nt footprints were included in the analysis. 3′ alignments of the footprints were generated and used for subsequent analysis. Reads per million (rpm) was computed by normalizing the read count at each nucleotide position by the total number of mapped reads and then multiplying that value by 10^6.

Metagene analysis. Metagene plots were constructed by calculating the average (normalized by the total rpm in a window around the site of interest of the 100 nt centered at the middle codon of the motif. Genes with features smaller than the window size were excluded.

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Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support this study are available from the corresponding authors upon reasonable request. The cryo-EM and associated molecular model for the S. cerevisiae 80S ribosome complexed with telomycin is available from the EMDB (EMDB-11951 [https://www.ebi.ac.uk/pdb/entry/emdb/EMDB-11951]) and PDB (7AZY [https://doi.org/10.2210/pdb7AZY/pdb]), respectively. Ribo-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession code GSE164275. Source data is provided with this paper.

Code availability

The software used for the analysis of Ribo-seq data is available from https://github.com/guydoshlab/Yeastcode1.

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