Recognition of a start codon by the initiator aminoacyl-tRNA determines the reading frame of messenger RNA (mRNA) translation by the ribosome. In eukaryotes, the GTPase eIF5B collaborates in the correct positioning of the initiator Met-tRNA\textsuperscript{Met} on the ribosome in the later stages of translation initiation, gating entrance into elongation. Leveraging the long residence time of eIF5B on the ribosome recently identified by single-molecule fluorescence measurements, we determine the cryoEM structure of the naturally long-lived ribosome complex with eIF5B and Met-tRNA\textsuperscript{Met} immediately before transition into elongation. The structure uncovers an unexpected, eukaryotic specific and dynamic fidelity checkpoint implemented by eIF5B in concert with components of the large ribosomal subunit.
Protein synthesis starts with the assembly of a ribosomal complex at the start site on the mRNA. Eukaryotes employ numerous translation initiation factors (eIFs) to achieve the 80S initiation complex (IC) with the initiator aminoacyl-tRNA (Met-tRNA\textsubscript{Met}) and the AUG start codon of the mRNA programmed in the ribosomal peptidyl-tRNA site (P site). The 40S small ribosomal subunit, accompanied by eIFs and Met-tRNA\textsubscript{Met}, is recruited to the 5′-untranslated region (5′-UTR) of the 7-methylguanosine-capped mRNA. This is followed by a dynamic inspection of the 5′-UTR in search of the correct AUG codon as a start site, a process termed “scanning”\textsuperscript{4,5}. Upon AUG recognition by the Met-tRNA\textsubscript{Met} anticodon, a series of eIF reorganizations and departures coupled to ribosomal conformational rearrangements occurs, resulting in a post-scanning 48S preinitiation complex (PIC)\textsuperscript{6-8}. This 48S PIC is then joined by the 60S large ribosomal subunit to form the 80S IC, catalyzed by the universally conserved GTPase eIF5B (Fig. 1a)\textsuperscript{9,10}. Using single-molecule measurements, we performed cryo-EM analysis of the on-pathway initiation complexes to provide high-resolution information on the molecular mechanism by which eIF5B assists on the positioning of the initiator tRNA into the ribosomal P site and gates the transition from initiation to elongation.

### Results and discussion

#### Architecture of the eIF5B-bound 80S IC

Leveraging the slow dissociation of eIF5B from the 80S IC in the native initiation...
pathway (average lifetime of the eIF5B-bound 80S state is 30–60 s at 20 °C)\textsuperscript{[11]}, we prepared and froze samples at a pre-steady-state reaction timepoint (40 s, Fig. 1b, Supplementary Fig. 1, and “Methods”) corresponding to when ~60% of the assembled 80S should be bound with eIF5B (80S IC) after mixing 48S PICs with eIF5B-GTP and 60S subunits. Image processing and 3D classification in Relion\textsuperscript{[12,18]} identified a homogeneous class of ~70% of the total pool of 80S particles, allowing the reconstruction of a 3D map with a global resolution of 2.9 Å (Supplementary Figs. 2–4). The reconstruction shows clear density for a tRNA in the P-site region and density for all domains of eIF5B (Fig. 1c). Further analysis of the map revealed excellent densities for domain III (DIII), the connecting α-helix 12 (Fig. 1c, d, h\textsuperscript{[19]}) and especially for domain IV (DIV) of eIF5B, as well as for the Met-tRNA\textsubscript{Met} and the start codon of the mRNA (Fig. 1c, d, and Supplementary Fig. 4). Less well-resolved were domains II and G of eIF5B and the elbow region of Met-tRNA\textsubscript{Met} (Supplementary Fig. 3).

eIF5B DIV stabilizes the acceptor stem of Met-tRNA\textsubscript{Met} near the PTC. In our reconstruction, DIV of eIF5B is tightly packed against the peptidyl transferase center (PTC) of the large subunit where it contacts the \textsubscript{73}ACCA\textsubscript{76}-Met end of Met-tRNA\textsubscript{Met} (Fig. 2). The close interaction of eIF5B DIV with the acceptor stem of Met-tRNA\textsubscript{Met} enforces a change in the trajectory of the \textsubscript{73}ACCA\textsubscript{76}-Met end when compared with the position adopted by the acceptor stem on an elongation peptidyl-tRNA in a canonical pathway (average lifetime of the eIF5B-bound 80S state is 30–60 s at 20 °C)\textsuperscript{[11]}, we prepared and froze samples at a pre-steady-state reaction timepoint (40 s, Fig. 1b, Supplementary Fig. 1, and “Methods”) corresponding to when ~60% of the assembled 80S should be bound with eIF5B (80S IC) after mixing 48S PICs with eIF5B-GTP and 60S subunits. Image processing and 3D classification in Relion\textsuperscript{[12,18]} identified a homogeneous class of ~70% of the total pool of 80S particles, allowing the reconstruction of a 3D map with a global resolution of 2.9 Å (Supplementary Figs. 2–4). The reconstruction shows clear density for a tRNA in the P-site region and density for all domains of eIF5B (Fig. 1c). Further analysis of the map revealed excellent densities for domain III (DIII), the connecting α-helix 12 (Fig. 1c, d, h\textsuperscript{[19]}) and especially for domain IV (DIV) of eIF5B, as well as for the Met-tRNA\textsubscript{Met} and the start codon of the mRNA (Fig. 1c, d, and Supplementary Fig. 4). Less well-resolved were domains II and G of eIF5B and the elbow region of Met-tRNA\textsubscript{Met} (Supplementary Fig. 3).

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A eukaryotic specific Met-tRNA\textsubscript{Met} recognition mechanism. The high quality of the map around the PTC region revealed specific contacts of residues of eIF5B DIV with the four-terminal bases of the Met-tRNA\textsubscript{Met} as well as with the ribose and phosphate backbone atoms (Fig. 3). Specifically, the base of Met-tRNA\textsubscript{Met} nucleotide A76 is tightly monitored by eIF5B residues Glu921 and His924, which anchors the adenine moiety to eIF5B DIV (Fig. 3b, c). In this orientation, the methionyl group esterified to the 3’OH of the A76 ribose is directed towards a hydrophobic “pocket” formed by the surface of eIF5B around residue Ile874 (Fig. 3c, d, and Supplementary Fig. 4c). Intriguingly, this “hydrophobic pocket” is capped by a loop of ribosomal protein uL16, a component of the 60S (Fig. 3d), which is conserved in yeast and humans (Supplementary Fig. 6). This uL16 loop, formed by residues 100 to 120, is disordered in reported elongation 80S complexes, but is well-resolved in our reconstruction, allowing its modeling and refinement. Notably, this loop is stringently checked at the last step of 60S biogenesis by sophisticated cellular machinery that blocks 60S exporting to the cytoplasm if its integrity is compromised\textsuperscript{[22]}. No clear function has been assigned for this loop that would justify such a conserved and costly cellular machinery\textsuperscript{[23]}.

Here, residues Ser104 to Arg110 of this loop are located in close proximity to the \textsubscript{73}ACCA\textsubscript{76}-Met of Met-tRNA\textsubscript{Met}, in an almost parallel configuration to the phosphate backbone of the Met-tRNA\textsubscript{Met} (Fig. 3c). In addition, uL16 residues Leu103 and Ser104 tightly approach the eIF5B residues around Leu871 to define a narrow and hydrophobic cavity where the methionine residue of Met-tRNA\textsubscript{Met} is hosted (Fig. 3d and Supplementary Fig. 6).
contacts between the methionine moiety and either eIF5B or uL16 residues could be observed, which suggests that the recognition of the amino acid attached to an initiator tRNA is not achieved via a specific interaction with eIF5B or uL16 residues. To explore the determinants that govern such recognition mechanism, we systematically mis-acylated tRNA\textsubscript{iMet} with five different amino acids with increasing side chain volumes and hydrophobicity or introducing a positive charge (Fig. 4)\textsuperscript{24}.

All the mis-acylated tRNAs are active in 48S PIC and 80S formation (Fig. 4). However, the 60S subunit joining rate is altered in a way that reflects a predilection for larger side chains: the stepwise increase of the side chain size from Gly to Ala, Val, Leu, and even the positively charged Lys resulted in increasing 60S joining rates, progressively approaching the values observed for Met (Fig. 4a, c). In addition, the mean times for the transition to elongation all decreased substantially to a similar level (by ~3–5-fold) due to the mis-acylation. Both the altered 60S arrival effects a predilection for larger side chains: 60S joining rates, progressively approaching the values observed for Met (Fig. 4a, c).

**Fig. 2 Role of the G–C base-pairs clusters in Met-tRNA\textsubscript{iMet} conformation.**

a Sequence diagram of the *S. cerevisiae* Met-tRNA\textsubscript{iMet} with initiator-specific base pairs colored in orange and modified nucleotides marked with asterisks. b Overview of the conformation adopted by Met-tRNA\textsubscript{iMet} on the 80S-IC complex with the initiator-specific G–C base pairs colored in orange. c Superposition of Met-tRNA\textsubscript{iMet} (green) with a P site classical-state elongation tRNA (gray) reveals a distorted configuration of the acceptor stem of Met-tRNA\textsubscript{iMet} when compared with an elongation peptidyl-tRNA. This distortion prevents the \textsubscript{74}CCA\textsubscript{76}-Met end of Met-tRNA\textsubscript{iMet} from reaching the peptidyl transferase center (PTC, blue) on the 60S. d In contrast, the ASL of Met-tRNA\textsubscript{iMet} has reached its final position when compared with an elongation tRNA in the P site. e The arginine residue 955 at DIV of eIF5B specifically recognizes the Hoogsteen edge of the base of nucleotide G70 of Met-tRNA\textsubscript{iMet} (red arrow, R955). f Cryo-EM density for side chains and individual bases in the area around eIF5B R955. On the right, chemical diagram with contacts and distances indicated. g-i Yeast growth and GCN4-lacZ reporter assays of strains lacking eIF5B or expressing wild-type or R955A mutant. Results are the average and standard deviation of six independent transformants combined from two independent experiments with three replicates each; p values were calculated using two-sided Student’s t test. The p values for panel (h) are eIF5B vs. eIF5B\textsubscript{Δ}: 7.57349 \times 10^{-12}, eIF5B vs. eIF5B-R955A: 6.30158 \times 10^{-9} and for panel (i) are eIF5B vs. eIF5B\textsubscript{Δ}: 1.23215 \times 10^{-5}, eIF5B vs. eIF5B-R955A: 1.3313 \times 10^{-6}.
cumulative probability distributions of the dwell times for 60S joining (Mg<sup>2+</sup> state (Fig. 5, Supplementary Fig. 5, and Supplementary Movie 1))<sup>20</sup>. To that described for a peptidyl-tRNA in an elongation, canonical AUG recognition by the Met-tRNA<sub>Met</sub> anticodon. The anticodon arm of Met-tRNA<sub>Met</sub> features a configuration very similar to that described for a peptidyl-tRNA in an elongation, canonical state (Fig. 5, Supplementary Fig. 5, and Supplementary Movie 1)<sup>20</sup>. Anticodon bases C34, A35, and U36 have reached their final elongation position, which would allow a productive transition into elongation. The G–C cluster of base pairs in the ASL seems to play an important role, allowing a local distortion that guarantees an ideal positioning of the anticodon bases to maximize the interaction with the AUG codon<sup>15</sup>, while allowing the tRNA to bend at the elbow region of T-loop/D-loop tertiary interaction to prevent accommodation of the aminoacyl-acceptor stem to the elongation state (Fig. 2b–d). In addition, the A minor type interactions between 40S residues at the P site and the Met-tRNA<sub>Met</sub> ASL at the G–C base-pair cluster are similar to those described to be important in bacterial initiation, highlighting the functional importance of the G–C base-pair cluster in the ASL of tRNA<sub>Met</sub> throughout evolution (Supplementary Fig. 5c)<sup>25</sup>. Thus, both G–C base-pair clusters at the acceptor stem and the anticodon arm of Met-tRNA<sub>Met</sub> are essential to allow a specific conformation of the Met-tRNA<sub>Met</sub> on the P site in both the early<sup>21</sup> and late stages of initiation<sup>14</sup>.

For the mRNA, we could unambiguously identify six nucleotides including the AUG start codon and the three nucleotides immediately upstream (Fig. 5). Only weak densities ascribable to the A-site codon could be observed, and no ordered RNA density could be identified at the entry or exit sites of the mRNA channel on the 40S. This is in marked contrast with 48S PIC structures from earlier initiation stages, in which long stretches of the mRNA from the entry to the exit sites were well resolved<sup>3</sup>, pinpointing a less prominent role of mRNA/40S interactions once initiation has progressed into its later stages.

The codon-anticodon interaction observed in our reconstruction is virtually identical to a canonical cognate pair (Fig. 5b, c)<sup>30</sup>. Nucleotides C1637 and U1191 of the 18S rRNA at the base of the 40S P-site bracket the base pair mRNA-3'C34-tRNA<sub>Met</sub> in a very similar configuration as in an elongation complex (Fig. 5c, d). In addition, ribosomal bases G1150 and G904 engage mRNA nucleotides at position −1 and −3, respectively, in stacking interactions in a similar configuration as in early initiation complexes in the 48S PIC context (Fig. 5c, d)<sup>6,7</sup>. Thus, in the later stages of initiation, prior to entrance into elongation, the start codon and its flanking residues present a hybrid configuration with bases at position −1/−3 retaining key contacts with RNA bases that are instrumental in the early positioning of the mRNA on the 40S and, at the same time, with the start-codon features characteristic of a standard conformation of an elongation state<sup>20</sup>. Hence, the start codon-surrounding sequence, especially the bases at positions −3/−1, plays an essential role along all initiation, from early “scanning” to later entrance into elongation<sup>29</sup>.

**AUG recognition by the Met-tRNA<sub>Met</sub> anticodon.** The anticodon arm of Met-tRNA<sub>Met</sub> features a configuration very similar to that described for a peptidyl-tRNA in an elongation, canonical state (Fig. 5, Supplementary Fig. 5, and Supplementary Movie 1)<sup>20</sup>. Anticodon bases C34, A35, and U36 have reached their final elongation position, which would allow a productive transition into elongation. The G–C cluster of base pairs in the ASL seems to play an important role, allowing a local distortion that guarantees an ideal positioning of the anticodon bases to maximize the interaction with the AUG codon<sup>15</sup>, while allowing the tRNA to bend at the elbow region of T-loop/D-loop tertiary interaction to prevent accommodation of the aminoacyl-acceptor stem to the elongation state (Fig. 2b–d). In addition, the A minor type interactions between 40S residues at the P site and the Met-tRNA<sub>Met</sub> ASL at the G–C base-pair cluster are similar to those described to be important in bacterial initiation, highlighting the functional importance of the G–C base-pair cluster in the ASL of tRNA<sub>Met</sub> throughout evolution (Supplementary Fig. 5c)<sup>25</sup>. Thus, both G–C base-pair clusters at the acceptor stem and the anticodon arm of Met-tRNA<sub>Met</sub> are essential to allow a specific conformation of the Met-tRNA<sub>Met</sub> on the P site in both the early<sup>21</sup> and late stages of initiation<sup>14</sup>.

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**Release of eIF5B-GDP limits the rate of the transition into elongation.** Analysis of the G domain of eIF5B that binds GTP reveals clear density for a bound nucleotide that, however, lacks features compatible with the presence of a γ-phosphate, implying that the nucleotide state is either GDP or GDP-Pi (Supplementary Fig. 4d)<sup>27,28</sup>. The absence of density ascribable to the γ-phosphate even at a low threshold (Supplementary Fig. 4d, right) points towards a nucleotide state with GDP. This is additionally supported by the fact that the switch I of eIF5B is disordered, which differs from the ordered state prior to GTP hydrolysis<sup>27,29</sup>. Thus, our reconstruction represents an intermediate of the 80S IC right before its transition to the elongation-competent state, but post GTP hydrolysis. This is further supported by the fact that the ribosomal inter-subunit configuration observed here is different from the 80S IC state prior to GTP hydrolysis (Supplementary Fig. 7). A ~3° counterclockwise rotation of the 40S in relation to the 60S was observed in the pre-GTP hydrolysis state, which was coupled with apparent 40S head swivel (Supplementary Fig. 7)<sup>12,13,30</sup>. However, the eIF5B-bound 80S IC observed here presents a configuration very similar to a canonical non-rotated 80S complex, with virtually no rotation of the small subunit and minimal swiveling of the 40S head (Supplementary Fig. 7). Thus, the 80S IC will reconfigure its conformation after GTP hydrolysis to a state that is more similar to the non-rotated 80S IC.
Our results demonstrated that GTP hydrolysis and Pi release are not the rate-limiting steps for eIF5B dissociation from the 80S IC, but the subsequent eIF5B-GDP dissociation. Notably, our structure describes stable interactions among Met-tRNAiMet, eIF5B DIV, and uL16 after GTP hydrolysis by eIF5B (Fig. 6). Disruption of this network of contacts is required for release of eIF5B, perhaps explaining the slow eIF5B departure rate post GTP hydrolysis. Such cooperation among Met-tRNAiMet, an initiation factor and the 60S subunit highlights a eukaryote-specific mechanism to control the progression of the initiation complex into the elongation phase.

**Methods**

**Materials.** All the yeast Saccharomyces cerevisiae 40S and 60S ribosomal subunits, initiation factors eIF1, 1A, 2, 5, and 5B (residues 396–1002), and mRNA were prepared and characterized as described11. The model mRNA, with the sequence: GG(UUC)UCAAAAUGUUCAAA(AUC)U, was an uncapped, unstructured model mRNA containing the optimal yeast 5′ context consensus sequence (underlined) with a biotin covalently linked to the 3′end.11 Native yeast methionylated initiator tRNA (Met-tRNAiMet) was purchased from tRNA Probes, LLC (MI-60).

**80S/eIF5B complex assembly on model mRNA.** Previously, by applying single-molecule fluorescence microscopy methods to a purified, reconstituted yeast translation system, we have revealed that eIF5B is the gating factor during the transition from euukaryotic translation initiation to elongation.11 We performed post-scanning 48S preinitiation complexes (48S PICs, wherein 40S was labeled with a Cy3 dye) which were immobilized on zero-mode waveguides (ZMWs) imaging surface via a biotin at the 3′end of the mRNAs (Supplementary Fig. 1a)11. After washing away unbound components, 60S (Cy5-labeled), eIF5B (Cy5.5-labeled), and the first elongator Phe-tRNA(Phe) (Cy3.5-labeled) in ternary complex with the elongation factor eEF1A and GTP (Phe-TC) were delivered along with required eIFs to start the reaction. By directly illuminating all the fluorescent dyes, we could monitor, in real time, the order of molecular events occurring during the late translation initiation stages and its transition to elongation (Supplementary Fig. 1b)11. This allowed us to measure and determine the occupancy times of eIF5B on newly formed 80S complexes on the native reaction pathway. In particular, for the model mRNA, the mean time of 60S joining to form an 80S was ~16 s with the eIF5B occupancy time on the 80S ~34 s at 20 °C and 3 mM free Mg2+; whereas these values were ~3.6 s and 68.7 s at 20 °C and 10 mM free Mg2+, respectively11. Simulating the kinetic curves from these two reactions provided the information about the time-evolution of the populations of different complexes (Supplementary Fig. 1c).11 To aid the reconstruction of a high-resolution structure of the on-pathway eIF5B-bound 80S prior to its transition to elongation without the need of non-hydrolysable GTP analogs or mutants, we decided to prepare and freeze our sample at timepoint 40 s at 20 °C and 10 mM free Mg2+, where we would expect the eIF5B-bound 80S population accounts ~60% of the total 80S particles (Supplementary Fig. 1c).11 Samples were prepared in the buffer containing 30 mM HEPES-KOH pH 7.5, 100 mM KOAc, 10 mM Mg(OAc)2, and 1 mM GTP:Mg2+. First, a ternary complex mixture was prepared by pre-incubating 3.8 μM eIF2 at 30 °C for 10 min followed by another 5 min incubation at 30 °C after addition of 2.8 μM Met-tRNAi. Next, this ternary complex mixture was diluted to one-third of the concentration, and incubated at 30 °C together with 1 μM eIF1, 1 μM eIF1A, 0.5 μM model mRNA, and 0.3 μM 40S for 15 min, resulting a 48S PIC mixture. Separately, a 60S mixture was prepared by mixing 1 μM eIF5, 1 μM eIF6B, and 0.15 μM 60S. The 48S PIC and 60S mixtures were kept on ice before sample freezing. In parallel, the 200-mesh Quantifoil R2/1 grids (Electron Microscopy Sciences, Q250AR1) were glow-discharged for 25 s in a PELCO EasyGlow glow discharger (Ted Pella, Inc., conditions: negative charge, 15 mA, 0.4 mBar, 25 s). After warming the samples to room temperature, 3.5 μL of the 48S PIC mixture was mixed with 3.5 μL of the 60S mixture. A 3 μL sample from the resulting mixture was applied to each grid at 21 °C and 95% humidity. The sample was vitrified by plunging into liquid ethane after 2.5 s blotting using a Leica EM GP (Leica Microsystems) plunger. The total time from combining the 48S PIC and 60S mixtures to grid freezing was ~40 s.

**Single-molecule experiments with mis-acylated tRNAiMet.** Native yeast tRNAiMet was mis-acylated by the flexizyme dFx with Gly-, Ala-, Val-, Leu-, and Lys-DBE as described12,31. Real-time single-molecule experiments on the ZMW-based PacBio RSII instrumentation and data analyses were performed with exactly the same methodology as previously described11. The mean times of 60S arrival to the immobilized 48S PICs (60S arrival time) and the subsequent transition to elongation were determined in experiments performed with the model mRNA and unlabeled eIF5B at 3 mM free Mg2+ and 20 °C.
The start codon is recognized, eIF5B in its GTP-bound form is able to transiently stabilize the aminoacyl-tRNA in the P site and catalyze the recruitment of the large subunit (60S, blue). Proper positioning of Met-tRNAiMet, eIF5B, and the 60S would lead to the structuring of the uL16 loop (residues 100-112) implemented in two steps, beginning with the super-domain G/II and followed by the detachment of DIV-h12-DIII. The data that support this study are available from the corresponding authors upon reasonable request. Cryo-EM maps have been deposited in the Electron Microscopy Bank under accession code 6WOO. Source data for Fig. 2h, i are included as Supplementary Table 2.

References

1. Frank, J. & Gonzalez, R. L. Jr. Structure and dynamics of a processive Brownian motor: the translating ribosome. *Ann Rev Biochem* 79, 381–412 (2010).
2. Aitken, C. E. & Lorsch, J. R. A mechanistic overview of translation initiation in eukaryotes. *Nat. Struct. Mol. Biol.* 19, 568–576 (2012).
3. Jackson, R. J., Hellen, C. U. & Pestova, T. V. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat. Rev. Mol. Cell Biol.* 11, 113–127 (2010).
4. Hinnebusch, A. G. The scanning mechanism of eukaryotic translation initiation. *Ann Rev Biochem* 83, 779–812 (2014).
5. Hinnebusch, A. G. Structural Insights into the Mechanism of Scanning and Start Codon Recognition in Eukaryotic Translation Initiation. *Trends Biochem Sci.* 42, 589–611 (2017).
6. Llacer, J. I. et al. Translational initiation factor eIF5 replaces eIF1 on the 40S ribosomal subunit to promote start-codon recognition. *Elife* 7, https://doi.org/10.7554/elife.39273 (2018).
7. Hussain, T. et al. Structural changes enable start codon recognition by the eukaryotic translation initiation complex. *Cell* 159, 597–607 (2014).
8. Hashem, Y. & Frank, J. The jigsaw puzzle of mRNA translation initiation in eukaryotes: a decade of structures unraveling the mechanics of the process. *Ann. Rev. Biophys.* https://doi.org/10.1146/annurev-biophys-070818-034034 (2018).
9. Pestova, T. V. et al. The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature* 403, 332–335 (2000).
10. Dever, T. E. et al. Universal translation initiation factor IF2/eIF5B. *Cold Spring Harb. Symp. Quant. Biol.* 66, 417–424 (2001).
11. Wang, J. et al. eIF5B gates the transition from translation initiation to elongation. *Nature* 573, 605–608 (2019).
12. Fernandez, I. S. et al. Molecular architecture of a eukaryotic translational initiation complex. *Science* 342, 1240585 (2013).
13. Yamamoto, H. et al. Structure of the mammalian 80S initiation complex with initiation factor 5B on HCV-IRE5 RNA. *Nat. Struct. Mol. Biol.* 21, 721–727 (2014).
14. Kolitz, S. E. & Lorsch, J. R. Eukaryotic initiator tRNA: finely tuned and ready for action. *FEBS Lett.* 584, 396–404 (2010).
15. Kolitz, S. E., Takacs, J. E. & Lorsch, J. R. Kinetic and thermodynamic analysis of the role of start codon/anticodon base pairing during eukaryotic translation initiation. *RNA* 15, 138–152 (2009).
16. Acker, M. G. et al. Kinetic analysis of late steps of eukaryotic translation initiation. *J. Mol. Biol.* 385, 491–506 (2009).
17. Scheres, S. H. Processing of structurally heterogeneous Cryo-EM Data in RELION. *Methods Enzymol.* 579, 125–157 (2016).
18. Zhang, J. et al. New tools for automated high-resolution cryo-EM structure determination in RELION-3. *Elife* 7, https://doi.org/10.7554/elife.42166 (2018).

Fig. 6 A structural view of the on-ribosome eIF5B catalysis. After Met-tRNA<sub>Met</sub> (green) is delivered to the P site of the small subunit (40S, yellow) and the start codon is recognized, eIF5B in its GTP-bound form is able to transiently stabilize the aminoacyl-tRNA in the P site and catalyze the recruitment of the large subunit (60S, blue). Proper positioning of Met-tRNA<sub>Met</sub>, eIF5B, and the 60S would lead to the structuring of the uL16 loop (residues 100–112) which in turn anchors eIF5B DIV solidly to the 60S in the vicinity of the PTC. The successful establishment of these interactions signals for eIF5B GTPase activation and GTP hydrolysis, which happens in the G domain of eIF5B placed near the GTPase activation center of the 60S. GTP hydrolysis by eIF5B is followed by the de-structuring of switch I, which reduces the affinity of the factor to the ribosome. The final departure of eIF5B from the ribosome is likely implemented in two steps, beginning with the super-domain G/II and followed by the detachment of DIV-h12-DIII.
19. Shin, B. S. et al. Structural integrity of [alpha]-helix H12 in translation initiation factor eIF5B is critical for 80S complex stability. RNA 17, 687–696 (2011).
20. Shao, S. et al. Decoding mammalian ribosome-mRNA states by translational GTPase complexes. Cell 167, 1229–1240 e1215 (2016).
21. Dong, J. et al. Conserved residues in yeast initiator tRNA calibrate initiation accuracy by regulating preinitiation complex stability at the start codon. Genes Dev. 28, 502–520 (2014).
22. Sulima, S. O. et al. Bypass of the pre-60S ribosomal quality control as a pathway to oncogenesis. Proc. Natl Acad. Sci. USA 111, 5640–5645 (2014).
23. Sulima, S. O. et al. Eukaryotic rpl10 drives ribosomal rotation. Nucleic Acids Res. 42, 2049–2063 (2014).
24. Murakami, H., Ohta, A., Ashigai, H. & Suga, H. A highly flexible tRNA activation model for non-natural polypeptide synthesis. Nat. Methods 3, 357–359 (2006).
25. Lancaster, L. & Noller, H. F. Involvement of 16S rRNA nucleotides G1338 and A1339 in discrimination of initiator tRNA. Mol. Cell 20, 623–632 (2005).
26. Kozak, M. The scanning model for translation: an update. J. Cell Biol. 108, 229–241 (1989).
27. Huang, B. Y. & Fernandez, I. S. Long-range interdomain communications in eIF5B regulate GTP hydrolysis and translation initiation. Proc. Natl Acad. Sci. USA 117, 1429–1437 (2020).
28. Kuhle, B. & Ficner, R. eIF5B employs a novel domain release mechanism to catalyze ribosomal subunit joining. Mol. Cell Biol. 34, 1605–1612 (2014).
29. Shin, B. S. et al. tRNA suppressor of a eukaryotic translation initiation factor 5B/translation initiation factor 2 mutant reveals a binding site for translational GTPases on the small ribosomal subunit. Mol. Cell 29, 808–821 (2009).
30. Sprink, T. et al. Structures of ribosome-bound initiation factor 2 reveal the mechanism of subunit association. Sci. Adv. 2, e1501502 (2016).
31. Peacock, J. R. et al. Amino acid-dependent stability of the acyl linkage in aminocyl-tRNA. RNA 20, 758–764 (2014).
32. Shin, B. S. et al. Intragenic suppressor mutations restore GTPase and translation functions of a eukaryotic initiation factor 5B switch II mutant. Mol. Cell Biol. 27, 1677–1685 (2007).
33. Hinnebusch, A. G. A hierarchy of trans-acting factors modulates translation of an activator of amino acid biosynthetic genes in Saccharomyces cerevisiae. Mol. Cell Biol. 5, 2349–2360 (1985).
34. Grant, C. M., Miller, P. F. & Hinnebusch, A. G. Requirements for intercistronic distance and level of eukaryotic initiation factor 2 activity in reinitiation on GCN4 mRNA vary with the downstream cistron. Mol. Cell Biol. 14, 2616–2628 (1994).
35. Shin, B. S. et al. Uncoupling of initiation factor eIF5B/eIF2 GTPase and translational activities by mutations that lower ribosome affinity. Cell 111, 1015–1025 (2002).
36. Rohou, A. & Grigorieff, N. CTFFIND4: fast and accurate defocus estimation from electron micrographs. J. Struct. Biol. 192, 216–221 (2015).
37. Zivanov, J., Nakane, T. & Scheres, S. H. W. Estimation of high-order aberrations and anisotropic magnification from cryo-EM data sets in RELION-3.1. IUCrJ 7, 253–267 (2020).
38. Kucukelbir, A., Sigworth, F. J. & Tagare, H. D. Quantifying the local resolution of cryo-EM density maps. Nat. Methods 11, 63–65 (2014).
39. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).
40. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D. Biol. Crystallogr. 60, 2126–2132 (2004).
41. Afonine, P. V. et al. Real-space refinement in PHENIX for cryo-EM and crystallography. Acta Crystallogr. D. Struct. Biol. 74, 531–544 (2018).
42. Nicholls, R. A., Long, F. & Murshudov, G. N. Low-resolution refinement tools in REFMAC5. Acta Crystallogr. D. Biol. Crystallogr. 68, 404–417 (2012).
43. Brown, A. et al. Tools for macromolecular model building and refinement into electron cryo-microscopy reconstructions. Acta Crystallogr. D. Biol. Crystallogr. 71, 136–153 (2015).
44. Scheres, S. H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol. 180, 519–530 (2012).

Acknowledgements
We are grateful to Dr. Dong-Hua Chen for technical support for sample freezing, the cryo-EM facility staff at Columbia University Irving Medical Center (CUIMC) for technical assistance in cryo-EM data acquisition, and Dr. Ya-Ming Hou for the kind gifts of the flexizyme substrates. This work was supported by the US National Institutes of Health (NIH) grants GM011378 and AI047365 to J.D.P.; a Knut and Alice Wallenberg Foundation postdoctoral scholarship to J.W. (KAW 2015.0406); the Intramural Research Program of the NIH (T.E.D.).

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Jinfan W. prepared the cryo-EM samples and performed single-molecule experiments; Jing W. and I.S.F. acquired and processed the cryo-EM data and built the structural models; B.S.S. and J.R.K. performed the yeast growth and reporter experiments; T.E.D., J.D.P. and I.S.F. supervised the work; Jinfan W., B.S.S., T.E.D., J.D.P., and I.S.F. wrote the paper with input from all the other authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-18829-3.

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Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work.

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