A conformational switch in initiation factor 2 controls the fidelity of translation initiation in bacteria

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Initiation factor (IF) 2 controls the fidelity of translation initiation by selectively increasing the rate of 50S ribosomal subunit joining to 30S initiation complexes (ICs) that carry an N-formyl-methionyl-tRNA (fMet-tRNA\textsuperscript{fMet}). Previous studies suggest that rapid 50S subunit joining involves a GTP- and fMet-tRNA\textsuperscript{fMet}-dependent “activation” of IF2, but a lack of data on the structure and conformational dynamics of 30S IC-bound IF2 has precluded a mechanistic understanding of this process. Here, using an IF2-tRNA single-molecule fluorescence resonance energy transfer signal, we directly observe the conformational switch that is associated with IF2 activation within 30S ICs that lack IF3. Based on these results, we propose a model of IF2 activation that reveals how GTP, fMet-tRNA\textsuperscript{fMet}, and specific structural elements of IF2 drive and regulate this conformational switch. Notably, we find that domain III of IF2 plays a pivotal, allosteric, role in IF2 activation, suggesting that this domain can be targeted for the development of novel antibiotics.
Initiation of bacterial protein synthesis, or translation, proceeds along a multi-step pathway that begins with the assembly of a 30S initiation complex (IC) (Supplementary Fig. 1a). The 30S IC is composed of the small (30S) ribosomal subunit, initiation factor (IF) 1, the guanosine triphosphatase (GTPase) IF2, IF3, initiator N-formyl-methionyl-transfer RNA (fMet-tRNAfMet), and messenger RNA (mRNA). Although 30S IC assembly can occur via multiple pathways, a kinetically favored pathway has been identified in which the three IFs bind to the 30S subunit and synergistically regulate the kinetics of tRNA binding. Consequently, fMet-tRNAfMet is preferentially selected into the peptidyl-tRNA-binding (P) site of the 30S subunit, where it base-pairs to the start codon of an mRNA that can bind to the 30S subunit before, during, or after the IFs bind. The IFs further enhance the accuracy of translation by cooperatively regulating the rate with which the large (50S) ribosomal subunit joins to the 30S IC and by modulating the stability of the resulting 70S IC.

70S IC formation triggers GTP hydrolysis by IF2, which subsequently drives a series of maturation steps that enable the 70S IC to enter the elongation stage of protein synthesis. IF2 plays central roles throughout the initiation pathway that ensure accurate fMet-tRNAfMet selection. During 30S IC assembly, IF2 specifies fMet-tRNAfMet selection by interacting with the N-formyl-methionine and aminoacyl acceptor stem of fMet-tRNAfMet. IF2 further ensures the accuracy of fMet-tRNAfMet selection by preferentially accelerating the rate with which the 50S subunit joins to a 30S IC carrying fMet-tRNAfMet.

Indeed, 50S subunit joining to 30S ICs carrying GTP-bound IF2 (IF2(GTP)) and P site-bound fMet-tRNAfMet is up to two orders of magnitude faster than to 30S ICs in which GTP has been substituted with GDP or to “pseudo” 30S ICs in which fMet-tRNAfMet has been substituted with an unformylated Met-tRNAfMet, unacylated tRNAfMet, or no tRNA at all.

IF2 consists of four conserved structural domains, referred to here as domains I-IV (dI-IV) in the nomenclature of Roll-Mecak et al., but also referred to as domains G2 (dI), G3 (dII), C1 (dIII), and C2 (dIV) in the nomenclature of Gualerzi et al. or as domains dIV (dI), dV (dII), dVI-1 (dIII), and dVI-2 (dIV) in the nomenclature of Mortensen et al. The arrangement of these domains is such that dII and dIII separate the guanine nucleotide-binding domain, dI, from the fMet-tRNAfMet-binding domain, dIV. Structural studies of non-ribosome-associated IF2 strongly suggest that the spatial positions of dII and dIV are flexible relative to dI and dII, allowing IF2 to adopt increasingly extended conformations upon transitions from nucleotide-free IF2 to IF2(GDP) and IF2(GTP). Within the context of the 30S IC, dI helps anchor IF2(GTP) to the 30S IC by interacting with 16S ribosomal RNA (rRNA) helices h5 and h14. Moreover, dIII and dIV adopt positions relative to dI and dII that enable dIV to interact with the P site-bound fMet-tRNAfMet. These interactions, which might be further stabilized by the interactions of dII with ribosomal protein S12, result in the formation of an IF2(GTP)-tRNA sub-complex on the inter-subunit surface of the 30S IC.

Previously, Andersson and colleagues identified IF2 variants containing single amino acid substitutions within domain III (mutIF2s) that, remarkably, enable mutIF2(GDP)-bound pseudos to catalyze rapid 50S subunit joining to 30S ICs and mutIF2(GTP)-bound pseudos to catalyze rapid 50S subunit joining to pseudo 30S ICs. Based on these results, we have proposed that IF2 is “activated” for rapid 50S subunit joining by a GTP- and fMet-tRNAfMet-dependent conformational switch that is rendered GTP- and fMet-tRNAfMet-independent by the “activating” mutations in dIII of the mutIF2s. Nonetheless, due to a lack of experimental data on the structure of IF2(GDP)-bound 30S ICs, IF2(GTP)-bound pseudos 30S ICs, mutIF2(GDP)-bound 30S ICs, and/or mutIF2(GTP)-bound pseudos 30S ICs, as well as on the GTP- and fMet-tRNAfMet-dependent conformational dynamics of 30S IC-bound IF2 and mutIF2, the structural basis and molecular mechanism of IF2 activation have remained unknown.

To close this gap in our understanding of how IF2 helps regulate the fidelity of translation initiation, here we report an investigation of the structural dynamics of GTP- and fMet-tRNAfMet-dependent conformational dynamics of 30S IC-bound IF2 and mutIF2, the structural basis and molecular mechanism of IF2 activation have remained unknown.

Fig. 1 Effect of GTP and fMet-tRNAfMet. smFRET measurements of (a) wtIF2(GTP) and (b) mutIF2(GTP) interacting with 30S ICwT and 30S ICmT, respectively. First row: cartoon illustrations depicting 30S ICwT-bound wtIF2(GTP) (light purple) and 30S ICmT-bound mutIF2(GTP) (dark purple). Second row: representative Cy3 (green) and Cy5 (red) emission intensities vs. time trajectories. Third row: corresponding E_FRET vs. time trajectories. Fifth row: post-synchronized surface contour plots of the time evolution of population FRET. Surface contour plots were generated by superimposing hundreds of individual IF2-binding events. “N” indicates the total number of E_FRET vs. time trajectories for each 30S IC and “n” indicates the total number of individual IF2-binding events. The surface contours were plotted from tan (lowest population plotted) to red (highest population plotted) as indicated in the population color bar.
highlighting dIII as an attractive target for the development of novel antibiotics that function as allosteric inhibitors of IF2.

**Results**

*Escherichia coli* mutIF2 catalyzes rapid 50S subunit joining. MutIF2s were initially selected in *Salmonella (S) typhimurium* on the basis of their ability to complement the slow growth phenotype arising from a Met-tRNA^Met^ formylation deficiency. One such *S. typhimurium* mutIF2 contains a Ser755Tyr mutation in dIII and has been shown to strongly compensate for a Met-tRNA^Met^ formylation deficiency both in vivo and in vitro. Here, we generated the homologous *E. coli* Ser753Tyr mutIF2 (Supplementary Fig. 2), purified it, and confirmed its ability to catalyze rapid 50S subunit joining to both 30S ICs and pseudo 30S ICs using ensemble kinetic studies of subunit joining (Supplementary Fig. 3). Importantly, a Gly810Cy5 mutation in dIV, previously used to label *E. coli* IF2 with a FRET acceptor fluorophore (ref. 28 and vide infra), did not alter the kinetic performance of either *E. coli* IF2(GTP) or *E. coli* Ser753Tyr mutIF2(GTP). We further validated the biochemical activities of our unlabeled IF2 variants using a standard, biochemical IF2 activity assay that is based on primer extension inhibition, to investigate the effects that the activating mutation in dIII highlighting dIII as an attractive target for the development of novel antibiotics that function as allosteric inhibitors of IF2.

| 30S IC               | IF2       | Nucleotide | $k_a$ (μM$^{-1}$s$^{-1}$)$^a$ | $k_d$ (s$^{-1}$)$^a$ | $K_d$ (nM)$^a$ |
|----------------------|-----------|------------|-----------------------------|-----------------|----------------|
| 30S ICwT             | wtIF2     | GTP        | 2.0 ± 0.13$^b$              | 0.041 ± 0.01$^b$ | 21 ± 6         |
| 30S ICwT             | mutIF2    | GTP        | 2.2 ± 0.4$^b$              | 0.013 ± 0.001$^b$ | 6.4 ± 1.3     |
| 30S ICwD             | wtIF2     | GDP        | 2.1 ± 0.12                 | 1.32 ± 0.09     | 622 ± 28      |
| 30S ICwD             | mutIF2    | GDP        | 1.2 ± 0.10                 | 0.13 ± 0.01     | 102 ± 9       |
| 30S ICwT,Met         | wtIF2     | GTP        | 0.52 ± 0.02$^c$            | 1.2 ± 0.2       | 2328 ± 294   |
| 30S ICwT,Met         | mutIF2    | GTP        | 0.77 ± 0.05                | 0.11 ± 0.01     | 136 ± 7       |
| 30S ICwT,OH          | wtIF2     | GTP        | 0.38 ± 0.02$^c$            | 2.2 ± 0.5       | 5842 ± 1469  |
| 30S ICwT,OH          | mutIF2    | GTP        | 0.74 ± 0.02                | 0.14 ± 0.02     | 185 ± 18      |

$^a$k$_a$, $k_d$, and $K_d$ were obtained from three independently collected data sets (mean ± SE) using a transition probability matrix-based population decay analysis as described previously$^{28}$ and in Methods section.

$^b$k$_a$ and $k_d$ were corrected for the effects of Cy3 photobleaching.

$^c$k$_a$ was corrected for the effects of Cy5 photobleaching.
Fig. 1a and b). Consistent with this, $K_{d,wT}$ is approximately threefold smaller than $K_{d,wD}$ (Table 1), demonstrating that the activating mutation confers a higher affinity of mutIF2(GTP) for 30S IC$_{wT}$ than the affinity of wtIF2(GTP) for 30S IC$_{wT}$. Interestingly, the distribution of $E_{\text{FRET}}$ values for the mutIF2(GTP)-bound state of 30S IC$_{wT}$ (Fig. 1b and Supplementary Fig. 5b) was composed of a single non-zero $E_{\text{FRET}}$ peak that was centered at an $<E_{\text{FRET}}>\approx0.85\pm0.01$ that is within error of that observed for the wtIF2(GTP)-bound state of 30S IC$_{wT}$ ($p=0.2$, Supplementary Table 1). This indicates that the conformation of 30S IC$_{wT}$-bound mutIF2(GTP) is not significantly altered by the activating mutation and is very similar to that of a 30S IC$_{wT}$-bound wtIF2(GTP). Previously, we have used ensemble kinetic experiments to show that wtIF2(GTP) and mutIF2(GTP) can catalyze rapid 50S subunit joining to 30S IC$_{wT}$* and 30S IC$_{wT}$ (where the asterisk denotes the analogous 30S IC in the kinetic studies) 4,5,20,21. We therefore interpret the observed $<E_{\text{FRET}}>\approx0.85$ and $<E_{\text{FRET}}>\approx0.87$ as corresponding to a conformation of the IF2(GTP)•tRNA sub-complex in which IF2(GTP) is active for rapid 50S subunit joining.

GTP allosterically positions dIV closer to the P-site tRNA. We next performed smFRET experiments using wtIF2[Cy5]$_{dIV}$(GDP) and 30S IC$_{wD}$ (where the “D” subscript denotes GDP) to explore if and how the affinity of IF2 for the 30S IC and the conformation of 30S IC-bound IF2 depend on the guanine nucleotide that is bound to IF2. These experiments reveal that excursions to the 30S IC$_{wD}$ $E_{\text{FRET}}$ trajectories are more transient than those to the wtIF2(GTP)-bound state in the 30S IC$_{wT}$ $E_{\text{FRET}}$ trajectories (compare Figs. 2a and 1a). Correspondingly, we observe a $K_{d,wT}$ value that is ~30-fold larger than the $K_{d,wD}$ Value (Table 1), demonstrating that the affinity of wtIF2 binding to the 30S IC is much higher when GTP, rather than GDP, is bound to IF2. In addition, we found that the distribution of $E_{\text{FRET}}$ values for the wtIF2(GDP)-bound state of 30S IC$_{wD}$ (Fig. 2a and Supplementary Fig. 5c) exhibited two non-zero $E_{\text{FRET}}$ peaks. One of the peaks encompassed a minor, 18 ± 1.5%, subpopulation of 30S IC$_{wD}$-bound wtIF2(GDP) and was centered at an $<E_{\text{FRET}}>\approx0.89\pm0.01$ that is within error of that observed for 30S IC$_{wT}$-bound wtIF2(GTP) ($p=0.2$, Supplementary Table 1). The other peak encompassed a major, 82 ± 1.5%, subpopulation of 30S IC$_{wD}$-bound wtIF2(GDP) and was centered at an $<E_{\text{FRET}}>\approx0.67\pm0.01$ that is notably lower than that observed for 30S IC$_{wT}$-bound wtIF2(GTP) ($p=0.002$, Supplementary Table 1).

Previously, we have used ensemble kinetic experiments to show that 30S IC$_{wD}$* exhibits a drastic, ~60-fold smaller rate of 50S subunit joining than 30S IC$_{wT}$* 21. Based on the values of $K_{d,wD}$ and $K_{d,wT}$ determined here (622 nM and 21 nM, respectively) and the IF2 and 30S IC concentrations employed in our previous kinetic studies of 50S subunit joining 21, we estimate that the occupancy of wtIF2(GDP) on 30S IC$_{wD}$* in our previous studies was only twofold lower than the occupancy of wtIF2(GTP) on 30S IC$_{wT}$* (Supplementary Table 2). Thus, this occupancy difference is insufficient to account for the decreased rate of 50S subunit joining to 30S IC$_{wD}$*. Instead, we conclude that the decreased rate of 50S subunit joining primarily arises from the stabilization of a major subpopulation of 30S IC$_{wD}$-bound wtIF2 (GDP) in a conformation that is inactive for rapid 50S subunit joining and that, given our measured $<E_{\text{FRET}}>\approx0.67$ features, values for the second subpopulation of our labeling positions that is ~9 Å longer than what is in a 30S IC$_{wT}$-bound wtIF2(GTP) that is active for rapid 50S subunit joining. Given that dIV is connected to dIII via a potentially flexible linker 24, this ~9 Å increase in the distance between dIV and the P-site tRNA can arise from two different scenarios. In the first scenario, dIV adopts a single, fixed position that is ~49 Å from the P-site tRNA. In the alternative scenario, dIV adopts multiple positions that interconvert on a timescale that is faster than the acquisition time of our TIRF microscope (i.e., 0.1 s per frame), yielding a time-averaged position that is ~49 Å from the P-site tRNA. Such a difference between the conformations of the GDP- and GTP-bound forms of IF2 is consistent with comparative structural analyses of non-ribosome-associated IF2(GDP) and IF2(GTP) 26 and of 70S IC-bound IF2(GDP) 31 and IF2(GTP) 27,31-33. Based on these analyses, we propose that the guanine nucleotide bound to dI of 30S IC-bound IF2 allosterically modulates the position of dIV relative to that of dIII and the P-site tRNA. Indeed, compared to the position of dIV in IF2(GDP) in these structures, dIV in IF2(GTP) is positioned further away from dI-dIII and closer to the P-site tRNA.

To validate this model, we developed a wtIF2 variant in which dIII was labeled with a Cy5 fluorophore (wtIF2[Cy5]$_{dIII}$, Methods section), and used wtIF2[Cy5]$_{dIII}$ to repeat the smFRET experiments described above. We found that the distributions of $E_{\text{FRET}}$ values for the wtIF2(GTP)-bound state of 30S IC$_{wT}$ and the wtIF2(GDP)-bound state of 30S IC$_{wD}$ were both composed of only a single non-zero $E_{\text{FRET}}$ peak that was centered at an $<E_{\text{FRET}}>\approx0.3$ and an average distance between our labeling positions of ~63 Å (Supplementary Fig. 6). These results strongly suggest that the relative distance between dIII and the P-site tRNA is similar in the minor and major subpopulations of 30S IC$_{wT}$-bound wtIF2(GDP) and that this distance is comparable to the corresponding distance in 30S IC$_{wT}$-bound wtIF2(GTP).
The activating mutation in dIII allosterically positions dIV. To determine whether and how the activating mutation in dIII modulates the affinity of IF2(GDP) for the 30S IC and the conformation of 30S IC-bound IF2(GDP), we performed smFRET experiments using mutIF2[Cy5]dIV(GTP) and 30S ICwT. The results show that excursions to the mutIF2(GDP)-bound state in the 30S ICwT EFRET trajectories are significantly longer than those to the wtIF2(GDP)-bound state in the 30S ICwT EFRET trajectories (compare Fig. 2a and b). In line with this, we find that the value of $K_w$ is approximately sixfold smaller than that of $K_w$ (Table 1). Thus, the activating mutation in dIII enables mutIF2(GDP) to adopt a conformation that closely resembles that observed for a 30S ICwT-bound wtIF2(GTP) that is active for rapid 50S subunit joining.

Previously, we have used ensemble kinetic experiments to show that the rate of 50S subunit joining to 30S ICwT* is ~40-fold higher than to 30S ICwD*. Thus, the activating mutation in dIII enables mutIF2(GDP) to catalyze 50S subunit joining to 30S ICwD* at a rate similar to that observed for 50S subunit joining to 30S ICwT*. Based on the results reported here, we propose that the activating mutation in dIII enables rapid 50S subunit joining by stabilizing a conformation of dI-dIII that increases the affinity of mutIF2(GDP) for 30S ICwD and that enables mutIF2(GDP) to position dIV closer to the P site such that it can interact with the P site tRNA and controls the activation of 30S IC-bound IF2 for rapid 50S subunit joining.

### Fig. 3 Effect of substituting fMet-tRNAfMet with Met-tRNAfMet or tRNAfMet, interacting with (a, c) 30S ICwT,Met or 30S ICwT,OH, respectively, and to (b, d) 30S ICAwT,OH or 30S ICAwT,OH, respectively. Data are displayed as in Fig. 1

Notably, however, we were able to unambiguously identify two kinetically distinguishable subpopulations of the wtIF2(GDP)-bound state of 30S ICwT whose kinetic properties were equivalent to those of the minor and major subpopulations of the wtIF2(GDP)-bound state of 30S ICwD that we identified using wtIF2(Cy5)dIV (Supplementary Fig. 7). Collectively, the data obtained using wtIF2(Cy5)dIV and wtIF2(Cy5)dIV allows us to validate and extend the structural model described above.

### Table 1

| Subpopulation | 30S ICwD | 30S ICwT |
|---------------|---------|---------|
| wtIF2(GDP)    | $N = 586, n = 808$ | $N = 418, n = 503$ |
| mutIF2(GDP)   | $N = 723, n = 960$ | $N = 865, n = 1272$ |

fMet-tRNAfMet stabilizes the active conformation of wtIF2. Next, we performed smFRET experiments using wtIF2[Cy5]dIV(GTP) and analogs of 30S ICwT in which the fMet-tRNAfMet has been substituted with Met-tRNAfMet (30S ICAwT,Met) or tRNAfMet (30S ICAwT,OH) to investigate if and how the affinity of IF2 for the 30S IC and the conformation of 30S IC-bound IF2 depend on the N-formyl moiety and/or methionine of the 30S IC-
bound fMet-tRNA^Met. Consistent with our previous smFRET studies, we found that the 30S IC_{wT,Met} and 30S IC_{wT,OH} E_{FRET} trajectories exhibit excursions to the wtIF2(GTP)-bound state that are much shorter lived than those of 30S IC_{wT} (compare Fig. 3a, b with Fig. 1a). In line with this, K_{d,wT,Met} is ~100-fold and K_{d,wT,OH} is ~300-fold larger than K_{d,wT} (Table 1). These results suggest that the absence of just the N-formyl moiety or the N-formyl-methionine from the 30S IC-bound fMet-tRNA^Met is enough to disrupt interactions between dIV and fMet-tRNA^Met that significantly contribute to anchoring wtIF2(GTP) to the 30S IC.

Consistent with our previous smFRET studies, we find that the distribution of E_{FRET} values for the wtIF2(GTP)-bound state of 30S IC_{wT,Met} (Fig. 3a and Supplementary Fig. 5e) is very broad, with values in the 0.2-1.0 range that encompass two non-zero E_{FRET} peaks. The peak corresponding to the larger, ~3 ± 12%, subpopulation of 30S IC_{wT,Met}-bound wtIF2(GTP) was centered at an <E_{FRET}> of 0.81 ± 0.01 that is outside the error of that observed for 30S IC_{wT}-bound wtIF2(GTP) (<p value = 0.08, Supplementary Table 1). This observation suggests that the separation between our labeling positions is ~43 Å in this subpopulation of 30S IC_{wT}-bound wtIF2(GTP), a separation that is ~3 Å longer than what it is in a 30S IC_{wT}-bound wtIF2(GTP) that is active for rapid 50S subunit joining. The peak corresponding to the smaller, ~44 ± 12%, subpopulation of 30S IC_{wT,Met}-bound wtIF2(GTP) was centered at an even lower <E_{FRET}> of 0.55 ± 0.01, indicating that the distance between our labeling positions is ~53 Å, ~13 Å longer than what it is in 30S IC_{wT}-bound wtIF2(GTP) that is active for rapid 50S subunit joining. Even more dramatic results are obtained for the distribution of E_{FRET} values for the wtIF2(GTP)-bound state of 30S IC_{wT,OH} (Fig. 3b and Supplementary Fig. 5g) in that the distribution exhibits only a single non-zero E_{FRET} peak that is centered at an <E_{FRET}> of 0.53 ± 0.02 that is within error of that observed for the smaller subpopulation of 30S IC_{wT,Met}-bound wtIF2(GTP) (<p value = 0.4, Supplementary Table 1).

Our previous ensemble kinetic studies have shown that the rates of 50S subunit joining to 30S IC_{wT,Met}* and 30S IC_{wT,OH}* are approximately fourfold and ~15-fold lower, respectively, than that to 30S IC_{wT,0}^{22}. Given the values of K_{d,wT,Met} and K_{d,wT,OH} determined here and of the wtIF2 and 30S IC_{wT,Met} and 30S IC_{wT,OH}* concentrations used in our previous kinetic studies, we estimate that the occupancy of wtIF2(GTP) on 30S IC_{wT,Met}* and 30S IC_{wT,OH}* in our previous kinetic studies was approximately fivefold and ~10-fold lower, respectively, than that on 30S IC_{wT} (Supplementary Table 2). It is notable that these estimated decreases in the occupancies of wtIF2(GTP) on 30S IC_{wT,Met}* and 30S IC_{wT,OH}* closely approximate the decreases in the rates of 50S subunit joining to 30S IC_{wT,Met}* and 30S IC_{wT,OH}* that is inactive for rapid 50S subunit joining. The possibility of an N-formyl moiety or N-formyl-methionine on 30S IC-bound Met-tRNA^Met or tRNA^Met decreases the rate of 50S subunit joining by reducing the occupancy of wtIF2(GTP) on these pseudo 30S ICs to ~19% and ~9%, respectively (Supplementary Table 2), rather than by stabilizing wtIF2(GTP) in an inactive conformation at an occupancy of nearly 100% on these pseudo 30S ICs, as we have previously suggested. The key question therefore becomes whether the activation of IF2(GTP) for rapid 50S subunit joining merely involves an fMet-tRNA^Met-dependent increase in the affinity of IF2(GTP) for the 30S IC or whether, in addition, there is an fMet-tRNA^Met-dependent change in the conformation of 30S IC-bound IF2(GTP).

To address this question, we performed ensemble kinetic experiments to measure the rate of 50S subunit joining to 30S IC_{wT,OH} as a function of wtIF2(GTP) concentrations that were high enough to saturate 30S IC_{wT,OH} with wtIF2(GTP). As a reference, we measured the maximal rate of 50S subunit joining to 30S IC_{wT} using a wtIF2(GTP) concentration of 1.0 µM and obtained a rate of ~80 s^{-1} (Fig. 4). A result that, in excellent agreement with our previous studies, is ~14-fold faster than the rate of 50S subunit joining to 30S IC_{wT,OH} measured at the same wtIF2(GTP) concentration. Titrating the concentration of wtIF2(GTP) from 0.6 to 10 µM using 30S IC_{wT,OH} resulted in a small, ~1.5-fold increase in the rate of 50S subunit joining, suggesting that, at the 0.6 µM concentrations of wtIF2(GTP) used in the previous studies, 30S IC_{wT,OH} was not saturated with wtIF2(GTP). Nonetheless, we find that the rate of 50S subunit joining to 30S IC_{wT,OH} plateaus at a wtIF2(GTP) concentration of ~2.5 µM, indicating that at wtIF2(GTP) concentrations above ~2.5 µM, 30S IC_{wT,OH} is saturated with wtIF2(GTP). Interestingly, we find that, even when 30S IC_{wT,OH} is saturated with wtIF2(GTP), the rate of 50S subunit joining is still ~11-fold lower than the maximal rate of 50S subunit joining to 30S IC_{wT}. Based on these results, we conclude that the decreased rate of 50S subunit joining originates from a conformation of 30S IC_{wT,OH}-bound wtIF2(GTP) that is inactive for rapid 50S subunit joining.

The conformation of dI-dIII confers rapid subunit joining. To investigate whether and how the activating mutation in dIII modulates the affinity of IF2(GTP) for pseudo 30S ICs and the conformation of the resulting pseudo 30S IC-bound IF2(GTP), we performed smFRET experiments using mutIF2[C_{3T},353G1(T)] and 30S IC_{wT,Met} or 30S IC_{wT,OH}. The results of these experiments demonstrate that excursions to the mutIF2(GTP)-bound
Fig. 5 Structural model for the GTP and tMet-tRNA^{tMet}-dependent activation of 30S IC-bound IF2. 30S IC-bound IF2 can occupy at least four distinct conformational states relative to the P-site tRNA (denoted as conformations a-d). These conformational states are characterized by \( E_{\text{FRET}} \) values of 0.67 (a), 0.53 (b), 0.81 (c), and 0.87 (d). The dotted box highlights 30S ICs and pseudo 30S ICs studied in this work and their corresponding \( E_{\text{FRET}} \) values. 30S ICs and \( E_{\text{FRET}} \) values indicated outside of the dotted box are predicted conformational states of IF2. (Central panel) The specific binding of GTP to dII of IF2 is allosterically communicated through dIII and results in a repositioning of dIV closer to the P site of the 30S IC and further from dI-III. The specific recognition of the N-formyl-methionine of a P-site-bound fMet-tRNA^{fMet} by dIV of IF2(GTP) feeds back to dIII (dark purple), thereby stabilizing a conformation of d-III of IF2 that is active for rapid 50S subunit joining. In contrast, (top panel) the binding of GDP to dI of IF2, or (bottom panel) the presence of an unformylated Met-tRNA^{tMet} or an elongator tRNA in the P site fails to stabilize the active conformation of IF2, instead leaving IF2 in a conformation(s) that are inactive for rapid 50S subunit joining

Interestingly, the distribution of \( E_{\text{FRET}} \) values for the mutIF2(GTP)-bound state of 30S IC_{mT,OH} is significantly more symmetrical than that for the wtIF2(GTP)-bound state of 30S IC_{wT,Met} and 30S IC_{wT,OH} (Fig. 3c, d with Fig. 3a, b). Consistent with this, we find that \( K_{dIII}^{\text{wt/Met}} \) and \( K_{dIV}^{\text{oh/Met}} \) are ~20-fold and ~30-fold smaller than \( K_{dIII}^{\text{wt/Met}} \) and \( K_{dIV}^{\text{oh/Met}} \), respectively (Table 1). The activating mutation in dIII therefore enables mutIF2(GTP) to bind to 30S IC_{mT,Met} and 30S IC_{mT,OH} with an affinity that is over an order of magnitude higher than that with which wtIF2(GTP) binds to 30S IC_{wT,Met} and 30S IC_{wT,OH}. This demonstrates that high-affinity binding of IF2(GTP) to the 30S IC does not necessarily require dIV to establish direct interactions with the N-formyl moiety or N-formyl-methionine of the P-site-bound fMet-tRNA^{tMet}. Rather, it is the conformation of d-III that contributes significantly to the affinity of IF2(GTP) for the 30S IC. Such a contribution could arise from direct interactions between dIII and S12 or some other component of the 30S IC and/or from allosteric modulation of the interactions that dII makes with h5 and h14 of 16S rRNA.

Interestingly, the distribution of \( E_{\text{FRET}} \) values for the mutIF2(GTP)-bound state of 30S IC_{mT,Met} and 30S IC_{mT,OH} is very similar to those for the wtIF2(GTP)-bound state of 30S IC_{wT,Met} and 30S IC_{wT,OH}. Specifically, the distribution of \( E_{\text{FRET}} \) values for the mutIF2(GTP)-bound state of 30S IC_{mT,Met} (Fig. 3c and Supplementary Fig. 5f) exhibited two non-zero \( E_{\text{FRET}} \) peaks. The first peak encompassed a smaller, 42 \pm 5.7%, subpopulation of the mutIF2(GTP)-bound state of 30S IC_{mT,Met} and was centered at an \( E_{\text{FRET}} \) of 0.83 \pm 0.04 that is within error of that observed for the larger subpopulation of the wtIF2(GTP)-bound state of 30S IC_{wT,Met} (\( p = 0.7 \), Supplementary Table 1). The second peak encompassed a larger, 58 \pm 5.7%, subpopulation of the mutIF2(GTP)-bound state of 30S IC_{mT,Met} and was centered at an \( E_{\text{FRET}} \) of 0.57 \pm 0.02 that is also within error of that observed for the smaller subpopulation of the wtIF2(GTP)-bound state of 30S IC_{wT,Met} (\( p = 0.4 \), Supplementary Table 1). Similarly, the distribution of \( E_{\text{FRET}} \) values for the wtIF2(GTP)-bound state of 30S IC_{mT,OH} (Fig. 3d and Supplementary Fig. 5h) exhibited a single non-zero \( E_{\text{FRET}} \) peak centered at an \( E_{\text{FRET}} \) of 0.57 \pm 0.01 that is within error of that observed for the larger subpopulation of the mutIF2(GTP)-bound state of 30S IC_{mT,OH} and the wtIF2(GTP)-bound state of 30S IC_{wT,OH} (\( p = 0.8 \), Supplementary Table 1). The fact that the \( E_{\text{FRET}} \) values for 30S IC_{mT,OH} and 30S IC_{mT,OH} are ~20-fold and ~30-fold smaller than those of dIV of wtIF2(GTP) in 30S IC_{mT,OH} relative to those of dIV of wtIF2(GTP) in 30S IC_{mT,OH} and 30S IC_{wT,OH} suggest that the activating mutation in dIII does not significantly alter the positions of dIV of wtIF2(GTP) in 30S IC_{mT,OH} and 30S IC_{mT,OH} relative to those of dIV of wtIF2(GTP) in 30S IC_{mT,OH} and 30S IC_{mT,OH}.

Previously, we have used ensemble kinetic experiments to show that the rates of 50S subunit joining to 30S IC_{mT,Met} and 30S IC_{mT,OH} are approximately fourfold and ~12-fold higher than those of 30S IC_{wT,Met} and 30S IC_{wT,OH}, respectively. The activating mutation in dIII enables mutIF2(GTP) to catalyze 50S subunit joining to 30S IC_{mT,Met} and 30S IC_{mT,OH} at rates that are within 30% of those observed for 30S IC_{mT,Met} and 30S IC_{mT,OH}. Based on the results reported here, we propose that the activating mutation in dIII enables mutIF2(GTP) to catalyze 50S subunit joining by stabilizing a conformation of d-III, that not only increases the affinity of mutIF2(GTP) for 30S IC_{mT,Met} and 30S IC_{mT,OH}, but that is optimized for the rapid recruitment of 30S subunits.
and/or docking of the 50S subunit onto 30S IC_{wT,Met} and 30S IC_{wT,OH}. We speculate that, in the context of wtIF2(GTP), this conformation of dI-dIII is rendered conditional on the direct interactions of dIV with the N-formyl-methionine.

**Discussion**

Here, we have used a combination of smFRET and ensemble kinetic studies of 50S subunit joining to elucidate the mechanism of IF2 activation for rapid 50S subunit joining to the 30S IC. Our results demonstrate how GTP and fMet-tRNA^{Met} stabilize the specific conformation of 30S IC-bound IF2 that confers high-affinity binding to the 30S IC and rapid 50S subunit joining. Based on our findings, we propose a model for IF2 activation during translation initiation (Fig. 5). In this model, four conformations of 30S IC-bound IF2, which we denote as conformations a–d in Fig. 5, play major roles. In conformation a, dI is not in the GTP-bound form, dII and dIII do not interact fully with the 30S subunit, and dIV is positioned closer to dI-dIII than to the free-bound tRNA. This conformation corresponds to the \( E_{\text{FRET}} \) of 0.67 ± 0.01 that we observe for 30S IC_{wT,GDP-bound} wtIF2(GTP) and it is inactive for rapid 50S subunit joining. In conformation b, dI is in the GTP-bound form, dII and dIII have established increased interactions with the 30S subunit, and dIV is positioned closer to the P site-bound tRNA than to dI-dIII. This GTP-dependent repositioning of dIV is driven by an allosteric mechanism in which binding of GTP to dI triggers a conformational change and/or repositioning of dI-dIII, which ultimately places dIV closer to the P site. Independent evidence in favor of our model comes from structural studies in which binding of GTP to dI results in a restructuring of dI-dIII that has been predicted to modulate the interactions of dI with h14 and h5 of 16S tRNA and of dII with S12. In conformation b, which corresponds to the \( E_{\text{FRET}} \) value of 0.53 ± 0.02 that we observe for 30S IC_{wT,OH-bound} wtIF2(GTP), dIV does not make any stabilizing contacts with fMet-tRNA^{Met}. Conformation c, which corresponds to the \( E_{\text{FRET}} \) of 0.81 ± 0.01 that we observe in 30S IC_{wT,OH-bound} wtIF2(GTP), is similar to the second conformation, except that dIV now makes partial interactions with the N-formyl-methionine of a P-site fMet-tRNA^{Met}. In conformation d, which corresponds to the \( E_{\text{FRET}} \) of 0.87 ± 0.02 that we observe in 30S IC_{wT,OH-bound} wtIF2(GTP), dIV interacts fully with the N-formyl-methionine of the P-site fMet-tRNA^{Met} and has adopted a position that allosterically feeds back and stabilizes the conformation of dI-dIII that is active for rapid 50S subunit joining. Independent evidence in support of the idea that the interactions between dIV and the N-formyl-methionine allosterically feed back to dI-dIII in order to stabilize a conformation that is active for rapid 50S subunit joining comes from previous measurements of the affinity of dI for GTP in which it was observed that the presence of a P-site fMet-tRNA^{Met} allosterically increases the affinity of dI for GTP.

A major finding of this study is that dIII integrates GTP binding to dI and fMet-tRNA^{Met} recognition by dIV in order to allosterically regulate the affinity of IF2 for the 30S IC and the conformation of the resulting 30S IC-bound IF2. This suggests that dIII may serve as a target for antibiotics that inhibit IF2 allosterically. To explore this option, further characterization of the mechanism of IF2 activation and, in particular, the dynamic interplay between dI-dIV will be necessary. This will require the development of additional, intramolecular labeling schemes to directly probe the interdomain dynamics of IF2. In addition, while low- to high-resolution cryo-EM structures of the active conformation of 30S IC-bound IF2 have been reported16–19, a comprehensive structural understanding of IF2 activation will undoubtedly require not only higher resolution cryo-EM or cryoelectron tomography, but also the active conformation(s) of 30S IC-bound IF2 (e.g., 30S IC_{wD}, 30S IC_{wT,Met}, or 30S IC_{wT,OH}).

**Methods**

**Preparation of 30S subunits.** Highly active 30S subunits were purified from the _E. coli_ strain MRE600 as described previously25. Briefly, clarified cell lysates were centrifuged through a sucrose cushion solution (20 mM Tris-HCl (pH 7.4), 720 mg/mL MgCl2, 0.5 mM EDTA, 60 mM βME, 37% sucrose) to isolate crude ribosomes. Next, crude ribosomes were centrifuged through a 10–40% sucrose density gradient to isolate tight-coupled 70S ribosomes. To promote the dissociation of tight-coupled 70S ribosomes into 30S and 50S subosomal ribosomes, the tight-coupled 70S ribosomes were dialyzed into ribosome dissociation buffer (10 mM Tris-OAc (pH 7.5), 7.5 mM NH4Cl, 1 mM MgCl2, 0.5 mM EDTA, 60 mM βME). To isolate 30S subunits, the dissociated ribosomes were centrifuged through a 30–40% sucrose gradient to prepare a 30S ribosomal dissociation buffer. Purified 30S subunits were pelleted by ultra-centrifugation, re-suspended in ribosome storage buffer (10 mM Tris-OAc (pH 7.5), 7.5 mM NH4Cl, 7.5 mM MgCl2)

Crystallographic structures of the active conformation of 30S IC-bound IF2, but also the inactive conformation(s) of 30S IC-bound IF2 (e.g., 30S IC_{wD}, 30S IC_{wT,Met}, or 30S IC_{wT,OH}).
mRNAAUG was chemically synthesized and mRNApri-ext was generated by in vitro transcription using T7 RNA polymerase as previously described. The mRNA sequence for 5′-bio-mRNAagg and mRNApri-ext are shown below. In both of the mRNA sequences, the Shine–Dalgarno (SD) sequence is underlined, the start codon is underlined and bolded, and the spacer sequence between the SD sequence and the start codon is italicized. In addition, for mRNApri-ext, the primer annealing site used to reverse transcribe the message is underlined, bolded, and italicized. The mMFTI mRNA used in our light-scattering experiments is also shown bolded and italicized. The mMFTI mRNA used in our light-scattering experiments is also shown bolded and italicized. The mMFTI mRNA used in our light-scattering experiments is also shown bolded and italicized.
The number of simulated $E_{\text{RET}}$ trajectories for each pseudo 30S IC was determined by taking the number of observed $E_{\text{RET}}$ trajectories that exhibited at least one IF2 binding event in 30S IC$_{\text{wT,Met}}$ or 30S IC$_{\text{wT,OH}}$ and multiplying by a correction factor given by:

Correction factor ($C = \frac{\% \text{ capable} – \% \text{ observed}}{\% \text{ observed}}$).

where "% capable" represents the fraction of $E_{\text{RET}}$ trajectories that exhibited at least one binding event in the analogous 30S IC formed with $\text{Met-tRNA}^{\text{Met}}$ (30S IC$_{\text{wT,OH}}$), and "% observed" represents the fraction of $E_{\text{RET}}$ trajectories that exhibited at least one binding event in 30S IC$_{\text{wT,Met}}$ or 30S IC$_{\text{wT,OH}}$. The length of the simulated $E_{\text{RET}}$ trajectories was set to the average length of the observed $E_{\text{RET}}$ trajectories for either 30S IC$_{\text{wT,Met}}$ or 30S IC$_{\text{wT,OH}}$ prior to Cy3 photobleaching.

Ensemble topegrning and kinetic experiments. The primer-extension inhibition- or "toeprinting"-based IF2 activity assay reports on the position of the 30S IC on mRNaAri-ex with single-nucleotide resolution and thus measures the ability of IF2 to direct the selection of the authentic initiator $\text{Met-tRNA}^{\text{Met}}$ into the P site of the 30S IC, over elongator tRNAs. Primer extension was performed using the Avian Myeloblastosis (AMV) reverse transcriptase as described previously.

Briefly, 25 μl reactions containing 5 μl of various 30S ICs, 1.2 mM ATP, 0.5 mM of each dNTP, and 6 units of AMV and Tris-Polyox with 10 mM MgOAc2 were incubated at 37 °C for 15 min. Following phenol-chloroform extraction and ethanol precipitation, complementary DNA (cDNA) pellets were re-suspended in formamide loading buffer, heat denatured at 95 °C for 5 min, and cDNA fragments were resolved on a 9% sequencing gel.

Rayleigh light scattering-based ensemble kinetic 50S subunit joining experiments were performed as described previously.

Briefly, 0.6–0.8 ml of 36 μM 30S subunits and 0.6–0.8 ml mixture containing 0.36 μM 30S IC assembled with IF1, $\text{Met-tRNA}^{\text{Met}}$, or $\text{tRNA}^{\text{Met}}$ (or no tRNA) and IF2 (wildtype or mutant, added in different concentrations) were pre-incubated for 20 min at 37 °C and loaded into the syringes of our stopped-flow instrument (SX-20 Applied Photophysics, Leatherhead, UK). The kinetics of 70S IC formation was monitored at 37 °C with light scattering after rapid mixing of equal volumes of the 30S IC mixture and the 50S subunits.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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**Author contributions**

K.C., M.P., M.E., and R.L.G. designed the research; K.C. performed the smFRET experiments and M.P. performed the ensemble rapid kinetic light scattering experiments; K.C., M.P., M.E., and R.L.G. analyzed the data; K.C., M.P., M.E., and R.L.G. wrote the manuscript; all four authors approved the final manuscript.

**Additional information**

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