

IF2 aptamers allosterically impair bacterial translation initiation

Lesia Tello

Universidad Peruana de Ciencias Aplicadas

Katherin Peñaranda

Universidad Peruana de Ciencias Aplicadas

Ana Sanchez-Castro

Universidad Peruana de Ciencias Aplicadas

Joaquin Rodriguez Trelles

Universidad Peruana de Ciencias Aplicadas

Victor Barrenechea

Universidad Peruana de Ciencias Aplicadas

Daria Vinogradova

Petersburg Nuclear Physics Institute

Pablo Dans

Universidad de la República de Uruguay

Alena Paleskava

Petersburg Nuclear Physics Institute

Andrey Konevega

Peter the Great St. Petersburg Polytechnic University

Roberto Spurio

Università di Camerino

Pohl Milon

`pmlon@upc.edu.pe`

Universidad Peruana de Ciencias Aplicadas

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Abstract

Translation initiation is a highly regulated step of protein synthesis. In prokaryotes, this process is orchestrated by three initiation factors, among which IF2 is the only universally conserved guanosine binding protein. IF2 facilitates recruitment of fMet–tRNA^{fMet} to the 30S initiation complex (30S IC), accelerates 50S subunit joining, and drives initiator tRNA accommodation on the 30S and 70S initiation complexes. Currently, no compounds are available to selectively halt IF2 functions during initiation complex assembly. Aptamers offer such a possibility; however, no IF2-targeting aptamers have been reported to date. In this study, we developed two DNA aptamers, Apt 311 and Apt 312, which showed strong binding and specificity toward IF2. Both aptamers exhibited low micromolar dissociation constants, with K_d of $0.5 \pm 0.1 \mu\text{M}$ for Apt 311 and $0.3 \pm 0.1 \mu\text{M}$ for Apt 312. We further characterized Apt 311 on translation initiation using rapid kinetic assays, revealing that the aptamer does not perturb the initial fMet–tRNA^{fMet} binding to the 30S complex, yet greatly halts 30S IC formation and the subsequent 50S subunit joining. The kinetic analysis of 30S IC formation shows that Apt 311 markedly impairs fMet–tRNA^{fMet} accommodation rather than the initial fMet–tRNA^{fMet} binding. Together with *in silico* modelling, our data suggests that both aptamers interact with the C1 and G domains, restraining essential IF2 dynamics that are required for initiator tRNA accommodation and, 30S and 70S ICs assembly. Altogether, our results establish Apt 311 and Apt 312 as new molecules that target transient, IF2-dependent, and essential reactions that ultimately lead to decoding the mRNA start site. In addition, this work entails a proof of concept for developing aptamers to probe function and dynamics of essential factors in protein synthesis.

Introduction

Translation initiation is a rate-limiting and highly regulated step of protein synthesis¹, making it a promising target for antibiotic development. During the initiation phase, the 30S ribosomal subunit together with initiation factors IF1, IF2, and IF3, establish the correct translational reading frame by promoting the initiation codon–anticodon duplex between the mRNA and fMet–tRNA^{fMet} (Fig. 1a)². The correct positioning of the initiation tRNA in the P-site of the ribosome and the recognition of the start codon kinetically stabilizes the complex, yielding the 30S initiation complex (30S IC)³. Subsequent joining of the 50S subunit and dissociation of the initiation factors produce the 70S initiation complex (70S IC), the functional platform for the elongation phase⁴. In prokaryotes, translation initiation requires all three initiation factors to coordinate, as kinetic effectors, the stepwise assembly of a competent ribosomal complex 70S IC, ensuring the appropriate speed and accuracy of the process^{2,5–7}.

Along all complex intermediates of translation initiation, IF2 plays a central role. It facilitates fMet–tRNA^{fMet} recruitment, promotes codon–anticodon pairing, and accelerates 50S subunit joining^{8–11}. These functions are distributed across a modular domain architecture; the N-terminal region contains domains N1 and N2, the C-terminal region (subdivided in C1 and C2) specifically recognizes fMet–tRNA^{fMet} and the central G-domain (subdivided in G1, G2 and G3) mediates GTP binding (Fig. 1b)¹². In *E.*

coli, IF2 naturally occurs as three co-expressed wild-type isoforms (IF2 α , IF2 β , and IF2 γ) translated from a single *infB* gene through three in-frame initiation codons; the shorter forms lack increasing portions of the N-terminal domain, and their combined presence is required for optimal growth^{13–15}.

IF2 is a conserved guanosine binding factor that requires GTP for productive 30S IC formation and GDP for rapid dissociation from the resulting 70S IC^{6,16}. Remarkably, GTP hydrolysis is not required *in vitro* provided that the interaction with fMet–tRNA^{fMet} is weakened by the R847G mutation in the C2 domain¹⁷. Also, the G3 mutation E571K results in abolishing GTP hydrolysis by IF2 while allowing *in vitro* translation and cell survival¹⁸. Thus, GTP hydrolysis can be dispensable both *in vitro* and *in vivo*. The alarmone (p)ppGpp can compete with GTP or GDP for IF2 reducing translation initiation efficiency under stringent response to stress^{16,19,20}. Thus, IF2 can transit between active and inactive conformations as a function of the guanosine nucleoside bound. Biochemical and structural work supports that at least two structural transitions of this factor exist off- and on the ribosome^{8,21}. Particularly, IF2 C2 positioning on the 70S IC is 35 Å away from the fMet–tRNA^{fMet} if GDP-bound, promoting IF2 compaction^{22,23}. How the long-range intramolecular contact network between the C2 and G2 (guanosine binding site) domains rearranges through the C1 and G3 domains remains elusive.

Among the known translation inhibitors, several influence IF2 indirectly or act on it without targeting translation initiation. For example, thiostrepton sterically perturbs the 50S subunit²⁴; lamotrigine binds IF2 directly but compromises ribosomal biogenesis and maturation, especially at low temperature, without detectably inhibiting productive initiation *in vitro* while impairing initiation fidelity *in vivo*^{25,26}; and evernimicin, binding to the 23S rRNA and protein L16 of the 50S subunit, inhibits the IF2-dependent formation of the 70S IC^{27,28}. Thus, although few translation initiation inhibitors exist²⁹, none of them directly and specifically targets IF2's functions during translation initiation. To fill this gap, we use aptamers as a precise and selective approach. Aptamers are short single-stranded nucleic acid sequences generated through SELEX (Fig. 1c) that can bind specific molecular targets with high affinity and specificity^{30,31}, offering a unique opportunity to selectively interfere with IF2. Beyond acting as inhibitors, aptamers can also serve as molecular tools³² to study the structure-function relationships of the targeted macromolecule.

In this study, we developed DNA aptamers against *E. coli* IF2 and investigated how their binding interferes with the factors function during translation initiation. By examining the effect of aptamers on the assembly of the 30S and 70S initiation complexes, this work provides mechanistic insights into IF2 and highlights how aptamers can be used as directed tools to study the functional landscape of essential translation factors.

Results

Aptamer selection and screening

The candidate aptamers were obtained from a 74-nt single-stranded DNA (ssDNA) library immobilized on polystyrene beads, consisting of a 40-nucleotide random central region flanked by two 17-nt constant regions required for PCR-based amplification. Four candidate aptamers (Apt 311-Apt 314) were enriched during SELEX and selected for further evaluation using a fluorescent pull-down assay (Table 1). Fluorescently labelled Apt 311 and Apt 312 exhibited the highest fluorescence signal after pull-down with IF2-coated beads, indicating greater recovery and suggesting stronger interaction with IF2, compared to Apt 313 and Apt 314 (**Supplementary Fig. S1**). Control reactions, in which the aptamers were incubated with an unrelated eukaryotic protein (1F8) or with uncoated Ni-NTA beads, showed low signal intensity with minimal variation between aptamers, supporting low levels of nonspecific binding. Altogether, these results identified Apt 311 and Apt 312 as the aptamers with the strongest apparent interaction with IF2.

Table 1
Selected aptamer candidates after SELEX methodology

Name code	Sequence
Apt 311	5′ TTTT TAGATCTCATACCAGTTGTCTCTTGCCCGTTTCCATCTAGTTTTT 3′
Apt 312	5′ TTTT TACTAGCCTGTTCTCCACTACGTTGCCGATTTCCTACTAGTTTTT 3′
Apt 313	5′ TTTT TACTAGCAATTTAGCCTGTATAGGGCATGTCGCCAGCTAGTTTTT 3′
Apt 314	5′ TTTT TAGATCCCATTTCTTCTAATCGGAGCCCTCTTGACTGATCTTTTT 3′

Apt 311 specifically binds IF2

To characterize the binding properties of Apt 311 and Apt 312, protein specificity was assessed by ELONA using biotinylated variants of the aptamers on protein coated microwell plates (Fig. 2a-b). Control experiments aimed at evaluating general nonspecific protein-aptamer interactions, were carried out using proteins unrelated to translation (1F8 and BSA); both produced only basal signals, corresponding to less than 14% of the signal displayed by IF2 (Fig. 2a-b). Specificity within the translation initiation machinery was then tested against the initiation factors IF1 and IF3. IF1 produced similar basal signals, whereas IF3 gave a signal higher than the other controls, although it was still lower than IF2 (~ 43% for Apt 311 and ~ 72% for Apt 312), likely due to its high positive charge (pI ~ 9.4). To assess nonspecific interactions of IF2 with nucleic acids, binding of a random oligonucleotide to IF2 was measured, yielding 5.8-fold and ~ 5.2-fold lower signals relative to specific IF2 binding for Apt 311 and Apt 312, respectively (**Supplementary Fig. S1c**). Finally, both aptamers showed comparable binding signals for both natural isoforms, the full-length IF2α and the shorter IF2γ, which lacks the N1 domain, indicating that neither Apt 311 nor Apt 312 interacts with the N1 domain of IF2. Since both isoforms were bound equivalently, hereafter "IF2" refers to IF2γ unless a specific isoform is indicated.

Then, we measured the binding parameters between IF2 and the aptamer with two different assays: steady-state affinity quantification, measured by micro-scale thermophoresis (MST), and real-time

binding kinetics, measured by stopped-flow technique. The affinity between each aptamer and IF2 α was quantified by titration of the protein against Cy5-labelled aptamer, yielding comparable MST traces and amplitudes for both aptamer candidates (Fig. 2c). Fitting to a one site binding model (Eq. 1; see Methods) resulted in dissociation constants (K_d) of $0.5 \pm 0.1 \mu\text{M}$ for Apt 311 and $0.3 \pm 0.1 \mu\text{M}$ for Apt 312. Then, we used Förster Resonance Energy Transfer (FRET) coupled to the stopped-flow technique (Fig. 2d), using a quencher-labelled IF2 (IF2^Q) at the C2 domain (**Supplementary Fig. S2**) and IF1 labelled with a donor fluorophore (IF1^{Flu}). IF2^Q binding to the complex produced a fluorescence amplitude corresponding to the binding and positioning of IF2 relative to IF1 in the 30S. This signal was comparable to that observed with a random oligonucleotide. In contrast, both Apt 311 and Apt 312 induced a greater decrease in fluorescence, indicating that: i) the aptamers do not inhibit IF2 binding to the 30S–IFs complex, and ii) the final relative orientation of IF2, particularly the C2 domain, appears in closer proximity to IF1 when aptamers are bound to IF2 (Fig. 2d).

Consistently, *in silico* structural analysis revealed high sequence and structural similarity between Apt 311 and Apt 312, with 58% identity and strong agreement in predicted secondary and tertiary structures (**Supplementary Fig. 3**). Together, these findings indicate that Apt 311 and Apt 312 are closely related aptamers, likely originating from the same selection cluster, and producing comparable effects on IF2. Therefore, Apt 311 was selected as a representative aptamer to further investigate the mechanism of aptamer binding to IF2 across different steps of bacterial translation initiation (Fig. 1a).

Apt 311 halts fMet–tRNA accommodation during 30S IC formation

IF2 has a critical role in anchoring fMet–tRNA^{fMet} to the P-site of the 30S pre-initiation complex (30S PIC) by recognizing the formyl group³³. Once the fMet–tRNA^{fMet} recognizes the start codon on the mRNA, a conformational rearrangement triggers the complex into a “locked” tRNA state, gaining over 70-fold affinity, and unlocking subsequent 50S subunit joining (Fig. 1a)⁷. To study how Apt 311 could interfere with IF2 during 30S IC formation, we measured the kinetics of fMet–tRNA^{fMet} binding in a stopped-flow system. We used a FRET signal between a fluorescein-labelled fMet–tRNA^{fMet} (fMet–tRNA^{Flu}) and an IF2 labelled with a quencher at the C2 domain, that was pre-bound to the 30S PIC. Upon fMet–tRNA^{Flu} binding, a large decrease of donor fluorescence is observed compared to the no acceptor control indicating the fluorescent tRNA gets into FRET range with the quencher located in IF2 (Fig. 3a). When Apt 311 was pre-incubated with the 30S PIC, the total amplitude related to fMet–tRNA^{Flu} binding was reduced if compared to aptamer absence or in the presence of a random DNA (Fig. 3b). The fluorescence change over time was biphasic, consistent with at least two steps being observed, fMet–tRNA^{Flu} initial binding and subsequent accommodation. The Apt 311 did not perturb either the amplitude or the rate associated with the initial tRNA binding, indicating that the aptamer does not perturb the IF2 C2–tRNA interaction. Conversely, the amplitude corresponding to the second phase was greatly affected by Apt 311, suggesting that the subsequent tRNA accommodation is compromised (Fig. 3b).

To further study the molecular mechanism of Apt 311 inhibition of tRNA recruitment, we titrated Apt 311 (from 0 to 2 μM) to a 30S PIC and fMet–tRNA^{fMet} binding was measured (Fig. 3c). The total FRET amplitude decreased with aptamer concentration reaching a plateau of about 40% if compared to the absence of the aptamer. The inhibitory behaviour yielded an IC_{50} of $0.3 \pm 0.1 \mu\text{M}$ (Fig. 3d), similar to the K_d measured by MST on the free IF2 (Fig. 2c). Analysis of each kinetic parameter as a function of aptamer concentration confirmed that the initial binding (Amplitude corresponding to apparent rate $k_{\text{app}1}$) is not affected by the aptamer (**Supplementary Fig. S4a**). Conversely, the amplitude corresponding the subsequent tRNA rearrangement largely decreased with aptamer concentration (**Supplementary Fig. S4a**). On the other hand, both apparent rates showed no major kinetic differences in the titration analysis (**Supplementary Fig. S4b**). The lack of major kinetic effects and the localized inhibition to the second step in tRNA binding is consistent with the hypothesis of Apt 311 inhibiting IF2 allosterically rather than competing with fMet–tRNA^{fMet} for the same binding site. Additionally, the observation that IC_{50} obtained from the total amplitude dependence resembles the K_d obtained with the isolated factor strongly suggests that Apt 311 triggers an IF2 conformation incompatible with complex progression. Altogether, these results indicate that Apt 311-bound IF2 precludes complex rearrangements required for 30S IC formation.

Apt 311 impairs the transition to 70S IC formation

To monitor fMet–tRNA^{fMet} positioning along 70S IC formation, we used two stopped-flow approaches: i) light scattering to directly monitor 70S formation^{34,35}; and ii) fluorescence changes of a BODIPY-labelled Met–tRNA^{fMet} (Bpy–Met–tRNA^{fMet}) as an indicator of the final fMet–tRNA^{fMet} accommodation step during 50S joining²⁰. Additionally, two mixing setups were used to investigate Apt 311 on the isolated step of 70S IC formation and under conditions of limiting 30S IC formation by fMet–tRNA^{fMet} arrival (Fig. 4).

When 70S IC formation was monitored by light scattering, the canonical reaction in the absence of Apt 311 and in the presence of a random DNA showed a signal increase, indicating efficient 50S joining to 30S IC and 70S IC formation (Fig. 4a). The addition of random DNA control shows that the 70S IC formation system is not affected by an unspecific DNA molecule. Additionally, the 30S PIC control gave no appreciable signal showing that in the absence of fMet–tRNA^{fMet}, there is no 70S IC formation (Fig. 4a). When Apt 311 was pre-incubated with the complex, 50S joining still proceeded with comparable kinetics but reached a lower amplitude, indicating that less 70S ICs were formed (Fig. 4a). Additionally, the accommodation of the fMet–tRNA^{fMet} was measured by the fluorescence change of Bpy–Met–tRNA^{fMet} upon 50S joining. For the no aptamer control and random DNA reactions, the fluorescence decreased, reporting a Bpy–Met–tRNA^{fMet} accommodation (Fig. 4b). With the Apt 311 pre-incubated with the 30S IC, the fluorescence amplitude was 2-folds smaller (Fig. 4b). Comparing the Bpy–

Met-tRNA^{fMet} and light scattering signals, the former shows greater inhibition, indicating that the 50S subunit can bind the 30S IC, but fMet-tRNA^{fMet} accommodation is still perturbed.

We next evaluated the concomitant binding of fMet-tRNA^{fMet} and the 50S subunit to a 30S PIC, coupling 30S IC to 70S IC formation. By light scattering, the reaction and the random DNA control reached maximal amplitude, indicating equivalent levels of 70S IC formation (Fig. 4c). When Apt 311 was pre-incubated, the total amplitude was about 2-fold lower (Fig. 4c). A two-exponential nonlinear fitting showed that the observed amplitude decrease originates in the first step, in which the difference is by 6-fold, approximately (**Supplementary Fig. S5a**). However, no changes were observed between the presence or absence of the Apt 311 in the second step amplitude (**Supplementary Fig. S5b**). The corresponding Bpy-Met-tRNA^{fMet} signal in the absence of Apt 311 or with a random DNA reactions equally decreased in time, indicating tRNA binding and accommodation of Bpy-Met-tRNA^{fMet} to the 30S IC and subsequently to the 70S IC (Fig. 4d). In the presence of Apt 311, the total fluorescence change was greatly reduced if compared to the absence of the aptamer (**Supplementary Fig. S5c**). Considering the regression analysis, we observed that the second step is lost in the presence of Apt 311, while the first step is reduced by 5.4-fold and is 2 times slower (**Supplementary Fig. S5c**, Table 2). We were unable to compare the amplitudes corresponding to the second phase of the reaction, since the fluorescence signal linearly depended over time on the presence of Apt 311 (Fig. 4d, Table 2). As in the isolated 50S-joining experiment, the inhibition of the Bpy-Met-tRNA^{fMet} signal greatly exceeded that of the light scattering, confirming that even if the 50S subunit binds the 30S complex, tRNA accommodation remains perturbed. Altogether, the data indicate that Apt 311 binding to IF2 restricts fMet-tRNA^{fMet} accommodation during late initiation: the 50S subunit can still join the small subunit, but the initiator tRNA fails to reach its fully accommodated configuration, possibly inducing the complex into a non-productive state.

Table 2
Apparent rates of initiation complex formation

Reaction		No Aptamer		Apt 311	
Complex	Ligand	$k_{app} 1$ (s^{-1})	$k_{app} 2$ (s^{-1})	$k_{app} 1$ (s^{-1})	$k_{app} 2$ (s^{-1})
30S-IF _{1/3} (FRET IF2 ^Q -IF1 ^{Flu})	IF2 ^Q	15.7 ± 0.2	0.45 ± 0.01	10.9 ± 0.10	0.66 ± 0.01
30S PIC ^{-tRNA} (FRET IF2 ^Q -fMet-tRNA ^{Flu})	fMet-tRNA ^{Flu}	0.68 ± 0.04	0.5 ± 0.01	0.73 ± 0.05	0.07 ± 0.01
30S IC (Light scattering)	50S	0.84 ± 0.19	0.09 ± 0.05	0.99 ± 0.16	0.08 ± 0.03
30S IC (Bpy-Met-tRNA ^{fMet} Fluorescence)	50S	0.79 ± 0.05	0.07 ± 0.01	0.71 ± 0.08	0.12 ± 0.02
30S PIC ^{-tRNA} (Light scattering)	50S + fMet-tRNA ^{fMet}	0.10 ± 0.01	0.02 ± 0.01	0.18 ± 0.06	0.01 ± 0.01
30S PIC ^{-tRNA} (Bpy-Met-tRNA ^{fMet} Fluorescence)	50S + Bpy-Met-tRNA ^{fMet}	0.05 ± 0.01	0.008 ± 0.01	0.026 ± 0.01	0.002 ± 0.01*
Shown values are mean and standard deviations from 5–7 replicates analysed independently. *This apparent rate was estimated with a linear regression fitting.					

Discussion

In this study, we identified two aptamers against *E. coli* IF2 that show similarities regarding the sequence, binding specificity, affinity, and predicted structure, indicating a shared binding model. Both aptamers are highly specific for IF2 over related (IF1, IF3) and unrelated (1F8, BSA) proteins, they bind with sub-micromolar affinity and shift the C2 domain of IF2 closer to IF1 within the 30S–IFs complex (Fig. 2). We selected Apt 311 as a representative aptamer to dissect the impairment of translation initiation in relation to IF2's functions. Overall, Apt 311 alters IF2's functions related to fMet-tRNA^{fMet} positioning (Fig. 3), thus interfering with the formation of the 30S IC and subsequent decreasing 70S IC formation (Fig. 4, Fig. 5a).

The main effect of Apt 311 binding is an impairment of coupled 30S IC–70S IC formation. When the 50S subunit arrives to a pre-formed 30S IC, the rate of 70S assembly is essentially unchanged, yet the amplitude of the signal is reduced (Fig. 4a). This dissociation between speed and yield suggests that the aptamer does not interfere with 50S joining itself but rather reduces the proportion of 30S ICs available. Also, Bpy–Met–tRNA^{fMet} quenching amplitude is reduced (Fig. 4b), showing that in complexes where 70S assembly initiates, the fMet–tRNA^{fMet} does not reach its final position in the ribosomal P-site. Together, these results indicate that the aptamer does not sterically block 50S binding; rather, it reduces the fraction of 30S ICs that complete the locking transition (Fig. 1). If 30S IC locking is indeed the limiting step, its perturbation should be more pronounced when 70S IC formation is limited by initiator tRNA binding, thus limited by 30S IC formation or locking. Consistent with this, when the fMet–tRNA^{fMet} and 50S arrive concomitantly at a 30S PIC, both effects are amplified: the 70S IC assembly amplitude is lower (Fig. 4c), and the Bpy–Met–tRNA^{fMet} signal shows a greater amplitude decrease (Fig. 4d). The aptamer induced alteration on IF2 uncouples subunit joining from correct fMet–tRNA^{fMet} accommodation creating a bottleneck that reduces both the speed and yield of 70S assembly. This is consistent with the kinetic partitioning model in which 30S ICs that do not reach the locked state dissociate rather than progress to 70S IC formation^{1,36}.

The mechanistic basis for these effects lies in the 30S IC. The total amplitude of the fMet–tRNA^{Flu} binding signal decreases markedly when Apt 311 is pre-incubated with the 30S PIC (Fig. 3b), and the rate of the fMet–tRNA^{fMet} binding remains similar (Fig. 3c). This indicates that fMet–tRNA^{fMet} is recruited at a similar speed but fails to reach its correct position within the complex. This pattern is consistent with the conformational heterogeneity of the 30S IC described³⁷, who used FRET between the fMet–tRNA^{fMet} and IF2 to show that the complex exists in distinct states that differ in tRNA accommodation depth. In that basis, a reduction in signal amplitude reflects a change in the population toward sub-accommodated states, where the tRNA has not reached its final P-site position. Apt 311 shifts IF2 closer to IF1 within the 30S complex (Fig. 2d), suggesting two alternative mechanisms that could explain the effect on fMet–tRNA^{fMet} positioning. In one, IF2 adopts a compact conformation where the C2 domain is closer to IF1, incorrectly positioning the fMet–tRNA^{fMet} upon binding. It could be plausible that this Apt 311-dependent conformation of IF2 is similar to that induced by GDP^{22,23}. In the other alternative, the aptamer reduces the conformational flexibility of IF2, limiting the degrees of freedom required for correct fMet–tRNA^{fMet} orientation³⁸. Thus, we propose that Apt 311 alters IF2 positioning and therefore impairs 30S IC locking, which hinders late events that drive IF2 dissociation and tRNA accommodation during 50S joining.

As for the structural context, we performed protein-aptamer docking analysis, where we identified the G and C1 domains as interaction hotspots (Fig. 5b, c). G2 and G3 form part of the switch regions that coordinate GTP binding and hydrolysis, while C1 connects them to the C2–fMet–tRNA^{fMet} interaction^{3,23}. None of the predicted contacts involve the key residues of the GTPase active site, indicating that the aptamer engages the G domain surface without directly occluding the guanosine binding and hydrolysis

machinery. We explored two models that could account for the aptamer's effects: a steric model, where the aptamer competes directly with the tRNA and sterically blocks 50S joining, and an allosteric model, where the aptamer disrupts the inter-domain dynamics important for tRNA positioning. Overall, our kinetic data favours the allosteric model. If the aptamer blocked tRNA or 50S binding sterically, kinetic differences would be expected in both assays (Fig. 3, Fig. 4a, Table 2); instead, we observed only amplitude reductions. Moreover, the IC_{50} calculated with the amplitude dependence on Apt 311 concentration ($0.3 \pm 0.1 \mu M$) closely matches the affinity determined from MST analysis ($0.5 \pm 0.1 \mu M$), indicating that the aptamer binds IF2 independently of tRNA and does not compete for the same surface. IF2 behaves like a molecular switch as a function of the bound guanosine nucleoside, propagating conformational changes towards the C2 domain and contributing to tRNA accommodation^{8,23}. NMR analysis has shown that this signalling depends on inter-domain flexibility between the GTPase center and the C-terminal region³⁸. We propose that Apt 311, by binding at both the G and C1 domains, restricts this inter-domain flexibility, obstructing the conformational dynamics that couple GTP hydrolysis to fMet-tRNA^{fMet} release and P-site accommodation.

It is worth noting that the docking model also suggests an additional structural explanation for the reduced amplitude of 70S IC formation (Fig. 5d). Superposed onto a native 30S subunit (PDB 5LMV), the IF2-Apt 311 model showed no steric clash, consistent with the aptamer-bound IF2 being able to bind the 30S subunit (Fig. 2d, **Supplementary Fig. S6a-c**). However, when superposed onto three 70S structures (*E. coli*, PDB 3JCN; *P. aeruginosa*, PDB 7UNR and 7UNV), the aptamer clashed with the 50S subunit (Fig. 5d, **Supplementary Fig. S6d-f**). Therefore, Apt 311 could also reduce or limit late 70S IC inter-subunit rotations in addition to allosterically inhibit IF2.

Beyond the mechanisms proposed above, we considered additional variables that could modulate the aptamer's effect. First, IF2 is a GTPase whose global conformation is guanosine nucleotide-dependent: the apo, GTP-, GDP-, and non-hydrolysable GTPγS-bound states stabilize distinct inter-domain arrangements (**Supplementary Fig. S7a**)^{8,39,40}. From these, the extended GTP/GTPγS-bound form that precedes GDP-dependent IF2 compaction is the one most relevant during initiation^{22,23,41}. Because our model proposes that Apt 311 contacts the G-domain that coordinates GTP binding and hydrolysis, we asked whether its affinity for IF2 depends on the guanosine nucleotide-bound state of the protein. To test that, Apt 311-Cy5 binding was evaluated as a function of the nucleotide bound to IF2 (apo, GTP-, GDP-, and GTPγS), revealing a comparable binding affinity across all variables, indicating that the recognition is independent of IF2 conformation or that binding overcomes guanosine-dependent IF2 conformations (**Supplementary Fig. S7b**).

Nucleotide-independent binding is consistent with an allosteric mechanism in which Apt 311 perturbs IF2 inter-domain dynamics rather than locking a single conformational state. Guided by the PLIP contact map (**Supplementary Fig. S8**), we introduced transversion mutations at predicted interface positions (A16C, G20T) and a control site (G8T), each paired with a compensatory base-pairing change. Relative residual activity, measured by final fluorescence amplitude in IF2^Q binding to 30S-IF3-IF1⁴⁸⁸ complexes,

showed that A16C reduced activity by ~ 55%, while G20T and G8T retained 81% and 71%, respectively. Restoration of base pairing further impaired function and abolished activity in the A16C-T35G double mutant, indicating that structural folding and the nature of the bases in the aptamer further occludes key contacts with IF2⁴².

Aptamers have been widely applied as antimicrobial and diagnostic reagents^{43–45}, but not to probe the mechanism and the dynamics of essential translation factors. Apt 311 fills this unexplored research gap as a functional probe of IF2 during initiation complex assembly. Its binding is reversible and concentration-dependent, allowing quantitative titration of the effect (Fig. 3d), and kinetic assays with the aptamer capture consequences across multiple assembly steps that static structures cannot evaluate. The specific features of Apt 311 contrast with lamotrigine, a small-molecule drug reported to bind IF2 and perturb ribosome biogenesis²⁶. Although this compound does not inhibit global protein synthesis, it has been shown to affect translation by compromising the fidelity of initiator tRNA recruitment²⁵. Apt 311 instead binds IF2 independently of its nucleotide state (**Supplementary Fig. S7**) and acts directly on the initiation-complex pathway. These findings establish Apt 311 as a functional probe of IF2 and show that perturbing a defined surface of the factor is sufficient to impair the correct progression from 30S IC to 70S IC assembly during translation initiation.

Methods

Aptamer Selection

High-affinity aptamers against IF2 were selected from a bead-linked ssDNA library containing a 40-nt random core flanked by two 17-nt constant regions for amplification, using the X-Aptamer Selection platform (AM Biotechnologies, LLC, USA) as reported before⁴⁶. Selection buffer consisted of 1× PBS (pH 7.4) (#524650-1EA, Sigma-Aldrich, USA), 1 mM MgCl₂ (#M8266-1KG, Sigma-Aldrich, USA), and 0.05% Tween-20 (#P1379, Sigma-Aldrich, USA), supplemented with 0.5% BSA where indicated. The bead-linked ssDNA library was rehydrated in a 1.3 mL selection buffer (without BSA), heat-denatured at 95°C for 5 min, and slowly annealed at room temperature (RT) for 30 min. The library was first subjected to a counter-selection (negative selection) against magnetic Ni-NTA beads alone; sequences that bound the beads were discarded, and the unbound pool was retained. For the primary positive selection, 15 µg of *E. coli* IF2 was immobilized on 25 µL Dynabeads His-Tag Isolation and Pull-Down beads (#10103D, Thermo Fisher Scientific, USA) and incubated with the unbound pool for 90 min at RT under rotation. Bound aptamers were eluted with 1 N NaOH (#106469, Merck, USA) at 65°C for 30 min, neutralized with 2 M Tris-HCl unbuffered (#T5941, Sigma-Aldrich, USA), and buffer-exchanged by column purification resulting in the Start Pool. A secondary positive selection was then performed in solution under three different conditions: the Start Pool alone, incubation with 100 nM IF2-His tag, and incubation with Ni-NTA beads alone, as a negative control. The three resulting libraries (Start Pool, IF2-enriched pool, and negative control) were amplified by PCR and sequenced on the Ion Torrent platform. Enrichment was

analysed in silico based on enrichment ratios (IF2/Start Pool and IF2/negative control). The four highest-enriched sequences were selected as candidate aptamers and are listed in Table 1.

Oligonucleotide synthesis and modifications

Selected aptamers Apt 311 and Apt 312 were synthesized and purified by MacroGen (USA). Aptamers were synthesized without the flanking Forward 5' CAG GGG ACG CAC CAA GG 3' and Reverse 5'CCA TGA CCC GCG TGC TG 3' annealing regions. Poly-(T) tails were added as linkers in each extreme region resulting in a 50 nt sequence that includes the 40 nt aptamer core region. Apt 311 and Apt 312 had a 5' biotin chemical modification for ELONAs (Enzyme-Linked Oligonucleotide Assay), a 5' Cy5 fluorophore modification for MST assays and were used unmodified in Stopped-Flow assays. A random DNA aptamer was used as a control: 5'CAC TCG GAC GCT ATC ATC CAT CGT GTA TGC CGT CAT 3'. Aptamer mutations were made guided by the PLIP Analysis (**Supplementary Fig. S8**). All substitutions exchanged purine for a pyrimidine or the reverse. Single mutations target the interaction zone (A16C in Zone I, G20T at the Zone II perimeter) and outside it (G8T). For each DNA variant, a compensatory mutation was added (A16C-T35G, G20T-C31A, and G8T-C42A).

Enzyme-Linked Oligonucleotide Assay (ELONA)

Proteins were immobilized at 1 μ M on a NUNC MaxiSorp plate (#442404, Thermo Fisher Scientific, USA) in carbonate buffer (pH 9.6; 100 μ L per well) overnight at 4°C. The proteins used for the assay included IF2 α , IF2 γ , IF1, IF3, BSA, and 1F8. Plates were blocked with PBS containing 0.05% Tween-20 (PBST) supplemented with 5% milk for 2 h at room temperature (RT). 1 μ M biotinylated aptamers were prepared in PBS buffer with 0.05% Tween and 1 mM MgCl₂, heat-denatured at 95°C for 10 min and allowed to refold at RT for 15 min. Aptamer solutions were supplemented with milk powder to a final concentration of 5% and incubated on the coated wells for 1 h at RT with shaking. Bound aptamers were detected using streptavidin-HRP (1:1,000 dilution), followed by development with 1-Step Slow TMB-ELISA substrate (#34024, Pierce Biotechnology, USA). The reaction was stopped after 30 min with 2 N sulfuric acid, and absorbance was measured at 450 nm using a Synergy H1 Multi-Mode Reader (BioTek Instruments, USA). Four washes with PBST (200 μ L per well) were performed between each incubation step. The 1F8 Chagas antigenic protein was obtained as previously described (Peñaranda et al., 2025), and BSA was purchased from Bio-Rad (#5000206, USA).

MST

Microscale Thermophoresis (MST) assays were performed using the Monolith X (NanoTemper Technologies, Germany) with Monolith NT.115 Series Capillaries (#MO-K022, NanoTemper Technologies, Germany). All reactions were done in TAKM₇ buffer (20 mM Tris-HCl, pH 7.4; 70 mM NH₄Ac; 30 mM KCl; 7 mM MgCl₂). To assess aptamer-target interaction, a 10-point titration of IF2 α was carried out with

concentrations ranging from 10 μ M to 19.53 nM, while aptamer concentration was kept constant at 100 nM. Samples were incubated for 10 min and centrifuged for 5 min before capillary loading. Measurements were performed at 25°C using medium MST power and 20% excitation power. Data was collected using a cold region from – 1 to 0 s and a hot region from 4 to 5 s. Assays in the presence of nucleotides (GTP, GDP, and GTP γ S) were performed under the same conditions, with the corresponding nucleotide included in the buffer at a constant concentration of 100 μ M.

Production of recombinant proteins

Competent BL21 *E. coli* cells (Mix & Go, Zymo Research, USA) were transformed using pET24c harboring: *infA* (for IF1), *infA* D5C (for mutated IF1 Asp5Cys), *infB* (for IF2 α), *infC* (for IF3). All vectors were acquired commercially (GenScript, USA). For IF2 γ constructs, pProEX-HTb plasmids encoding IF2 γ wild type (IF2 γ) and IF2 γ G810C were provided by the laboratory of Dr. Gonzalez (Columbia University, NY, USA)⁸. Translation factors IF1, IF1 D5C, IF2 α , and IF3 were obtained as previously described⁴⁷. Purified proteins were dialyzed in storage buffer (50 mM Tris-HCl, pH 7.1, 200 mM NH₄Cl, 10% glycerol, 6 mM β -mercaptoethanol) or labelling buffer (12 mM HEPES, pH 8, 60% DMSO) for IF1 D5C, quantified, and stored at – 80°C until use.

IF2 γ and IF2 γ G810C were recombinantly expressed in BL21 *E. coli* cells and induced with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG; #R0392, Thermo Fisher Scientific, USA) at OD₆₀₀ = 0.8 for 2 h at 37°C. Cells were harvested by centrifugation at 5,000 x g for 10 min at 4°C. Resuspended cells were lysed by freeze-thaw cycles in lysis buffer (20 mM Tris-HCl pH 7.3, 300 mM NaCl, 10 mM imidazole, 2 mM β -mercaptoethanol), supplemented with 100 μ g/mL lysozyme and 170 μ g/mL PMSF. During the final thawing step, 40 μ g/mL DNase I was added. Membrane debris were removed by centrifugation at 11,000 x g for 45 min at 4°C.

IF2 γ and IF2 γ G810C were purified using a HisTrap™ FF Crude column (#17-5247-01, Cytiva, USA), pre-equilibrated with binding buffer (20 mM Tris-HCl pH 7.3, 300 mM NaCl, 10 mM imidazole, 2 mM β -mercaptoethanol). The column was washed with 2 CVs (column volume) of the binding buffer. Then, the column was washed with 5 CVs of wash buffer (20 mM Tris-HCl pH 7.3, 300 mM NaCl, 30 mM imidazole, 2 mM β -mercaptoethanol). Subsequently, the column was washed with 5 CVs of binding buffer supplemented with 500 mM NaCl and 10 mM imidazole. Finally, the protein was eluted with the elution buffer (20 mM Tris-HCl, 200 mM NaCl, 250 mM imidazole, 2 mM β -mercaptoethanol, pH 7.3). Selected fractions were concentrated using Amicon® Ultra 50 kDa centrifugal filters (#UFC9050, Merck, USA). Subsequently, proteins were dialyzed in storage buffer (50 mM Tris-HCl, pH 7.1, 200 mM NH₄Cl, 10% glycerol, 6 mM β -mercaptoethanol) for 16 h using a D-Tube™ Dialyzer Maxi 12–14 kDa (#71510-M, Merck, USA). Afterwards, both proteins were cleaved with TEV protease (Mendoza-Rojas et al., 2021). Cleavage assays were performed using 0.5 mg/mL purified IF2 γ or IF2 γ G810C in cleavage buffer (50 mM Tris-HCl pH 7.3, 50 mM KCl, 0.1% Triton X-100, 2 mM β -mercaptoethanol) at a 1:20 molar ratio (TEV protease:IF2 γ) at 4°C for 12 h. The reaction mixture was subsequently loaded onto a HiTrap™ Capto™ SP

ImpRes cation-exchange column (#17546851, Cytiva, USA) pre-equilibrated with low-salt buffer (40 mM Tris-HCl pH 7.3, 30 mM NaCl, 40 mM NH₄Cl, 5 mM MgCl₂, 2 mM β-mercaptoethanol) using an ÄKTA™ Start FPLC system (Cytiva, USA). The column was washed with 2CV of low-salt buffer, and the cleaved IF2γ was eluted with high-salt buffer (40 mM Tris-HCl pH 7.3, 750 mM NaCl, 40 mM NH₄Cl, 5 mM MgCl₂, 2 mM β-mercaptoethanol) using a linear gradient from 0-100% over 30 CV. Fractions were collected at 4°C and analysed by 10% SDS-PAGE. Fractions containing IF2γ and IF2γ G810C proteins were selected based on quantity and purity and concentrated using Amicon® Ultra 50 kDa centrifugal filters (#UFC9050, Merck, USA). Subsequently, IF2γ was dialyzed in storage buffer, while IF2γ G810C was dialyzed in labelling buffer, both for 16 h. Protein concentration was determined using SDS-PAGE and Bradford assay analysis.

Protein Labelling

IF1 D5C was labelled using a 10-fold molar excess of Atto-488 maleimide (#A10254, Atto-Tec GmbH, Germany). The labelling reaction was carried out for 45 min at room temperature under gentle agitation and quenched by the addition of 6 mM β-mercaptoethanol. Excess unreacted dye was removed by cation-exchange chromatography on a HiTrap SP HP column, as described above for factor purification. IF2γ G810C was incubated with a 10-fold excess of TCEP (Tris(2-carboxyethyl)phosphine) at room temperature for 30 min and then mixed with 10-fold excess of Atto-540Q maleimide fluorophore (AD540Q, Atto-Tec GmbH, Germany). The labelling reaction, protected from light, was incubated at 4°C for 16 h and subsequently stopped by dilution in 6 mM β-mercaptoethanol low-salt buffer. The solution was loaded onto a HiTrap™ Capto™ SP ImpRes ion exchange chromatography column and eluted with high-salt buffer. The eluted fractions containing the quencher labelled protein were dialyzed in a Storage Buffer at 4°C for 16 h. Donor-labelled IF1 and quencher-labelled IF2γ (IF2^Q), were quantified by Bradford assay and 10% SDS-PAGE using ImageJ software (NIH), both using a BSA-based standard curve. The labelling efficiency was measured by absorbance on the Nanodrop One (Thermo Fisher Scientific, USA), and then stored at -80°C.

Ribosomal subunits, mRNA, fMet–tRNA^{fMet}, and fluorescent-labelled initiator tRNA

Ribosomal subunits (30S and 50S) and fMet–tRNA^{fMet} were obtained as detailed in Milón et al., 2007³⁵. The concentration of ribosomal subunits was determined by measuring the absorbance at 260 nm (A₂₆₀), using an extinction coefficient ε of 67 and 27 pmol/A₂₆₀ U for 30S and 50S, respectively. The mRNA model used was based on the *infC* gene and chemically synthesized (Microsynth, Switzerland).

The fMet–tRNA^{fMet} was aminoacylated and formylated with 5 mM ATP, 5 mM phosphoenolpyruvate, 0.025 µg/µl pyruvate kinase, 0.005 µg/µl methionine, methionyl-tRNA synthetase, formyl transferase, and 0.5 mM 10-formyl-tetrahydrofolate, pH 8, freshly prepared. The reaction was incubated at 37°C for 30

min and purified by salting out and chromatographic purification. fMet-tRNA^{fMet} was quantified by measurements at A₂₆₀. For fMet-tRNA^{Flu} labelling, fMet-tRNA^{fMet} was mixed with 35-fold excess of 5-iodoacetamidofluorescein (5-IAF) in labelling buffer (12 mM HEPES, pH 8, 60% DMSO) and incubated for 2 h at 50°C. The reaction was stopped with potassium acetate, pH 5.4, and the labelled tRNA was recovered by ethanol precipitation. The quantification was measured at A₂₃₃ nm considering an extinction coefficient of 25 mM⁻¹ cm⁻¹. Bpy-Met-tRNA^{fMet} were provided by the laboratory of Dr. Andrey Konevega and was prepared as Milon et al., 2007³⁵.

Rapid kinetics assays

Fluorescence and kinetic measurements were performed using the Stopped-Flow SX20 apparatus (Applied Photophysics, UK), which rapidly mixed equal volumes of each reactant solution. The excitation wavelength for donor fluorophores (Atto-488, fluorescein, Bodipy) was 470 nm, provided by the LED light source (Applied Photophysics, UK). The donor emission signal was detected by the photomultiplier through a 515 nm long-pass filter. All reactions were conducted in the TAKM₇ buffer (20 mM Tris-HCl, 70 mM NH₄Ac, 30 mM KCl, 7 mM MgCl₂, pH 7.4). The 30S subunits were activated in the TAKM₂₁ buffer (TAKM₇ with 21 mM MgCl₂) at 37°C for 30 min. The 30S subunits and donor fluorescent molecules were prepared at a 1:1 ratio at 0.1 μM per syringe. The remaining reaction components were used at a 3:1 ratio relative to the 30S subunit, except for GTP, which was prepared at equal concentrations of 100 μM in each syringe. Aptamers were denatured at 95°C for 5 min and then allowed to refold at room temperature for 15 min. Aptamer-containing reactant solutions were prepared at a concentration of 1 μM and were pre-incubated with IF2. All reactant solutions were centrifuged at 10,000 RPM for 10 min at 20°C before loading the instrument. Additionally, light scattering assays were performed in the Stopped-Flow apparatus, with the monochromator set at 430 nm and without an optical filter. A 3:1 molar ratio of 50S-30S ribosomes was considered, IFs, GTP, and aptamers were included as mentioned previously. A total of 1,000 data points were acquired per trace for all reactions. 5 to 7 replicates were recorded for each reaction and averaged prior to analysis.

Computational modelling

Computational modelling of the DNA aptamer was carried out in several stages. Because guanine-rich aptamers can adopt G-quadruplex folds that are not captured by duplex-based secondary-structure methods, the candidate sequence was first screened with several G-quadruplex prediction algorithms to rule out G-quadruplex formation⁴⁸. Having confirmed the absence of an appreciable G-quadruplex propensity, we predicted the secondary structure with VFold2D and the corresponding tertiary structure with VFold3D^{49,50}, using the default parameters throughout. Because the VFold programs are parameterized for RNA, the aptamer sequence was modelled as RNA during folding, and the resulting VFold3D model was subsequently converted from RNA back to DNA to provide the starting all-atom

structure. This structure was then refined by all-atom molecular dynamics in AMBER 24⁵¹, using the parmbsc1 force field for DNA⁵², the OPC water model⁵³, and the Li-Merz ion parameters⁵⁴. A single 1- μ s production trajectory was collected. Consensus secondary structure alignment was predicted using LocARNA under default parameters⁵⁵. Structural superposition between aptamer models was calculated using the Needleman-Wunsch-based alignment algorithm implemented in ChimeraX, minimizing the root mean square deviation (RMSD) between equivalent atomic pairs. The three-dimensional structure of IF2 was obtained from AlphaFold⁵⁶ using the NCBI reference sequence WP_000133044.1 as input. For docking and visualization, the first 290 N-terminal residues, comprising the entire N region, were removed (IF2 Δ N). Domain annotation, molecular visualization, and structural rendering were performed in UCSF ChimeraX (version 1.10.1).

Computational analysis

Protein-aptamer docking experiments were performed using the HDOCK Server⁵⁷ with default protein-nucleic acid docking parameters. Docking poses were ranked according to the HDOCK internal docking score, and the ten top-ranked models were selected for structural analysis. Models were visualized and aligned for structural comparison and interface inspection. The top-ranked model was structurally superposed onto ribosomal references using MatchMaker in UCSF ChimeraX, which aligns structures by sequence and secondary structure before fitting the matched backbone atoms. The IF2 chain of the docking model was superposed onto the IF2 chain of each reference, including the 30S initiation complex (PDB: 5LMV) and 70S ribosome structures from *Escherichia coli* (PDB: 3JCN) and *Pseudomonas aeruginosa* (PDB: 7UNR and 7UNV), to evaluate structural overlap.

Protein-aptamer contacts were analysed in PyMOL (Schrödinger, LLC) using a frequency-based residue contact approach across the ten selected docking models. Atomic contacts were defined using a distance cutoff of ≤ 4.0 Å. Residues showing at least one atomic contact in any docking model were classified as interaction points. Hotspots were defined using two selection criteria: (i) residues with contact occupancy $\geq 50\%$ across the docking ensemble (min_frac = 0.50), and (ii) the ten residues with the highest absolute contact frequencies (top_n = 10). The final hotspot set was obtained by combining both criteria. Noncovalent interactions were predicted from the top-ranked HDOCK model, used without further refinement, with the PLIP web server⁵⁸ under default parameters. Predicted hydrogen bonds, salt bridges, and π -cation contacts were mapped onto the aptamer sequence and grouped by position into three contact zones, I to III (**Supplementary Fig. S6**).

Data Analysis

Statistical analyses were performed in GraphPad Prism (version 10.6.1; GraphPad Software, USA). Group comparisons were analysed by one-way ANOVA followed by Dunnett's multiple-comparison test. Statistical significance was defined as $P < 0.0001$ (****). Binding affinities from microscale

thermophoresis (MST) assays were determined by fitting binding curves to a hyperbolic model: $Y = Y_0 + (B_{max} \cdot X) / (K_d + X)$ (Eq. 1).

Stopped-flow fluorescence traces were analysed by nonlinear regression using multi-exponential decay models (one or two-exponential terms, depending on the kinetic behaviour observed): $F = F_0 + F_1 \cdot \exp(-k_{app1} \cdot t)$ or $F = F_0 + F_1 \cdot \exp(-k_{app1} \cdot t) + F_2 \cdot \exp(-k_{app2} \cdot t)$; where,

F is the measured fluorescence at any given time (t);

F_0 is the initial fluorescence;

F_1 and F_2 are the fluorescence amplitudes corresponding to steps 1 and 2 respectively;

k_{app1} and k_{app2} are the apparent rates associated to steps 1 and 2 respectively; and

t is the time in seconds

Declarations

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Author contributions statement

P.M. and K.P. conceived the project. P.M., K.P., A.S-C., R.S., and D.S.V. acquired funding. A.L.K, A.P. and P.M. supervised the project. L.T., A.S-C., J. R., K.P., V.B., P.D., and D.S.V. conducted experiments/analysis. A.S-C, K.P., L.T., V.B., and J. R. prepared figures. All authors analysed and discussed the results. A.S-C., K.P., and P.M. wrote the original draft. All authors reviewed the manuscript. A.S-C., K.P., and P.M. wrote the final manuscript with the input from all authors.

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Data availability

All relevant data supporting the findings of this study, including the sequences of the aptamers identified in this work, are included in the manuscript and its Supplementary Information. The custom scripts used to identify the binding hotspots from the docking ensemble are available at https://github.com/PCMEKAPE/Laboratory-of-Biomolecules_GAPT. Additional methodological details are available upon request from the corresponding author.

Additional information

Competing interests: Ana Sanchez-Castro, Katherin Peñaranda, and Pohl Milón are inventors on patent 000428-2019/DIN (Peru) covering the aptamers described in this study. The remaining authors declare no competing interests.

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Figures

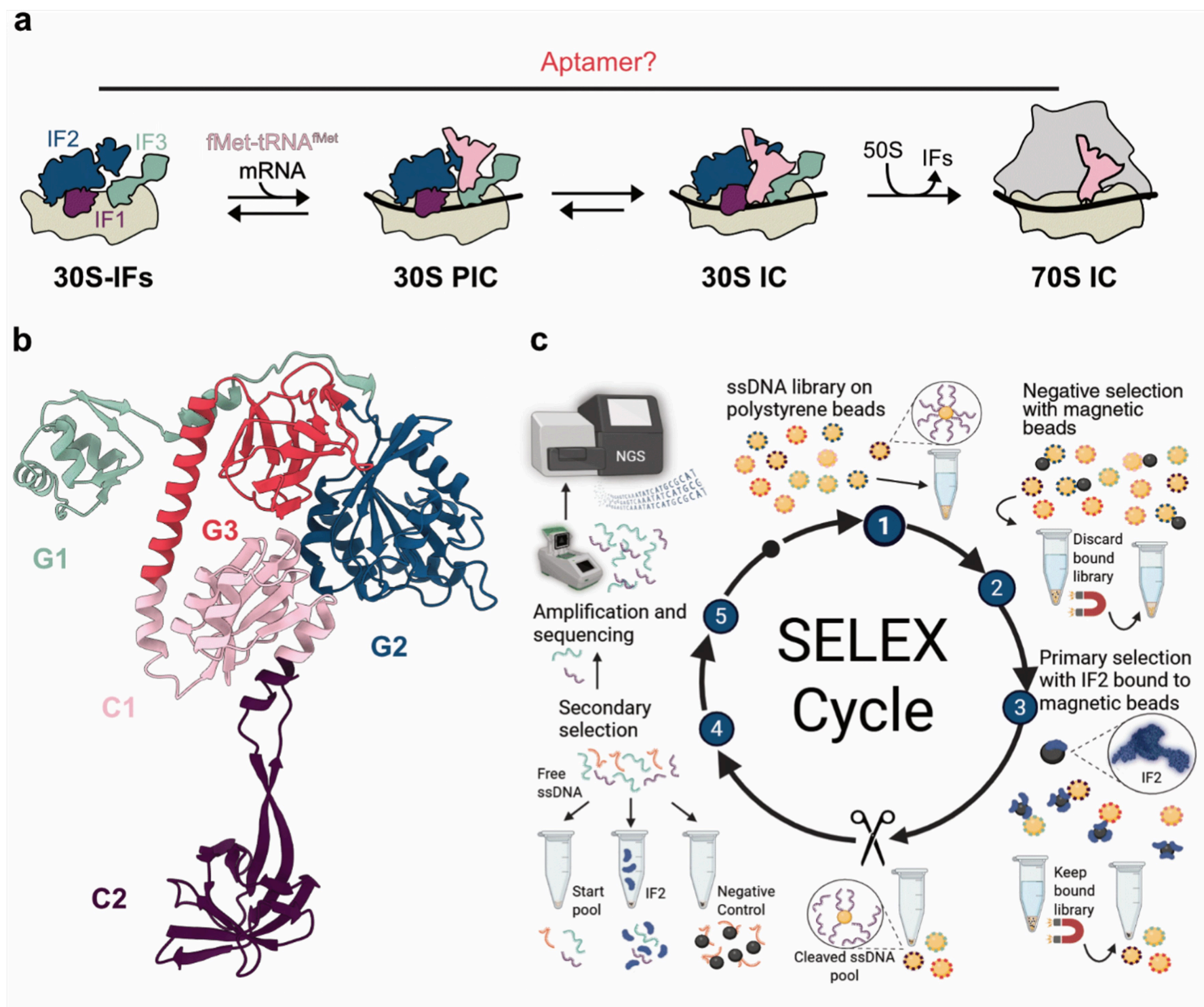


Figure 1

IF2 in bacterial translation initiation and SELEX strategy. (a) Representation of the main steps in bacterial translation initiation. The 30S–IFs complex is composed of the 30S ribosomal subunit (beige) and the initiation factors, IF1 (purple), IF2 (blue), and IF3 (green). Once the fMet–tRNA^{fMet} (pink), and the mRNA (black) bind, there are two distinct intermediates: the 30S PIC (pre-initiation complex) and the 30S IC differing only by pairing up the mRNA's initiation codon with the fMet–tRNA^{fMet}'s anticodon. The 30S IC intermediate unlocks 50S (grey) binding and IFs' dissociation, forming the 70S initiation complex (70S IC). (b) Schematic representation of *E. coli* IF2 lacking the N-terminal domain (IF2ΔN) and its domain architecture. The three-dimensional structure of the protein was generated using AlphaFold (<https://alphafold.ebi.ac.uk>), molecular visualization and rendering were performed using UCSF ChimeraX version 1.10.1 (<https://www.rbvi.ucsf.edu/chimerax/>). (c) Aptamer selection and identification by SELEX. (1) A ssDNA library bound to polystyrene beads (yellow) is used for three consecutive steps

during the selection. (2) A negative selection with magnetic Ni–NTA beads (black), in which bound library beads are discarded. (3) A primary positive selection with the protein of interest, 6XHis–IF2 (green), in which library beads that bind to the protein are retained. (4) A secondary positive selection in solution under three different conditions using cleaved libraries (indicated by the scissor). The start pool does not include IF2–His tag or Ni–NTA beads, while the negative control evaluates nonspecific binding to Ni–NTA beads. (5) Finally, all DNA fragments obtained by the selections steps were amplified by polymerase chain reaction (PCR) and sequenced by next-generation sequencing (NGS). The relative abundance of each sequence along SELEX was used to rank and identify aptamer candidates. This illustration was created in BioRender. <https://BioRender.com/yfhl44s>.

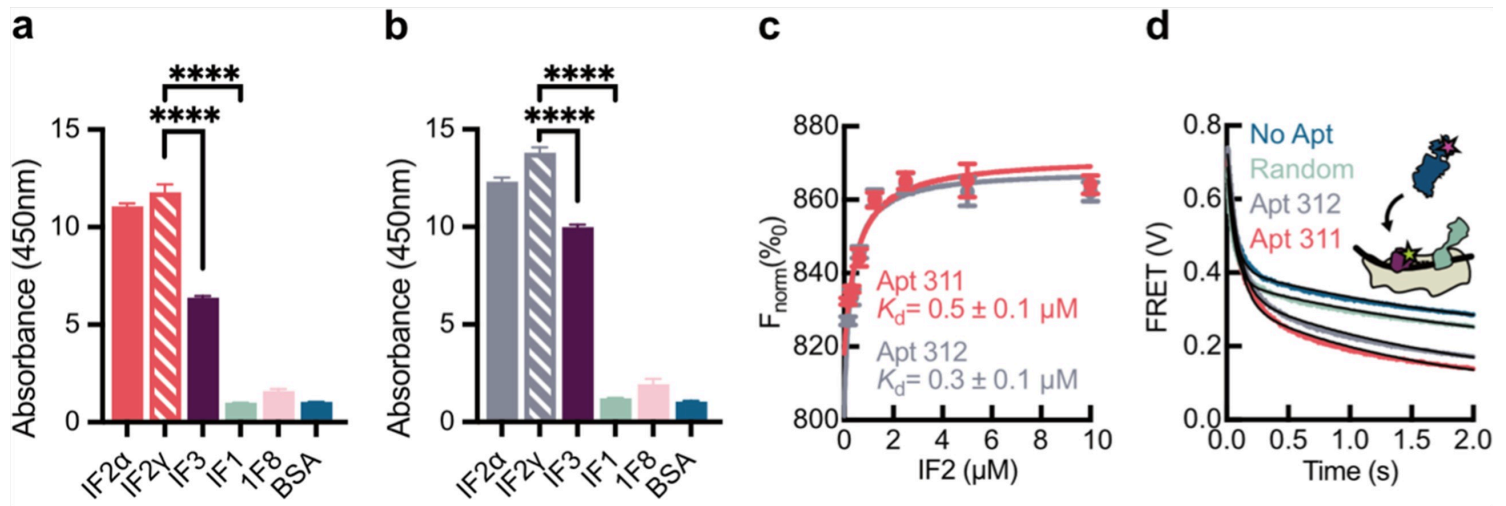


Figure 2

Affinity and specificity evaluation of aptamers 311 and 312. (a) Evaluation of binding of aptamer 311 to different IF2 protein isoforms, IF2 α and IF2 γ . In this assay, 1 μ M of purified proteins was immobilized onto a solid surface, and aptamer binding was detected through a streptavidin–HRP enzymatic reaction using TMB (3,3',5,5'-tetramethylbenzidine) as substrate. Negative control proteins included the bacterial initiation factors IF1 and IF3, as well as proteins unrelated to translation (1F8 and BSA). Absorbance was measured at 450 nm after 30 min of incubation, and it was normalized to the non-aptamer control. Data represent mean $\pm$ s.d. from triplicate experiments. Statistical analysis was performed using one-way ANOVA (**** $p \leq 0.0001$) (b) As in (a) but using Apt 312. (c) Microscale Thermophoresis (MST) assays of Apt 311 and Apt 312 were performed by titrating IF2 α against Cy5-labelled aptamers. The normalized fluorescence ($F_{norm} \%$) was used for K_d determination by a non-linear hyperbolic fitting. Error bars represent standard deviations (s.d.) from 4 replicates. (d) Time traces of IF2 binding to a 30S–IF3–IF1 complex measured by Förster Resonance Energy Transfer (FRET). IF2 was labelled with a quencher fluorophore (fuchsia) and IF1 with a donor fluorophore (green). Apt 311 (red), Apt 312 (grey) or no aptamer (blue) were incubated with IF2 before mixing with the 30S–IF3–IF1 complex. A random DNA control (green) was included. Five to seven replicates were averaged.

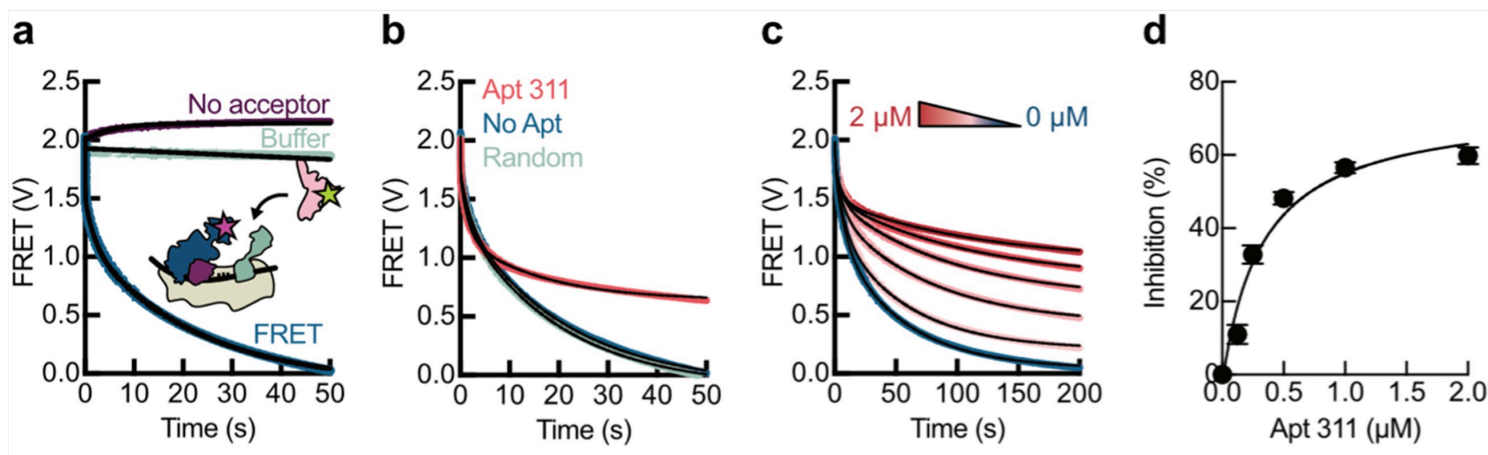


Figure 3

Aptamer 311 allosterically prevents IF2 from transitioning towards the 30S IC. (a) FRET signal controls for the fluorescently labelled fMet-tRNA^{fMet} (fMet-tRNA^{Flu}) binding to the 30S pre-initiation complex (30S PIC) containing a quencher-labelled IF2 (blue). A non-acceptor FRET control was performed using an unlabeled IF2 (purple), and a mixing signal control using only the donor mixed with buffer in the stopped flow apparatus (green). (b) Time traces of fMet-tRNA^{Flu} binding to the 30S PIC monitored by FRET. The 30S PIC was pre-incubated with Apt 311 (red) or a random DNA control (green) or in the absence of any (blue). (c) Initiator tRNA binding as a function of Apt 311 concentration. Time traces of fMet-tRNA^{Flu} binding to the 30S PIC as a function of Apt 311 concentration (0-2 μM). (d) fMet-tRNA^{Flu} inhibition as a function of Apt 311 concentration. A dose-response model was used to determine the IC₅₀. For panels a-c, 5-7 replicates were averaged and the non-linear fits are shown as black lines. Error bars in d represent standard deviations (s.d.) from 5-7 replicates.

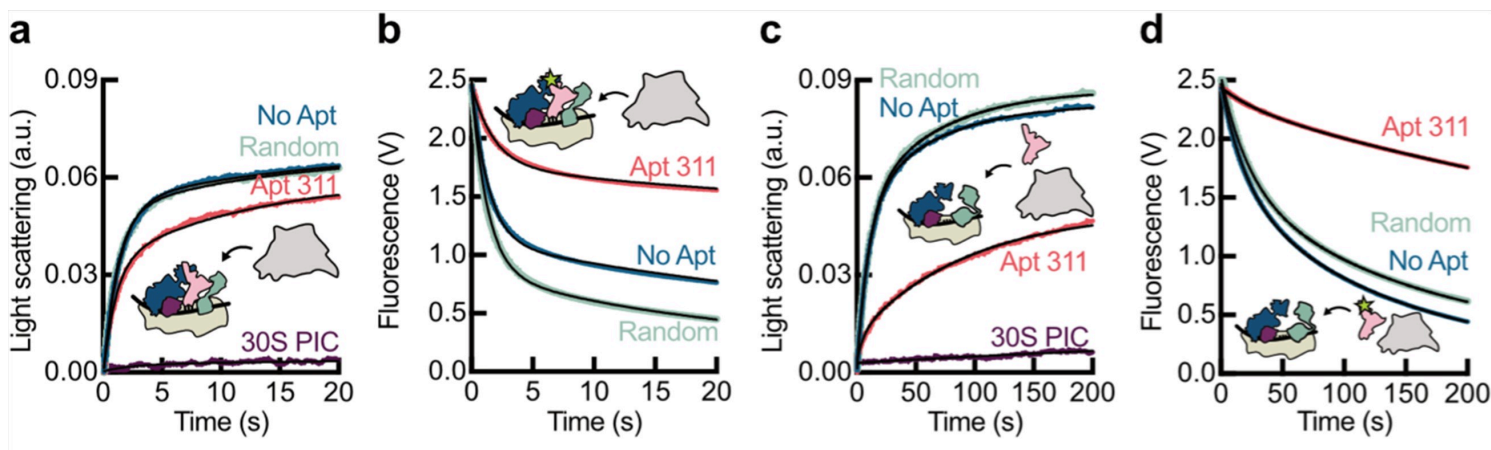


Figure 4

Aptamer 311 decreases IF2-dependent 70S IC formation. (a) Time traces of 50S subunit binding to a pre-formed 30S initiation complex (30S IC) monitored by light scattering. (b) Similar to the panel a to measure initiator tRNA accommodation, monitored by fluorescence using Bpy-Met-tRNA^{fMet} as the

reporter. **(c)** Time traces of the concomitant binding of fMet-tRNA^{fMet} and 50S subunit to the 30S pre-initiation complex (30S PIC, no fMet-tRNA^{fMet}), monitored by light scattering. **(d)** Similar to panel **c**, but monitored by fluorescence using Bpy-Met-tRNA^{fMet} as the reporter. For reactions containing Apt 311, the aptamer was pre-incubated with the 30S PIC or 30S IC (red). The reactions were compared to controls without aptamer (blue) or a random DNA (green). For all panels, 5-7 replicates were averaged and a two-exponential equation was used.

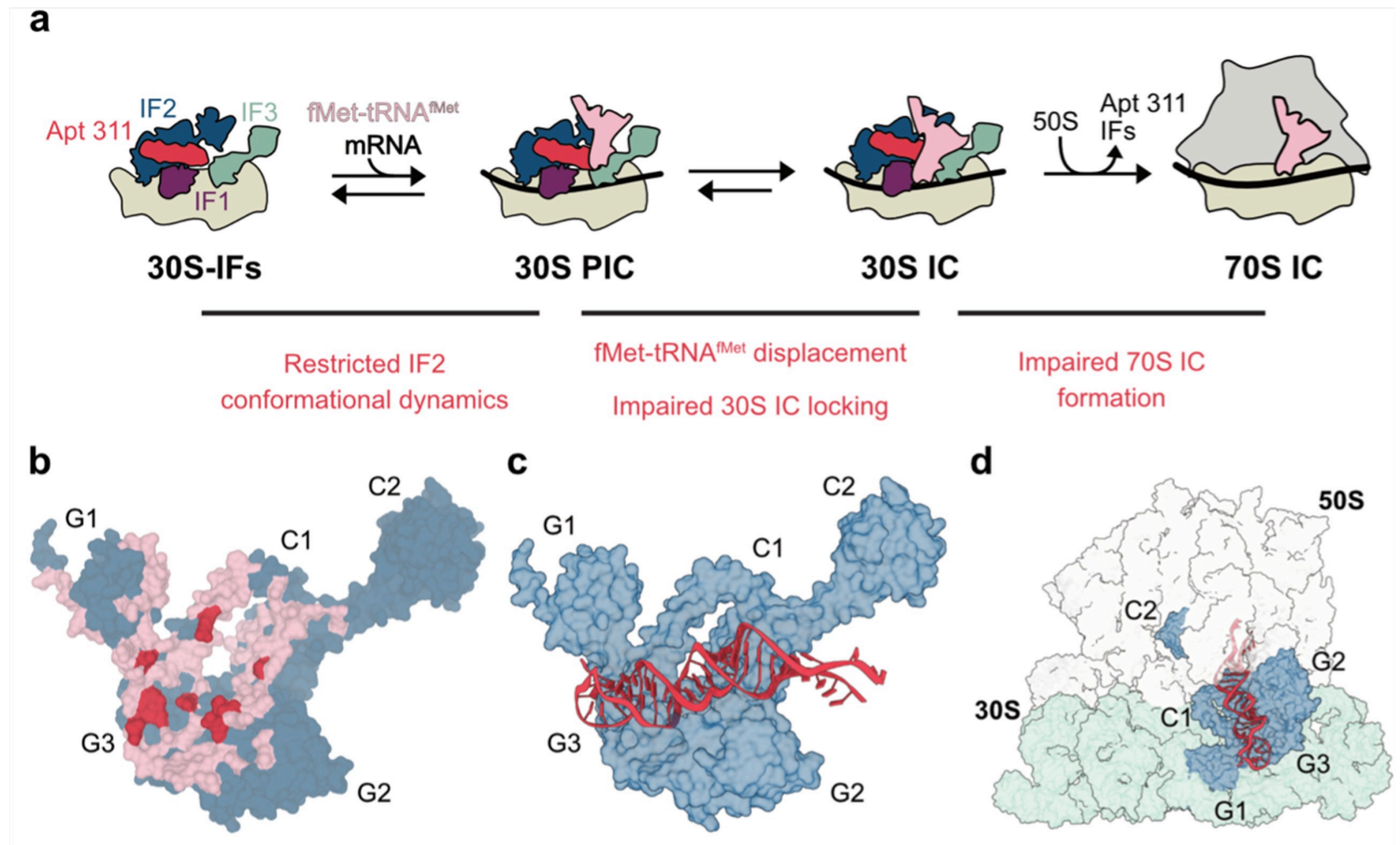


Figure 5

Interaction model and impact of Apt 311 on translation initiation dynamics. **(a)** Schematic representation of main Apt 311-mediated disruptions in different stages of translation initiation: 30S pre-initiation complex (30S PIC), 30S initiation complex (30S IC), and 70S initiation complex (70S IC). Components are coloured as in Fig. 1a, with Apt 311 in red. **(b)** Schematic representation of *E. coli* IF2 (blue) highlighting interaction points with partner molecules (pink) and predicted binding hotspots (red), mapping to the G domain and the C1 domain, derived from the top ten docking models. Structural representations and annotations were generated using PyMOL version 3.0 **(c)** Visualization of the highest-ranked docking interaction model between IF2 (blue) and aptamer 311 (red). The three-dimensional structure of (IF2 Δ N) was predicted using AlphaFold, based on the NCBI Reference Sequence WP_000133044.1 (<https://alphafold.ebi.ac.uk>). **(d)** Structural alignment of the top docking model with full 70S ribosome (PDB: 3JCN; pruned RMSD = 0.982 Å) reveals steric incompatibility with

the 50S subunit. In **(c)** and **(d)**, the complex was generated by molecular docking using HDOCK and visualized in UCSF ChimeraX (version 1.10.1).

Supplementary Files

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