Supplemental Information

Ribosome Assembly Defects Subvert Initiation Factor3 Mediated Scrutiny of Bona Fide Start Signal

Himanshu Sharma and B. Anand*

Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, INDIA

***Corresponding Author**

Email: banand@iitg.ac.in

Phone: +91-361-2582223

Fax: +91-361-2582249

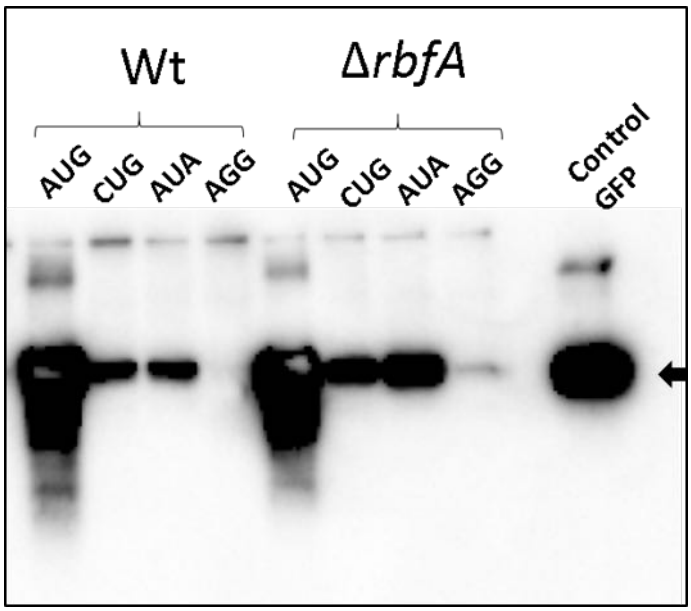

Figure S1: Assembly defects compromise the recognition of the start codon during translation initiation

Level of GFP production conferred by various start codons in Wt and Δ*rbfA*, measured by immunoblotting using an anti-GFP antibody is presented. The respective start codons are indicated on top of each lane and purified GFP is loaded in the control GFP lane. Bands corresponding to GFP are indicated with an arrow on the right. Cell lysates corresponding to a total of 30 µg protein was loaded in each lane after normalization.

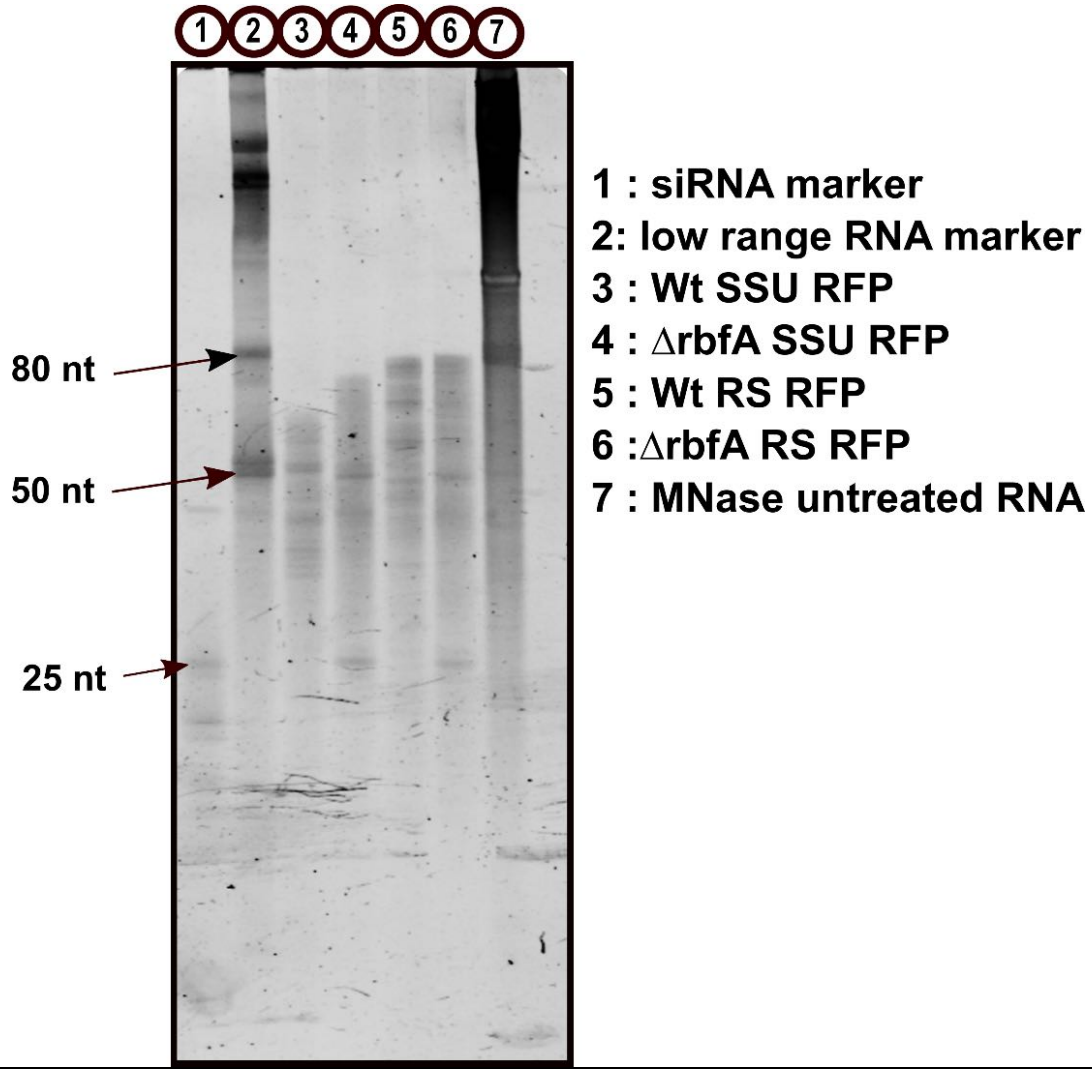

Figure S2: Purification of FPs from 30S and 70S fractions

Ribosome footprints purified from 30S and 70S fractions of WT and Δ*rbfA*. RNA size standards (siRNA Markers and low range marker) are loaded for comparison. RNA isolated from Wt 70S fraction not treated with MNase is also loaded for comparison in the last lane.

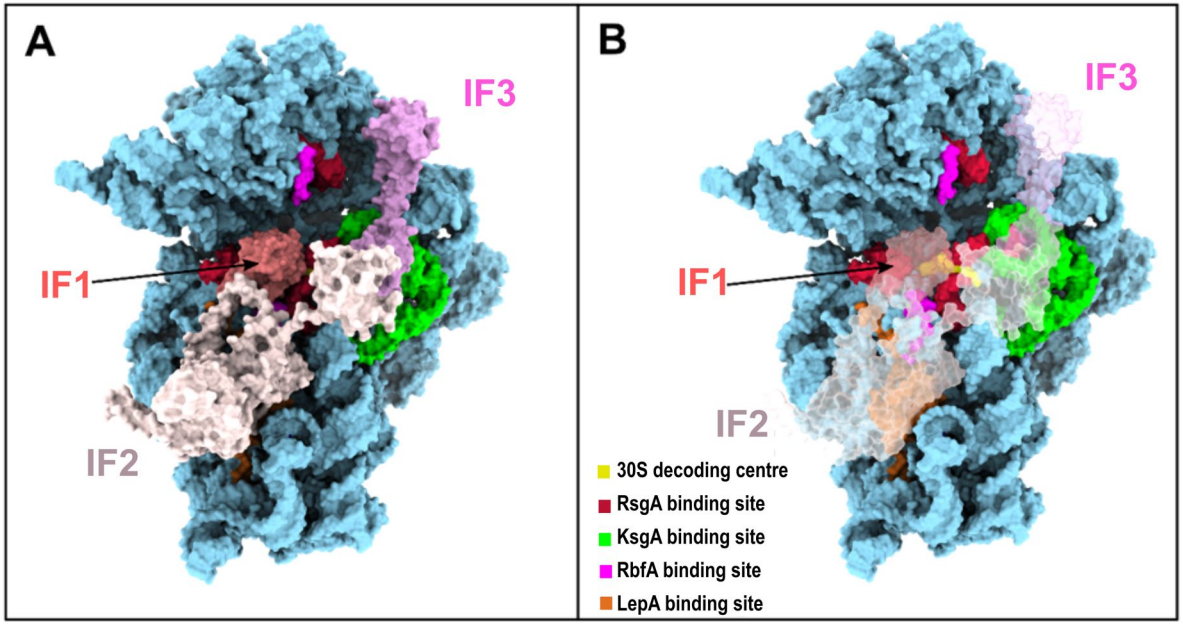

Figure S3: Assembly factors and Initiation factors bind to overlapping regions on the 30S subunit

(A) A surface representation of the 30S subunits (shown in sky blue) bound to the three initiation factors IF1(Coral red), IF2 (Beige) and IF3 (Rose Pink) as observed by Cryo-EM studies is presented (PDB ID: 5LMV) (1).

(B) The 30S subunit bound to initiation factors (PDB ID: 5LMV) is marked for the sites of late stage assembly factors and the 30S decoding center. The initiation factors are shown in a transparent surface representation and the colour coding for assembly factor binding sites are also indicated.

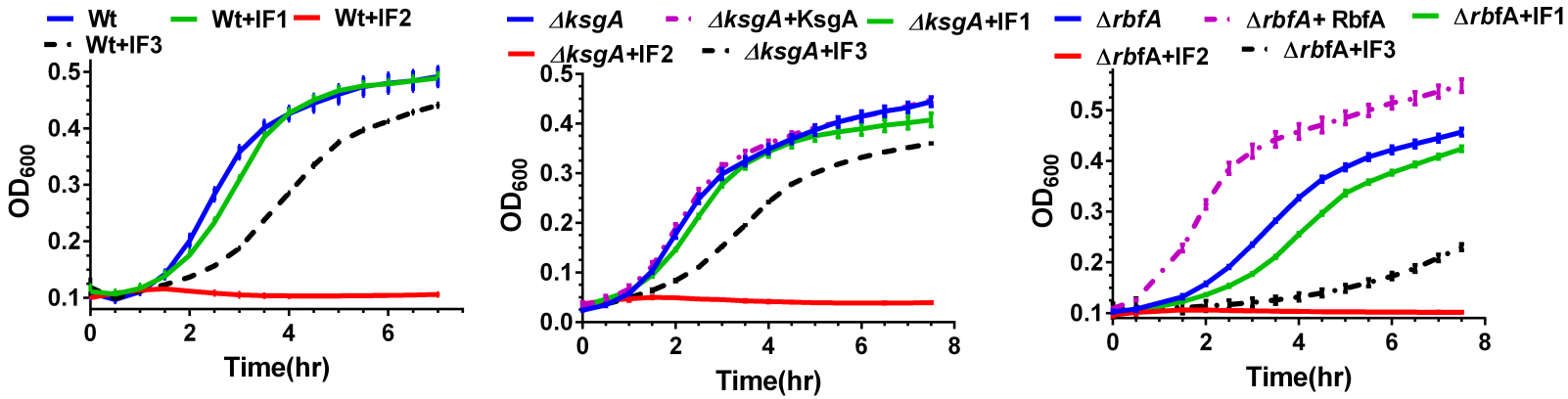

Figure S4: Overproduction of IFs does not provide a growth advantage

Growth comparison for Wt **(A)**, *ΔrbfA* **(B)** and *ΔksgA* **(C)** transformed with a vector carrying genes encoding IF1, IF2, IF3, RbfA and KsgA. Protein production from the respective plasmids was induced at time = 0.

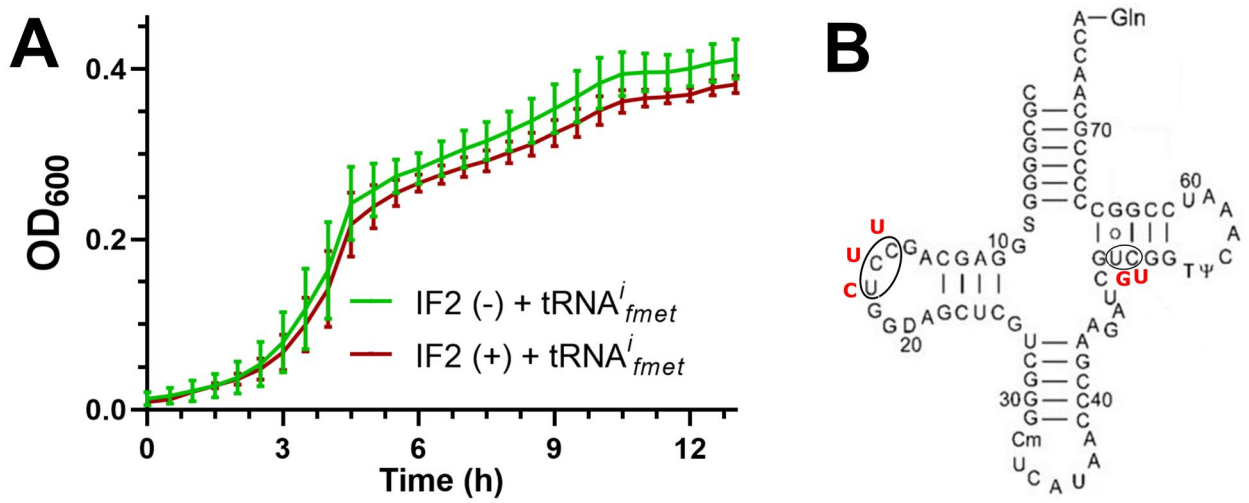

Figure S5: Suppressor mutations rescue the toxic effects of IF2 overproduction

(A) Growth analysis of Wt cells transformed with vectors isolated from strains rescued upon co-expression of genes encoding IF2 and tRNA$^{i}_{fmet}$. Transformed cells were induced for IF2 overproduction at t = 0, whereas tRNA$^{i}_{fmet}$ production was constitutive.

(B) Sequencing analysis of the plasmid borne gene encoding tRNA$^{i}_{fmet}$ isolated from rescued cells as used in (A). The Wt *E. coli* tRNA$^{i}_{fmet}$ is shown along with the mutated sequences. Mutated residues are highlighted with black outlines and the corresponding mutations are shown in red.

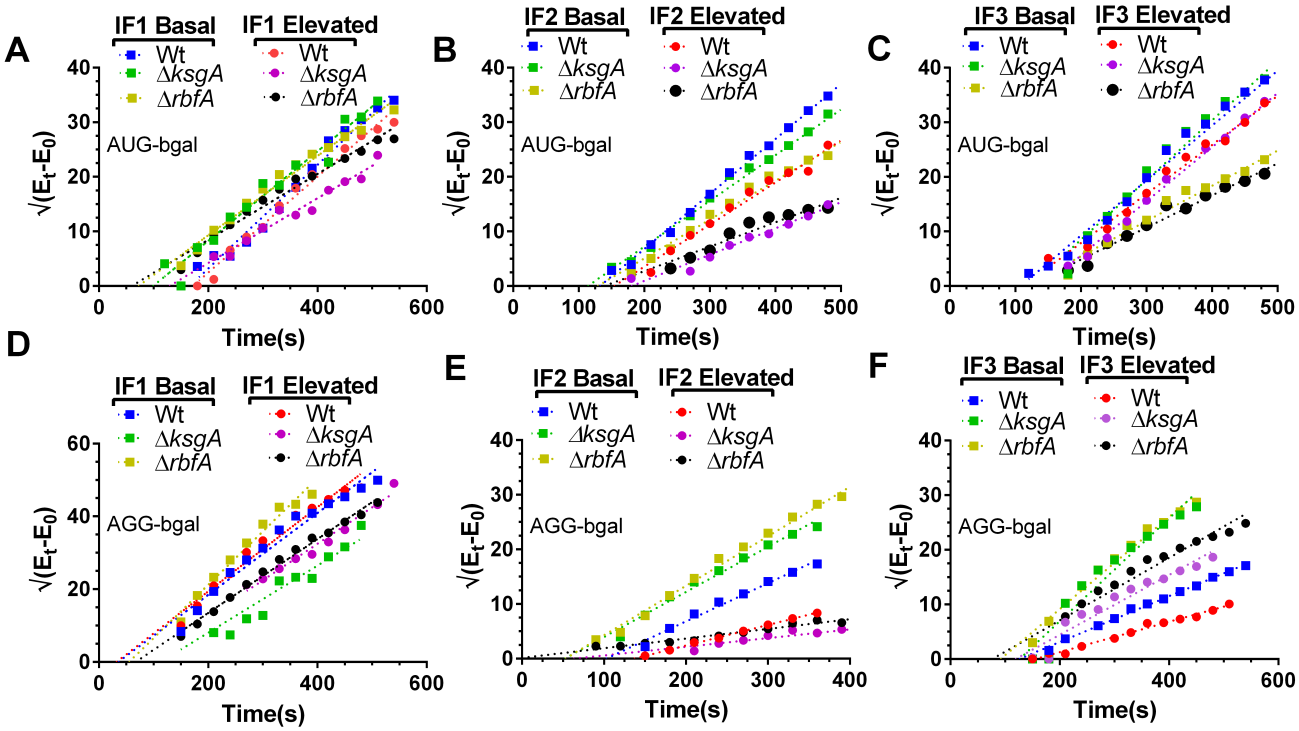

Figure S6: Elevated concentrations of Initiation factors prohibit translation initiation by premature ribosomes

Schleif plots for bgal translation kinetics from an AUG-bgal or AGG-bgal mRNA, measured in Wt, *ΔrbfA* and *ΔksgA* with basal or elevated cellular concentrations of IF1 (**A, D**), IF2 (**B, E**) and IF3 (**C, F**), respectively. Translation kinetics measurements were taken after elevating the cellular concentration of the respective IFs (*vide* Methods). The rise in bgal production over time was fitted to a linear regression model to derive the slope of each plot and this slope indicated the respective translation initiation rate (IR). The plots were drawn from the averaged measurement from three independent time course experiments. A ratio of the translation initiation rates derived from these plots are presented in Figure 7.

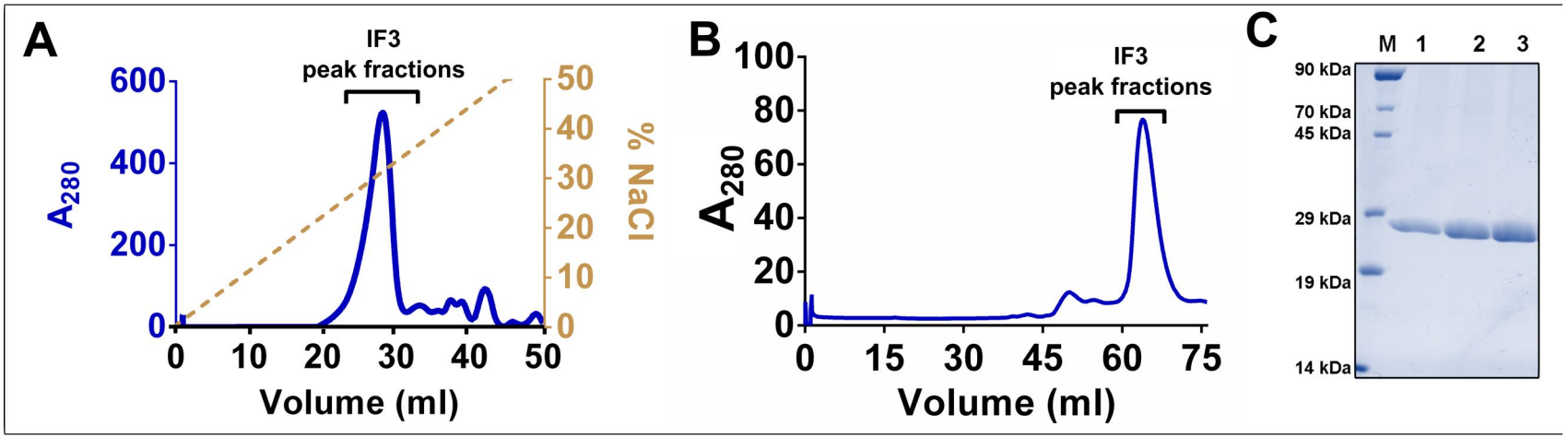

Figure S7: Purification of IF3

(A) A chromatogram depicting the purification of Initiation factor3 using ion exchange chromatography. The elution was performed using a linear NaCl gradient (*vide* Methods). The indicated peak fractions were pooled and further polished using gel filtration chromatography.

(B) A chromatogram representing size exclusion chromatography for pooled peak fractions of IF3 (from **A**). The indicated fractions were checked for homogeneity and used for binding with ribosomes.

(C) An SDS-PAGE analysis to check the purity of IF3 is presented. Purified fractions are indicated above the gel and protein size standard is loaded in the lane indicated as “M”. Molecular weights of the respective bands are indicated on the left.

Table S1. List of strains and plasmids used in this study

Strain/ Plasmids	Description	Source
BW25113 (Wt) (CGSC #7636)	*F-,Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Coli genetic stock centre (CGSC)
ΔrsgA	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔrsgA::kan*, *hsdR514*	This paper
ΔrbfA	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔrbfA::kan*, *hsdR514*	This paper
ΔksgA	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔksgA::kan*, *hsdR515*	This paper
ΔlepA	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔlepA::kan*, *hsdR516*	This paper
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU, galK, rpsL endA1, nupG, StrR	Invitrogen
B wild type (CGSC # 5365)	*lon-, malB-, dcm-*	Coli genetic stock centre
TKC	*E.coli* W3110 derivative, used for amplifying antibiotic selection markers	A kind gift from Donald. L.Court
pKD46 (CGSC#7739)	ori R101, repA101ts, AmpR, araC, expresses λ Red genes (gam-bet-exo) under the control of arabinose inducible promoter (pBAD).	Coli genetic stock centre (CGSC)
pET His6 GFP TEV LIC vector (1GFP) (Addgene plasmid #29663)	ori pMB1, KanR, lacI, expresses an enhanced version of *gfp* under the control of an IPTG inducible promoter (PT7lac).	Scott Gradia , Addgene plasmid #29663
pBAD Strep TEV LIC vector (8R) (Addgene plasmid # 37506)	ori pBR322,AmpR ,araC, araO2O1, N terminal strep tag fused to protein of interest expressed under control of pBAD promoter	Scott Gradia , Addgene plasmid
pAUG-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with* AUG start codon expressed under control of pBAD promoter	This paper
pCUG-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with C*UG start codon expressed under control of pBAD promoter	This paper
pAUA-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with* AUA start codon expressed under control of pBAD promoter	This paper

pAGG-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with* AGG start codon expressed under control of pBAD promoter	This paper
pPlus1-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with* AUG start codon and a +1 frameshift at 7th codon, expressed under control of pBAD promoter	This paper
pMinus1-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with* AUG start codon and a -1 frameshift at 7th codon, expressed under control of pBAD promoter	This paper
pUAA7-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with* AUG start codon and a premature UAA stop codon at the 7th codon, expressed under control of pBAD promoter	This paper
pUAG8-GFP-8R	ori pBR322,AmpR ,araC, araO2O1, *gfp with* AUG start codon and a premature UAG stop codon at the 8th codon, expressed under control of pBAD promoter	This paper
pQE2	ori ColE1, AmpR, expresses gene of interest to synthesize N-terminal 6xHis tagged protein under the IPTG inducible T5 promoter.	Qiagen
pAUG-bgal	pQE2 carrying a *lacz* gene with a AUG start codon under the IPTG inducible T5 promoter.	This paper
pAGG-bgal	pQE2 carrying a *lacz* gene with a AGG start codon under the IPTG inducible T5 promoter.	This paper
pRsgA-15A	ori p15A, CamR, tetR, *rsgA* under the control anhydrotetracycline inducible promoter (PLtetO-1).	This paper
pRbfA-15A	ori p15A, CamR, tetR, *rbfA* under the control anhydrotetracycline inducible promoter (PLtetO-1).	This paper
pKsgA-15A	ori p15A, CamR, tetR, *ksgA* under the control anhydrotetracycline inducible promoter (PLtetO-1).	This paper
pLepA-15A	ori p15A, CamR, tetR, *lepA* under the control anhydrotetracycline inducible promoter (PLtetO-1).	This paper
pIF-1-15A	ori p15A, CamR, tetR, *infa* under the control anhydrotetracycline inducible promoter (PLtetO-1).	This paper
pIF-2-15A	ori p15A, CamR, tetR, *infb* under the control anhydrotetracycline inducible promoter (PLtetO-1).	This paper
pIF-3-15A	ori p15A, CamR, tetR, *infc* under the control anhydrotetracycline inducible promoter (PLtetO-1).	This paper
pMetZ	pQE2 carrying the *metZ* gene under an *lpp* promoter.	This paper
p1R	T7-lac inducible, ColE1 origin plasmid for expressing genes with N-terminally fused Strep-II tag.	Scott Gradia Addgene plasmid #29664
p1R-IF3	T7-lac inducible, ColE1 origin plasmid for expressing *infC* with N-terminally fused Strep-II tag.	Scott Gradia Addgene plasmid #29664

Table S2. List of oligonucleotides used in this study

Oligo name	Sequence (5' to 3')	Description
C1FP_GFP_8R	TTTTTTGGGCTAACATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG GGTTCTTCTCACCATC	Amplifying *gfp* with an AUG start codon for cloning in 8R
URP_GFP_8R	CAAAACAGCCAAGGGATCCTTATCCACTTCCAATATTG	Amplifying *gfp* for cloning in 8R
C2FP_GFP_ 8R	TTTTTTTGGGCTAACATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATCT GGGTTCTTCTCACCATC	amplifying *gfp* with an CUG start codon for cloning in 8R
C3FP_GFP_8R	TTTTTTTGGGCTAACATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATAT AGGTTCTTCTCACCATC	Amplifying *gfp* with an AUA start codon for cloning in 8R
C4FP_GFP_8R	TTTTTTGGGCTAACATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATAGG GGTTCTTCTCACCATC	Amplifying *gfp* with an AGG start codon for cloning in 8R
C5FP_GFP_2	ATGGGTTCTTCTCACCATCATTAACACCATGGTTCTTCTGTGAGCA	Amplifying *gfp* with a premature UAA stop codon for cloning in 8R
C6FP_GFP_2	ATGGGTTCTTCTCACCATCATTAGCACCATGGTTCTTCTGTGAG	Amplifying *gfp* with a premature UAG stop codon for cloning in 8R

C7FP_GFP_2	ATGGGTTCTTCTCACCATCACGGATAAGGATCCCCGGGAATTCACATCACCATGGTTCTTCT	Amplifying *gfp* with a -1 frameshift mutation and cloning in 8R
C8FP_GFP_2	ATGGGTTCTTCTCACCATCACGTGTAGGGTTAGCGGCCCTAATTCACATCACCATGGTTCTTCT	Amplifying *gfp* with a +1 frameshift mutation and cloning in 8R
BGAL_PQE2_FP	ATCACCATCACCATCACCATATGATGACCATGATTACGGATTCAC	Amplifying *lacz* with an AUG start codon for cloning in pQE2
BGAL_PQE2_RP	TCCAAGCTCAGCTAATTAAGCTTTTATTTTTGACACCAGACCAAC	Amplifying *lacz* for cloning in pQE2
BGAL_AGG FP	GCGGATAACAATTTCACACAGAATTCATTAAAGAGGAGAAATTAACTAGGACCATGATTACGGATTCACT	Amplifying *lacz* with an AGG start codon for cloning in pQE2
INFA_P15A_FP	GATAGAGAAAAGAATTCAAAAGATCTAAAGAGGAGAAAGGATCTATGGCCAAAGAAGACAATATTG	Cloning *infA* into p15A vector backbone
INFA_P15A_RP	TGCCTGGAGATCCTTACTCGAGTCAGCGACTACGGAAGAC	

INFB_P15A_FP	GATAGAGAAAAGAATTCAAAAGATCTAAAGAGGAGAAAGGATCT ATGACAGATGTAACGATTAAAACG	Cloning *infb* into p15A vector backbone
INFB_P15A_RP	TGCCTGGAGATCCTTACTCGAGTTAAGCAATGGTACGTTGGAT	
INFC_P15A_FP	GATAGAGAAAAGAATTCAAAAGATCTAAAGAGGAGAAAGGATCT ATGAAAGGCGGAAAACGAGTTC	Cloning *infc* into p15A vector backbone
INFC_P15A_RP	TGCCTGGAGATCCTTACTCGAGTTACTGTTTCTTCTTAGGAGCG	
RSGA_P15A_FP	GTGATAGAGAAAAGAATTCAAAAGATCTAAAGAGGAGAAAGGATCTTTGAGTAAAAAT AAACTCTCCAAAG	Cloning *rsgA* into p15A vector backbone
RSGA_P15A_RP	GGAGATCCTTACTCGAGTCAGTCATCCGTATCAGAAAAG	
RBFA_P15A_FP	GTGATAGAGAAAAGAATTCAAAAGATCTAAAGAGGAGAAAGGATCTATGGCGAAAGAA TTTGGTCG	Cloning *rbfA* into p15A vector backbone
RBFA_P15A_RP	GGAGATCCTTACTCGAGTTAGTCCTCCTTGCTGTCGTCC	

KSGA_P15A_FP	GTGATAGAGAAAAGAATTCAAAAGATCTAAAGAGGAGAAAGGATCTATGAATAATCGAGTCCACCAGGG	Cloning *ksgA* into p15A vector backbone
KSGA_P15A_RP	GGAGATCCTTACTCGAGTTAACTCTCCTGCAAAGGCG	
LEPA_P15A_FP	GTGATAGAGAAAAGAATTCAAAAGATCTAAAGAGGAGAAAGGATCTATGAAGAATATACGTAACTTTTCGAT	Cloning *lepA* into p15A vector backbone
LEPA_P15A_RP	TGCCTGGAGATCCTTACTCGAGTTATTTGTTGTCTTTGCCGACGTG	
DELKSGA_FP	AATACACACTCGGGGCGAATTGATCATCGTTAACTCTCCTGCAAAGGCGCTTTAAGAAGGAGATATAGTTC	Used for creating knock-outs of *ksgA*
DELKSGA_RP	CATTGGGTGTTAACAATCATTTTGATGGCGAGATTAAGCGCCGTAATAAAGGATTGGAAGTAGAGGTTCTC	
DEL_RBFA_FP	GTCGCGACCGCGACGACGAGGACGACTCATTAGTCCTCCTTGCTGTCGTCTTTAAGAAGGAGATATAGTTC	Used for creating knock-outs of *rbfA*

DEL_RBFA_RP	CATAATAAATTCTCCTGACAAAAAAGGGGCTGTTAGCCCCTTTTTAAAATGGATTGGAA GTAGAGGTTCTC	
DEL_RSGA_FP	GACGATTCTAACGACGGTTAGCTTAATTGTCAGTCATCCGTATCAGAAAA CAAGAGGGTCATTATATTTCG	Used for creating knock-outs of *rsgA*
DEL_RSGA_RP	GTATCATAGATGTTTTGCCCATCAGGGGCGACCAGGAGTCTGTACGATTG TCCTAATTTTTGTTGACACTCTA	
DEL_LEPA_FP	ACTTCTAAAAGCCTGGTTAACCGGGCATTAAGGCACAATAATCATACTTTTTTAAGAAG GAGATATAGTTC	Used for creating knock-outs of *lepA*
DEL_LEPA_RP	GCAGCTCGACGTTACCGATCTGCTTCATGCGTTTCTTACCTTCTTTCTGCTTCTGCAG GGATTGGAAGTAGAGGTTCTC	
KSGA_200FLANK FP	TCCTTCGCCCTGGACTTC	Gene flanking primers used for screening of *ksgA* null mutants
KSGA_200FLANK RP	GCGATGTACCACGATCAGG	
RBFA_200FLANK FP	CTGGGAAAACTTCGTCGCTT	Gene flanking primers used for screening of *rbfA* null mutants
RBFA_200FLANK RP	ATTCGAAATCATCGAGATCCAACG	
RSGA_200FLANK FP	TCC ACA ATT GCG TGT ATCA	Gene flanking primers used for screening of *rsgA* null mutants
RSGA_200FLANK RP	CCTACATGCCGGATCCTG	
LEPA_200FLANK_FP	TGATTTCACTGGCTTTGTTGC	

LEPA_200FLANK_RP	TCCAGCCAGCCAGGCTTC	Gene flanking primers used for screening of *lepA* null mutants

References

1. Hussain, T., Llácer, J.L., Wimberly, B.T., Kieft, J.S. and Ramakrishnan, V. (2016) Large-Scale Movements of IF3 and tRNA during Bacterial Translation Initiation. *Cell*, **167**, 133-144.e113.