

Supplement to

RbfA and IF3 couple ribosome biogenesis and translation initiation to increase stress tolerance

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One (1) Table and eight (8) figures associated with main figures and discussion.

Table S1. List of strains, plasmids, and oligonucleotides used in this study.**Bacterial strains**

<i>E. coli</i> MRE600	Lab stock	N/A
<i>E. coli</i> BL21 (DE3)	New England Biolabs	Cat# C2527H
<i>E. coli</i> BX41 (Δ <i>rbfA</i>)	(1)	N/A
<i>E. coli</i> Δ <i>rsgA</i>	(2, 3)	N/A
<i>E. coli</i> BW25113	CGSC, Yale University	CGSC#7636
<i>E. coli</i> JW0050-3 (Δ <i>ksgA</i>)	(2)	CGSC#8292
<i>E. coli</i> TPR201	(4)	CGSC#8265
<i>E. coli</i> JK382 (parental)	(5)	CGSC#7503
<i>E. coli</i> JK378 (<i>infC362</i>)	(5)	CGSC#7501
<i>E. coli</i> WTZ19001 (<i>JK378</i> Δ <i>rsgA</i>)	This study	CGSC#14233

Plasmids

pET21b-RbfA-A2C	GenScript	N/A
p15BHA	(6)	N/A
p15B-RbfA-A2C-HA	This study	N/A
pMW118	(7)	N/A
pMWrbfA ⁺	(7)	N/A
pGEMX2His-RsgA	(7)	N/A
pD421-rsgA	Lab stock	N/A
pProEx-HTb-IF1	(8)	N/A
pET24b-IF2	(9)	N/A
pProEx-HTb-IF3	(8)	N/A
pProEx-HTb-IF3-K110L	This study	N/A
pProEx-HTb-IF3-Nter	This study	N/A
pProEx-HTb-IF3-Cter	This study	N/A
IF3.C65S.S38C.K97C	(10)	N/A
IF3-Y75N.C65S.S38C.K97C	(10)	N/A

Oligonucleotides

Primer 161	(11)	N/A
Primer 323	(11)	N/A
Primer 1523	(12)	N/A
Primer-5'end	(13)	N/A
Primer-3'end	(14)	N/A
Cy3-SA5	(15)	N/A

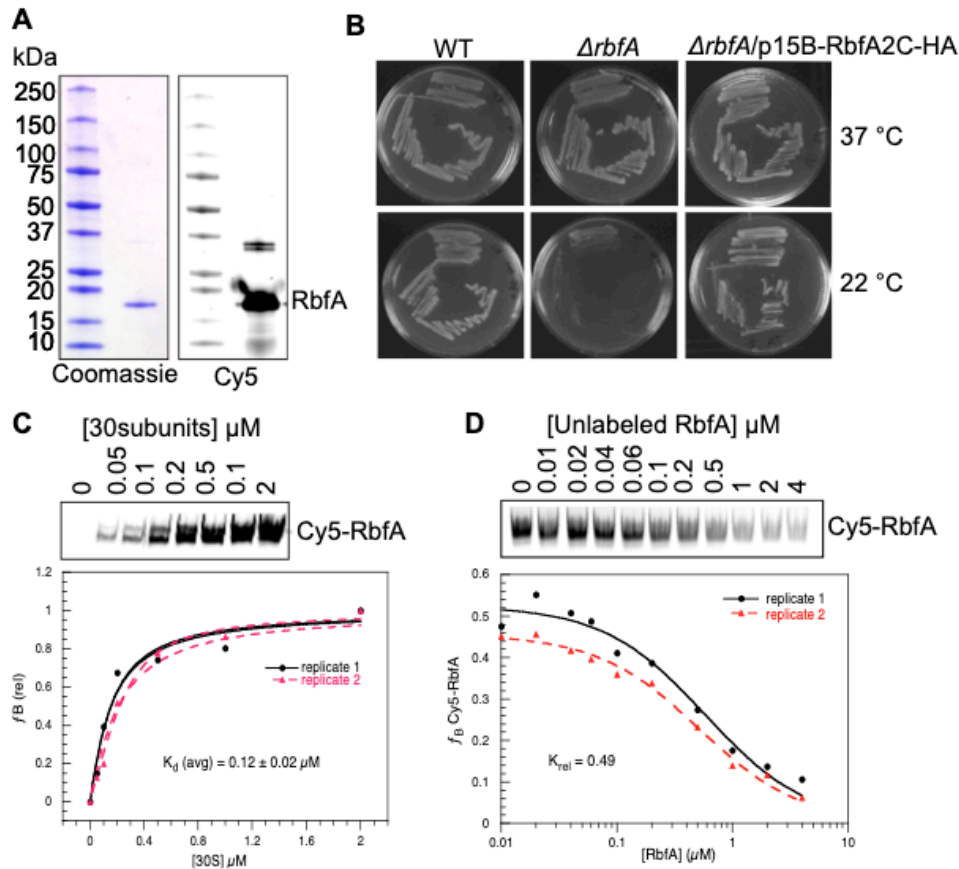


Figure S1. Preparation of Cy5-labeled RbfA.

(A) 4-20% SDS PAGE showing the purity of RbfA:A2C protein after labeling with Cy5-maleimide. (B) The *in vivo* function of RbfA:A2C was tested by complementation of the cold sensitive phenotype of BX41 $\Delta rbfA$ strain by expression of the *rbfA*:A2C mutant from plasmid p15B-RbfA2C-HA. The mutant RbfA was able to restore growth at 22 °C, comparable to an *rbfA*⁺ *E. coli* strain (MRE600). (C) Equilibrium binding of 100 nM Cy5-RbfA with 30S subunits, fit to a quadratic form of a single-site binding isotherm. (D) Competition between unlabeled RbfA and 40 nM Cy5-RbfA for a binding site on 0.17 μM 30S subunits. The fluorescence intensity was scaled to the fraction of Cy5-RbfA bound in the absence of competitor, assuming K_d for Cy5-RbfA = 0.12 μM . $K_{rel} = 0.49 \pm 0.09$ for two independent trials. Both (C) and (D) were performed by native 4% PAGE.

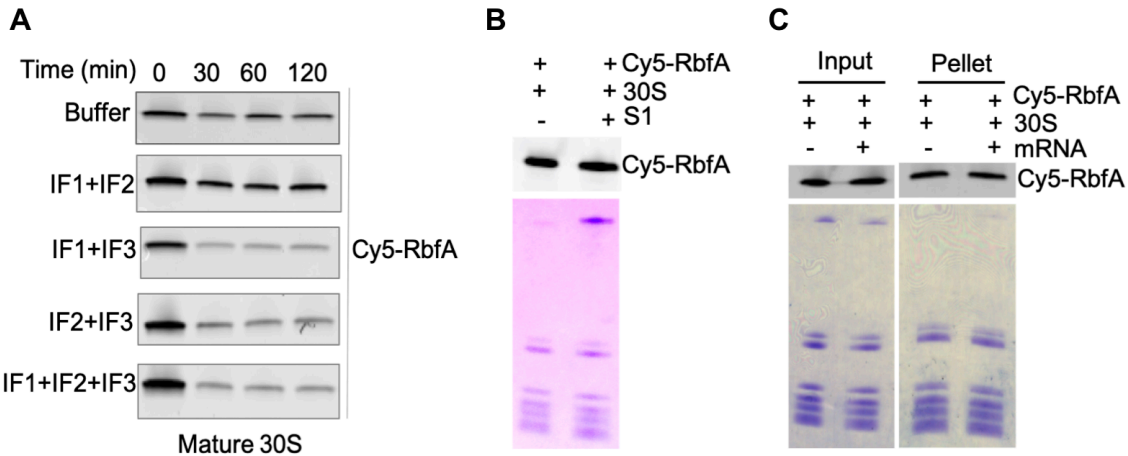


Figure S2. Other 30SIC components do not release RbfA.

(A) Ultrafiltration assays showing the kinetics of release of Cy5-RbfA from mature 30S subunits in the presence of buffer or combinations of IF1, IF2 and IF3. The retentates were analyzed by SDS-PAGE and scanned for Cy5 intensity as described in the Methods (also see Figure 1). The “0” min sample was filtered once to remove excess free Cy5-RbfA at the start of the assay. In the following lanes, the sample was filtered again at 30, 60 and 120 min to remove any RbfA that dissociated during this period. Top, the RbfA•30S complex is stable for 2 h in the absence of release factors. Lower panels, the presence of IF1 and IF2 had no effect on the release activity of IF3. (B) An ultrafiltration assay showing that addition of excess ribosomal protein bS1 (4 μ M) cannot displace Cy5-RbfA from 30S subunits. The retentate was resolved on a 4-20% SDS-PAGE and scanned for Cy5 intensity (top) or stained with Coomassie (bottom). (C) A pelleting assay showing that *sodB* mRNA (0.4 μ M), which contains a strong Shine-Dalgarno sequence, cannot displace Cy5-RbfA from 30S subunits.

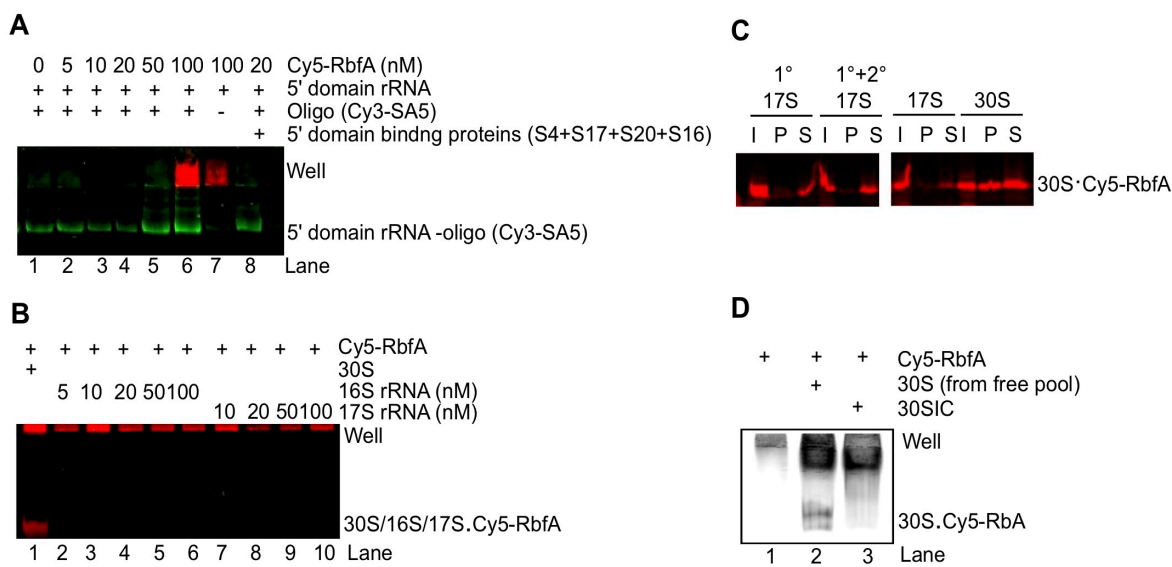


Figure S3. Early 30S assembly complexes are not recognized by RbfA.

(A) Native 4% PAGE showing that Cy5-RbfA cannot bind with the 5' domain of 16S rRNA. 5' domain RNA (15 nM) with a 3' extension was hybridized with a complementary Cy3-SA5 DNA oligomer (10 nM) as previously described (15, 16). 5' domain RNA•Cy3-SA5 complexes were incubated without or with an increasing concentration of Cy5-RbfA. Lane 8, reaction includes 2.5 μ M each 5' domain proteins S4, S17, S20, and S16 (17) to see if they could promote RbfA binding with 5' domain RNA. (B) Native 4% PAGE showing that Cy5-RbfA cannot bind with 16S rRNA (native) and 17S rRNA (transcribed). Lane 1, 30S subunits were used as a control, in which free Cy5-RbfA remains in the well and bound Cy5-RbfA migrates into the gel together with 30S complexes. (C) A pelleting assay showing that Cy5-RbfA cannot bind transcribed 17S rRNA reconstituted with primary (1°) or primary and secondary (1° + 2°) 30S proteins (18). (D) Native 4% PAGE showing that Cy5-RbfA binds free 30S subunits (lane 2) but not translation initiation complexes (30SIC) (lane 3). 30SIC was formed by incubating 30S subunits with initiation factors, mRNA (*sodB*), and fMet-tRNA^{fmet} as previously described (19).

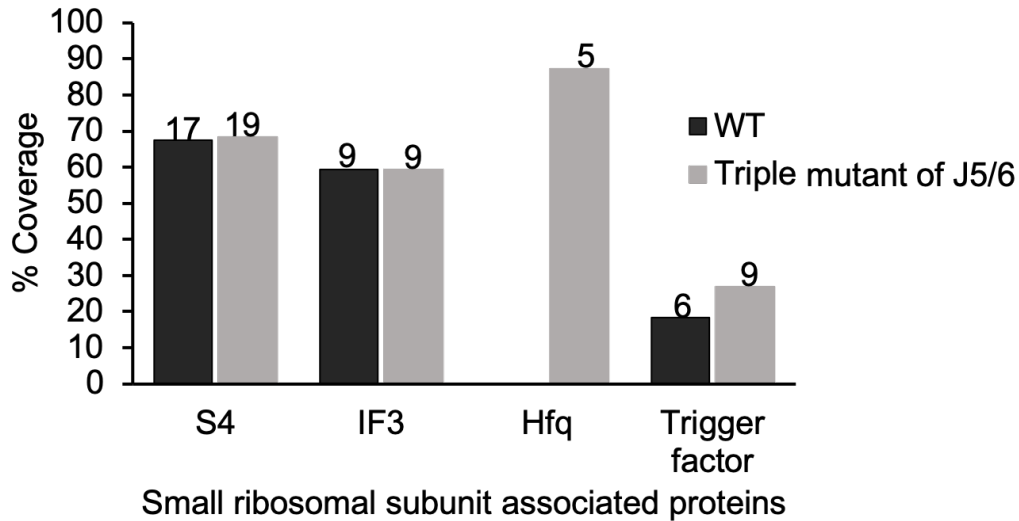


Figure S4. Detection of IF3 in a pre-30S complex.

Qualitative mass spectrometry data showing co-purification of IF3 with 30S (black bars) and Off-pathway MS2-tagged pre-30S particles (gray bars). These pre-30S subunits have three mutations (A59, 60C, G107C) in helix junction J5/6 of the 16S rRNA. Data reanalyzed from (20). The number of peptides observed for each protein are shown on the top of respective bar.

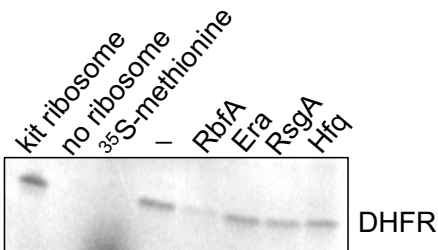


Figure S5. Measuring translation by pre-30S subunits in the presence of late stage binding 30S assembly factors.

In vitro translation using PURExpress Δ ribosome kit (NEB). 0.5 μ M pre-30S $_{\Delta rbfA}$ subunits were incubated with the components of the kit in a 15 μ l reaction mixture also containing 0.5 μ M purified 50S subunits, 1 μ Ci ³⁵S-methionine, and 3 μ M of the indicated assembly factor. Only RbfA inhibited translation by pre-30S subunits.

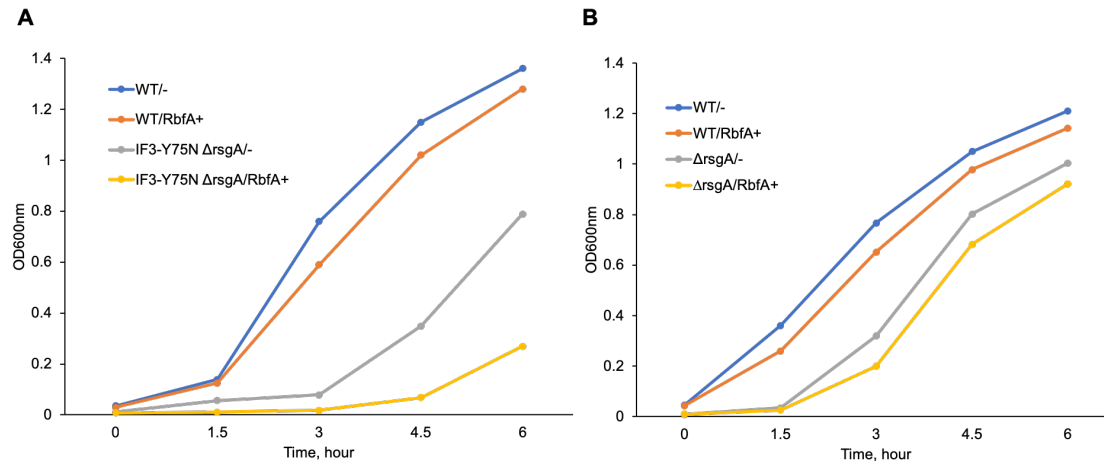


Figure S6. Genetic interactions between RsgA and RbfA. (A) Slow growth of *E. coli* IF3-Y75N Δ rsgA strain becomes even slower upon overexpression of RbfA. WT, parental; IF3-Y75N Δ rsgA, RsgA null mutation in JK378 strain; -, empty vector (pSE420); and RbfA⁺, + p15BHA (RbfA overexpression). (B) RbfA overexpression does not significantly affect cell growth in the absence of RsgA in the *E. coli* IF3 strain. WT, parental; Δ rsgA, RsgA null strain; -, empty vector (pMW118); and RbfA⁺, + pMWrbfA⁺ (RbfA overexpression). In all cases, cells were grown in LB media with shaking at 37 °C.

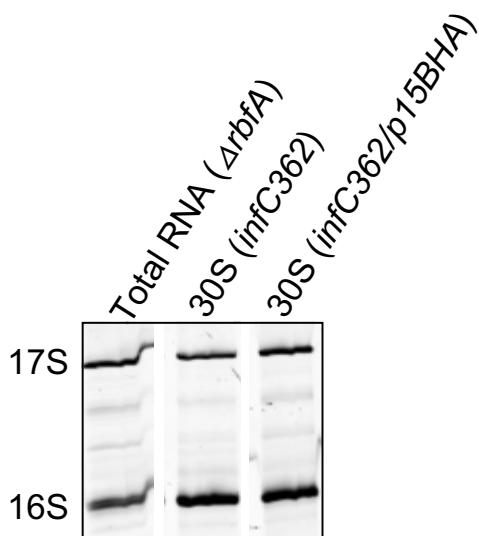


Figure S7. Processing of 16S rRNA is not impaired by overexpression of RbfA in *infC362* strain.

Processing of the 16S 5' end was analyzed by primer extension (Cy5-labeled primer 161) and denaturing 8% PAGE. The analysis was done with total cellular RNA (control) or 16S rRNA from 30S complexes (test). A similar proportion of 17S and 16S rRNA was obtained in 30S fractions from *infC362* and *infC362/p15BHA* strains (Figure 5E).

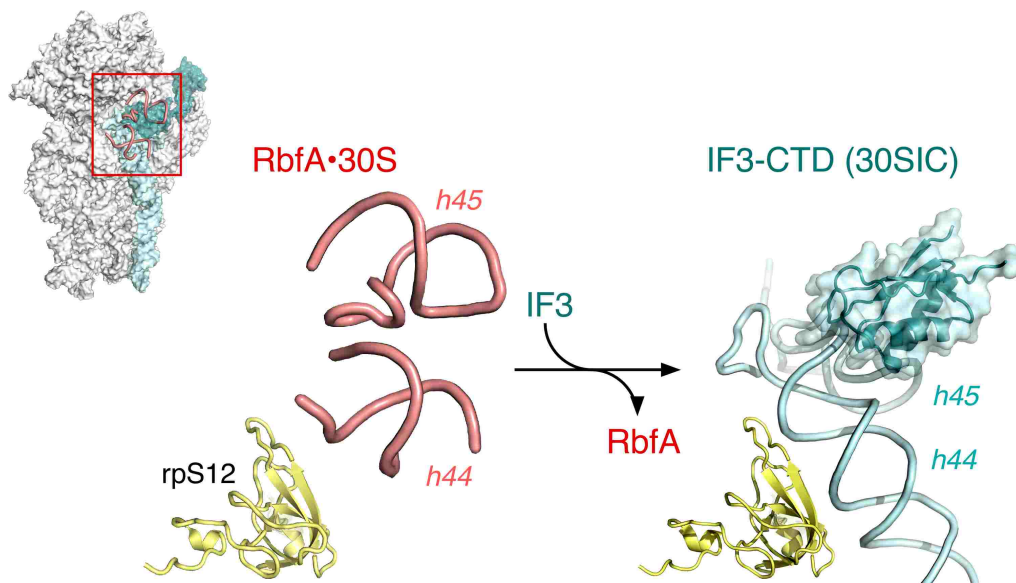


Figure S8. RbfA and IF3 modulate the conformation of 16S h44 and h45.

Binding of RbfA unfolds the top of 16S h44 and h45 (salmon) in a low-resolution cryo-EM model of the RbfA•30S complex (21). By contrast, binding of IF3 (teal) and extension of its CTD stabilizes the docked conformation of 16S h44 and h45 (light cyan) in structures of 30S initiation and pre-initiation complexes (22, 23). The opposing actions of RbfA and the IF3 CTD on h44 and h45 may explain how IF3 either promotes dissociation of RbfA or prevents RbfA from rebinding. Ribosomal protein uS12 (yellow) is shown for reference. Inset upper left: 16S fragments modeled into the cryo-EM density map of the 30S•RbfA complex (PDB: 2R1G; (21)) were superimposed on coordinates from a cryo-EM model of 30SIC2 (PDB: 5ME1; (23)). Atoms in 16S h1, h18, and h27 were aligned using PyMOL. Ribbons for IF1, IF2 and fMet-tRNA^{init} in the 30SIC2 are not shown for clarity.

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