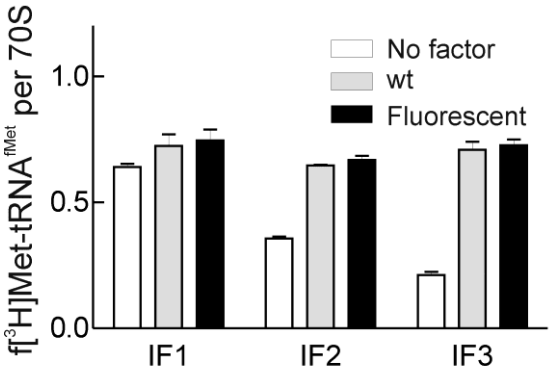
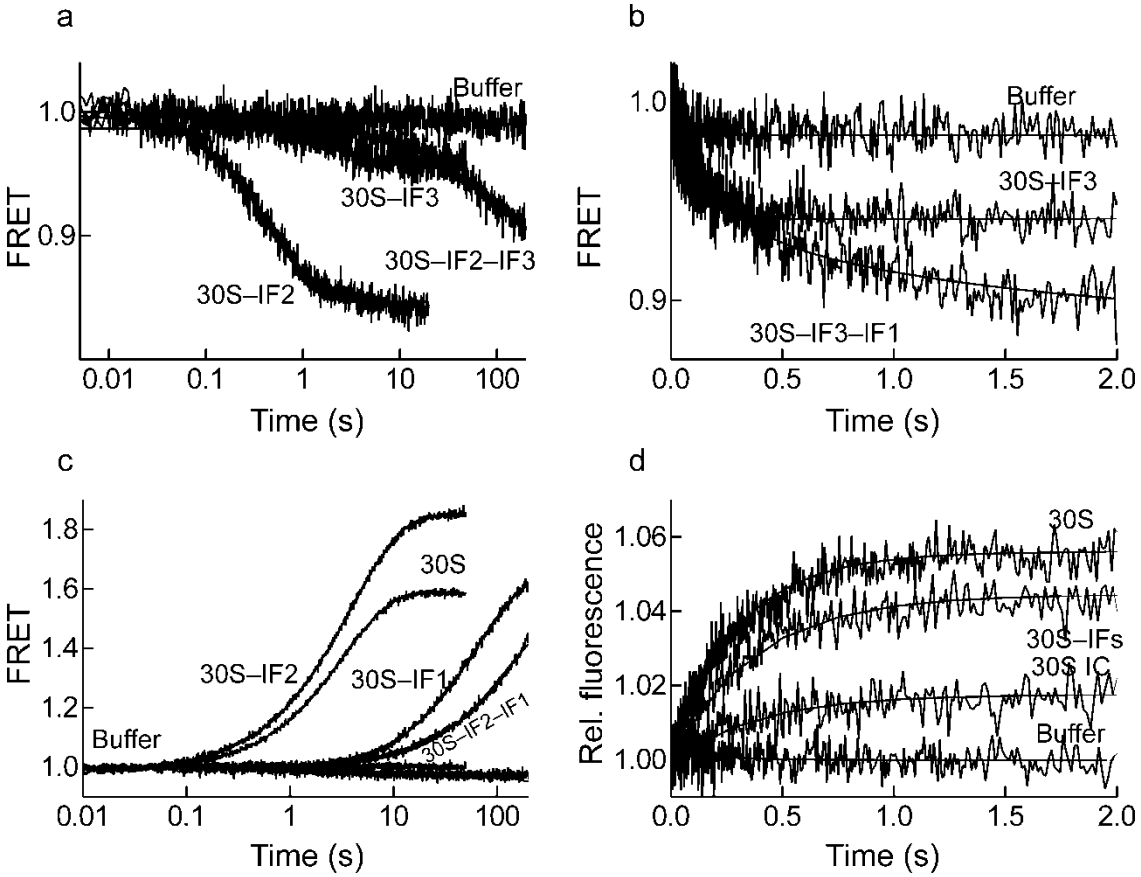
Supplementary Results

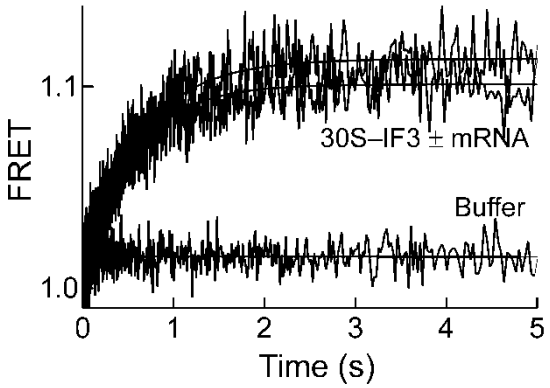


Supplementary Figure 1. Functional activity of fluorescent derivatives of initiation factors. 70S IC were formed with f[^{3}H]Met-tRNA^{fMet} and mRNA (m022) in the absence of the respective factor (white bars), in the presence of the respective unmodified (wt) factor (grey bars), or with fluorescence-labeled mutant initiation factor (black bars). The extent of complex formation was determined by nitrocellulose filtration.

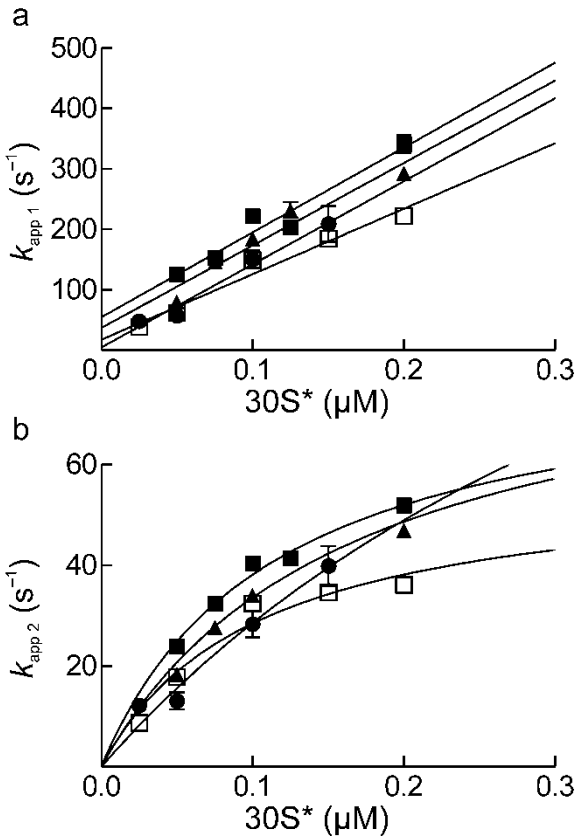


Supplementary Figure 2. Time courses of IF and mRNA dissociation. (**a**) Chase of IF1 from 30S complexes with IF2 or IF3 or IF2 + IF3. Complexes were formed using 30S subunits (0.05 μM), $IF1_4$(Alx555) (0.5 μM), $IF3_{166}$(Alx488) (0.06 μM), IF2 (0.15 μM) or $IF2_{757}$(Alx488) (0.11 μM), GTP (0.25 mM), as indicated; the dissociation was initiated by the addition of unlabeled IF1 (5 μM). (**b**) Chase of $IF2_{599}$(Atto465) from various 30S complexes was initiated by the addition of unlabeled IF2 (1.5 μM). 30S complexes (0.05 μM) were prepared with $IF2_{599}$(Atto465) (0.11μM), GTP (0.25 mM) in the absence or presence of IF1 (1 μM), $IF3_{166}$(Alx555) (0.1 μM), mRNA (0.4 μM). (**c**) Chase of IF3. Complexes were formed by incubating 30S subunits (0.1 μM) with $IF3_{166}$(Alx488) (0.01 μM), and mRNA(AttoQ) (0.4 μM) and where indicated IF1 (1 μM), IF2 (0.3 μM) with GTP (0.25 mM), and fMet-$tRNA^{fMet}$ (0.3 μM). The dissociation was initiated by addition of unlabeled IF3 (1 μM) to the 30S complex containing the FRET pair. (**d**) Chase of mRNA(Atto488) from 30S·mRNA complexes by excess of unlabeled mRNA. The dissociation of the fluorescent mRNA from the complexes (0.05 μM) was initiated by addition of unlabeled mRNA (1.5 μM).

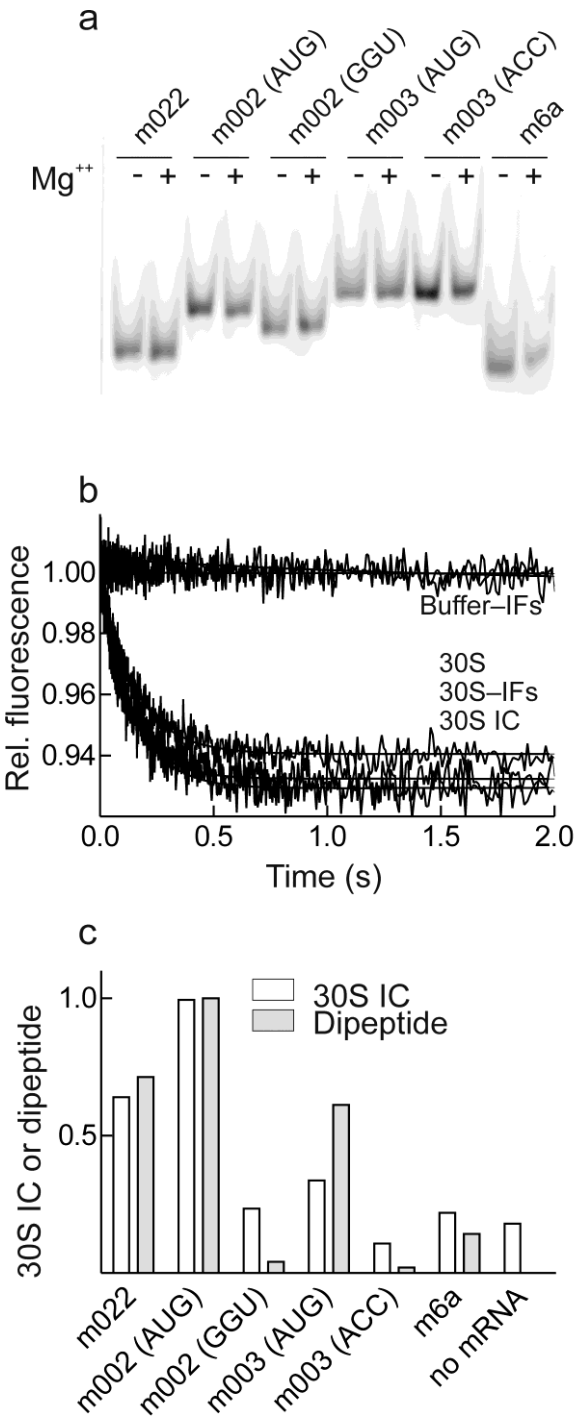
Nature Structural & Molecular Biology: doi:10.1038/nsmb.2285



Supplementary Figure 3. IF1 binding to 30S:IF3 complex in the presence or absence of mRNA. IF1(Alx555) (0.15 µM) was rapidly mixed with 30S subunits (0.05 µM) in complex with IF3(Alx488) (0.06 µM) in the absence or presence of mRNA (0.3 µM). Lower line, control without ribosomes.



Supplementary Figure 4. Concentration dependence of k_{app1} and k_{app2} values of IF3 binding to 30S complexes. (**a**) Linear dependence of k_{app1} values. (**b**) Hyperbolic dependence of k_{app2} values. The estimations of the elemental rate constants were used as initial parameters for global fitting. Experimental conditions are indicated in Fig. 4. Symbols: (■) 30S subunit; (●), with IF1; (▲) with IF2; (□) with IF1 and IF2.



Supplementary Figure 5. Binding, conformation and functional activities of mRNAs. (**a**) Mobility of mRNAs on a non-denaturing PAGE. 10 pmol of each fluorescent mRNA were loaded onto the gel after renaturation in the presence or absence of 7 mM magnesium acetate. Gels were run for 2.5 h at 4°C in TBE buffer and scanned using F7000 scanner with a fluorescence filter for Cy2 (GE Healthcare). (**b**) Time courses of mRNA(m022) binding to vacant 30S subunits, 30S with IF1, IF2, and IF3 (30S·IFs), or to the 30S·IFs·fMet-tRNA^{fMet}

complex; topmost curve, control without ribosomes. Complex formation was monitored by the fluorescence change of Atto488 attached to position +12 of the mRNA. In the presence of the highly structured m6a mRNA[1], no fluorescence intensity change was recorded (not shown) indicating that the signal observed for the other mRNAs originate from the association of single stranded segments of the constructs[1]. (**c**) 30S IC and dipeptide formation with mRNAs used in this study. The amount f[^{3}H]Met-tRNAf^{Met} bound to the 30S subunits was measured by nitrocellulose filtration and liquid scintillation counting. The relative values indicate efficiencies of fMet-tRNA^{fMet} recruitment for each mRNA normalized by the efficiency obtained when m002 mRNA was used, typically between 80-100%. Dipeptide formation was measured after addition of 50S subunits, EF-Tu, GTP, [^{14}C]Phe-tRNA^{Phe}, phosphoenol pyruvate, and pyruvate kinase to the 30S IC as described[2]. Relative efficiency values were calculated as for 30S IC formation measurements.

Supplementary Table 1. List of fluorescent reporters used in this work

Component	Modified residue	Dye	Comments	Figure
$IF1_4$(Alx555)	D4C	Alexa 555	FRET acceptor for $IF3_{166}$ (Alx488), $IF2_{757}$ (Alx488)	Fig. 2, 6e, S2
$IF2_{757}$(Alx488)	C599S/V757C	Alexa 488	FRET donor for $IF3_{166}$ (Alx555), $IF1_4$ (Alx555)	Fig. 2, S2
$IF2_{599}$(Atto465)	*wt* Cys599	Atto 465	FRET donor for $IF3_{166}$ (Alx555), $IF1_4$ (Alx555)	Fig. 3, 6e
$IF3_{166}$(Alx488)	C65S/E166C	Alexa 488	FRET donor for $IF1_4$ (Alx555), $tRNA^{Phe}$ (QSY), mRNA (AttoQ)	Fig. 2, 4, 5, 6e, S2, S3, S4
$IF3_{166}$(Alx555)	C65S/E166C	Alexa 555	FRET acceptor for $IF2_{757}$ (Alx488), $IF2_{599}$ (Atto465), fMet-$tRNA^{fMet}$ (Flu)	Fig. 3, S2
$tRNA^{Phe}$(QSY)	Thio-U8	QSY-35	FRET acceptor for $IF3_{166}$ (Alx488)	Fig. 5
mRNA(AttoQ)	5′ end	Atto 540Q	FRET acceptor for $IF3_{166}$ (Alx488)	Fig. 4, 6e S2
mRNA(Atto488)	3′ end (+12)	Atto 488	Fluorescence change	Fig. 6, S3, S4, S5

Supplementary Table 2. Dissociation rate constants of IF2 from 30S complexes.

IF2 **Complex**	k_{off}[a] **s^{-1}**
30S–IF3	15 ± 0.7
30S–IF3–mRNA	12 ± 0.6
30S–IF1–IF3	1 ± 0.1
30S–IF1–IF3–mRNA	2 ± 0.1
30S IC, no IF3	0.05 ± 0.005
30S IC, no IF1	0.17 ± 0.01
30S IC	0.04 ± 0.005

[a] Dissociation rate constant determined in chase experiments (**Fig. 3d**). To form 30S IC, m022 mRNA was used.

Supplementary Table 3. Association and dissociation rate constants of mRNA binding to 30S complexes.

		Vacant 30S		30S–IFs		30S IC	
20 °C	**T_m**	**k_{on}[a]**	**k_{off}[b]**	**k_{on}[c]**	**k_{off}[b]**	**k_{on}[c]**	**k_{off}[b]**
mRNA	**°C**	**$\mu M^{-1}s^{-1}$**	**s^{-1}**	**$\mu M^{-1}s^{-1}$**	**s^{-1}**	**$\mu M^{-1}s^{-1}$**	**s^{-1}**
m022	50	15 ± 2	3 ± 0.1	36 ± 1	2.5 ± 0.1	48 ± 1	n.d.
m002(AUG)	41	43 ± 6	0.05 ± 0.005	40 ± 1	0.06 ± 0.005	38 ± 1	n.d.
m002(GGU)	49	54 ± 1	0.05 ± 0.005	46 ± 1	0.04 ± 0.005	45 ± 1	0.04 ± 0.01
m003(AUG)	22	49 ± 3	0.06 ± 0.005	84 ± 1	0.06 ± 0.005	95 ± 1	0.09 ± 0.001
m003(ACC)	18	59 ± 4	0.05 ± 0.005	118 ± 2	0.05 ± 0.005	112 ± 2	0.05 ± 0.001

37 °C	**k_{on}[a]**	**k_{off}[b]**
mRNA	**$\mu M^{-1}s^{-1}$**	**s^{-1}**
m022	36 ± 4	13 ± 0.1
m002(AUG)	110 ± 7	1.2 ± 0.05
m002(GGU)	94 ± 11	1 ± 0.1
m003(AUG)	95 ± 6	2.8 ± 0.02

[a] Association rate constants determined from the slope of the linear concentration dependence of k_{app} (for a one-step mechanism).

[b] Dissociation rate constant determined in chase experiments. n.d., not detectable (too slow).

[c] Association rate constants estimated from $k_{app} = k_{on} \times [30S] + k_{off}$ for a one-step mechanism.

References

1. Studer, S.M. & Joseph, S. Unfolding of mRNA secondary structure by the bacterial translation initiation complex. *Mol. Cell* **22**, 105-115 (2006).

2. Milon, P. et al. The nucleotide-binding site of bacterial translation initiation factor 2 (IF2) as a metabolic sensor. *Proc. Natl. Acad. Sci. USA* **103**, 13962-13967 (2006).