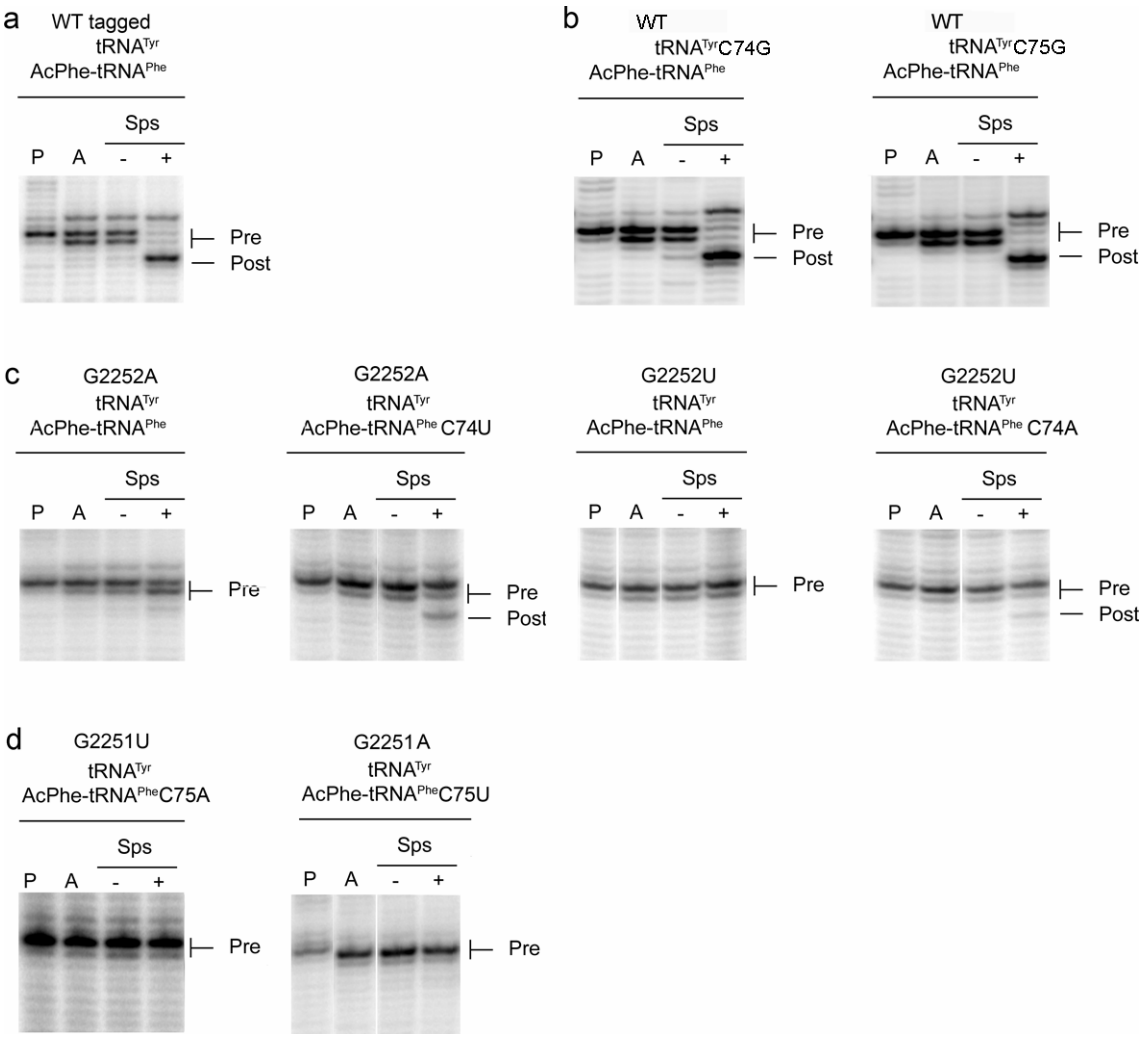
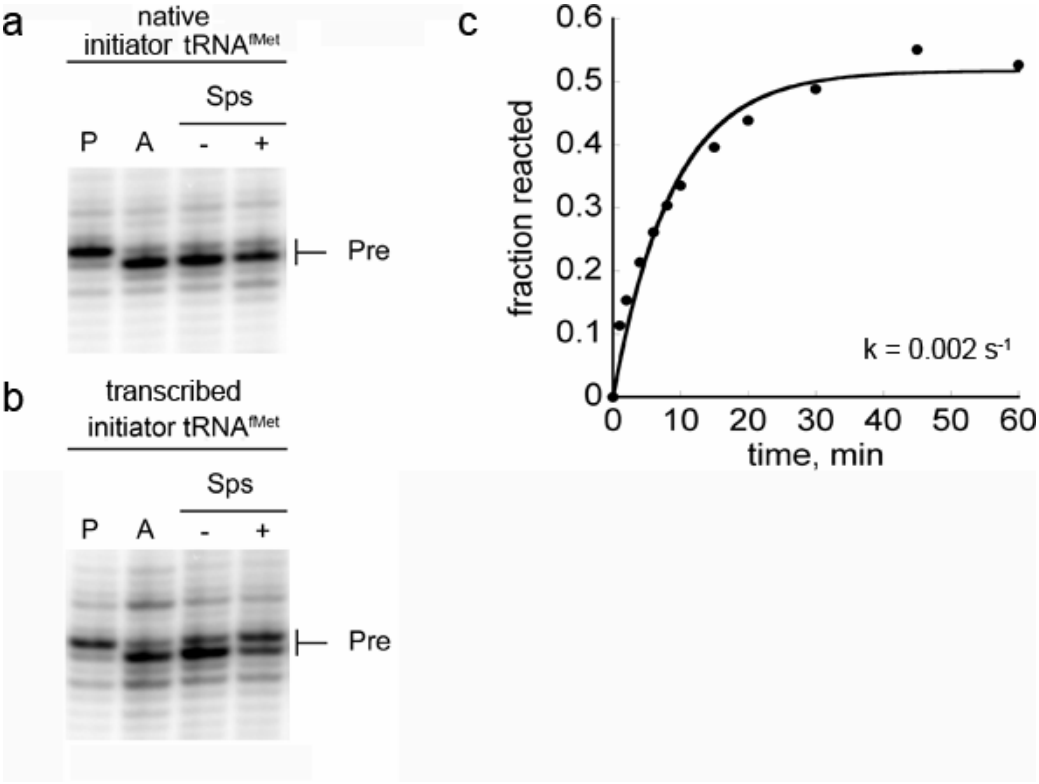
Supplementary Figure 1



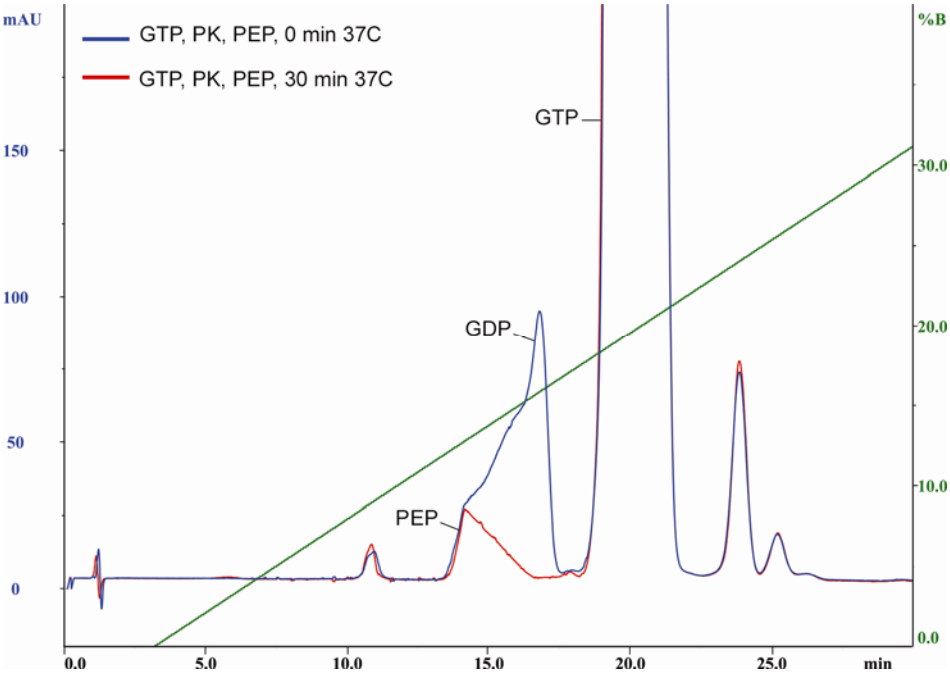
Supplementary Figure 1: Toeprint analysis of sparsomycin-mediated translocation on m301 messenger RNA. Pre-translocation complexes were assembled by binding deacylated $tRNA^{Tyr}$, $tRNA^{Tyr}C74G$, $tRNA^{Tyr}C75G$ to the P site (P lanes), followed by binding of N-Ac-Phe-$tRNA^{Phe}$, N-Ac-Phe-$tRNA^{Phe}$ C74U,A or N-Ac-Phe-$tRNA^{Phe}$ C75U,A to the A site (A lanes). Complexes were incubated with sparsomycin (+ Sps lane) or 2.5 % DMSO (- Sps lane). Ribosomes used were either (**a**) WT MS2-tagged (**b**) WT-MRE600 (**c**) G2251A or G2251U or (**d**) G2252A or G2252U. Reactions were carried out as described in Materials and Methods except that buffer C (20 mM HEPES, 4.5 mM $Mg(OAc)_2$, 150 mM NH_4OAc, 2 mM spermidine, 0.05 mM spermine, 4 mM 2-mercapthoethanol, pHed to 7.5 with KOH) was used which resulted in less efficient "rescues" than in buffer A.

Supplementary Figure 2



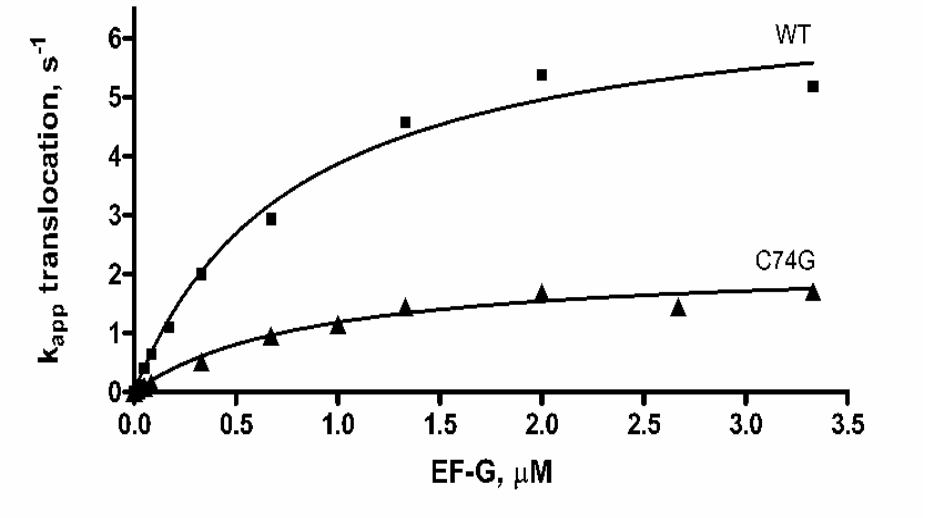
Supplementary Figure 2: Toeprint analysis of sparsomycin-mediated translocation and puromycin reactivity analysis comparing native and transcribed initiator tRNAs. Pre-translocation complexes were assembled by binding native (**a**) or transcribed (**b**) initiator $tRNA_f^{Met}$ to the P site (P lanes) of WT ribosomes, followed by binding of N-Ac-Phe-$tRNA^{Phe}$ to the A site (A lanes). Complexes were incubated with sparsomycin (+ Sps lane) or 2.5 % DMSO (- Sps lane). (**c**) Puromycin reactivity of pre-translocation complexes on m301 mRNA.

Supplementary Figure 3



Supplementary Fig. 3: FPLC analysis of GTP before and after incubation with PEP/PK. GTP (16 mM) was incubated with 40 mM 2-phospho(enol)pyruvate (PEP, potassium salt, pH 7.0, Roche) and 4 µg/ml rabbit-muscle pyruvate kinase (PK, Roche) in buffer B for 0 min (blue trace) or 30 min (red trace), 37º C. Reactions were separated on a ResourceQ column, with a 0 – 350 mM NaCl gradient in 20 mM Tris/Cl pH 7.5 over 30 min, 5 ml/min (see Material and Methods).

Supplementary Figure 4



Supplementary Figure 4: Pre-steady state kinetic analysis of EF-G-mediated translocation. Pre-translocation complexes of WT ribosomes with $tRNA_f^{Met}$ in the P site and f-Met-Phe-$tRNA^{Phe}$ in the A site were rapidly mixed with various concentrations of EF-G:GTP in the stopped-flow spectrophotometer and fluorescence measured as a function of time. The A site tRNAs used are indicated (either WT or C74G). $K_{1/2}$ values for EF-G were calculated in GraphPad prism using the hyperbolic Michaelis-Menten equation: Y=(x*m1)/(x+m2). $K_{1/2}$ for complexes with WT tRNA 0.8 ± 0.1 μM and with C74G tRNA 0.9 ± 0.3 μM.

Supplementary Table 1: Hybrid reactivity of dipeptidyl tRNA complexes with puromycin

P site	A site	WT	G2252C
WT	WT	0.06	n.r.
WT	C74G	n.r.	1.0

P site	A site	WT	G2251C
WT	WT	0.06	n.r.
WT	C75G	n.r.	0.06

Rates are expressed in min^{-1}, n.r. , no product after 60 min of incubation, SD were below 22%.

Pre-translocation complexes for analysis of puromycin reactivity were prepared as for stopped-flow experiments. For reaction with puromycin, complexes were incubated with 1 mM puromycin at 37ºC as described for Fig. 3.

Supplementary Table 2: EF-G dependent translocation with GTP, GTP or GTP/GDP

GTP, 0 mM	0.5
GTP, 1 mM	4.0
GTP, 2 mM	4.8
GTP +GDP each 1mM	4.6

Rates are expressed in s^{-1}, SD were below 20%

Translocation reactions were carried out on complexes containing MRE600 ribosomes, $tRNA^{fMet}$ in the P site and dipeptidyl fMetPhe-$tRNA^{Phe}$ in the A site.

Supplementary Table 3: EF-G dependent translocation with/without GTP and with/without energy regeneration system (PEP/PK).

GTP	PK/PEP	rate in s^{-1}
-	-	0.5
+	-	4.0
-	+	0.3
+	+	3.3

SDs were below 20 %

Reactions with PEP/PK: EF-G, 6 µM was preincubated with 2 mM GTP, 5 mM 2-phospho(enol)pyruvat (PEP, potassium salt, pH 7.0, Roche) and 4 µg/ml rabbit-muscle pyruvate kinase (PK, Roche) in buffer B for 30 min, 37º C. Control reactions were incubated without PEP/PK. This system was modified from Czworkowski J. and Moore P.B., Biochemistry 1997, 36, 10327-34. Translocation reactions were carried out on complexes containing MRE600 ribosomes, $tRNA^{fMet}$ in the P site and dipeptidyl fMetPhe-$tRNA^{Phe}$ in the A site.