SUPPLEMENTARY METHODS

eIF5 purification

The eIF5 expression vector with the N-terminal His- and MBP-tags and TEV protease site was transformed into *E.coli* BL21 (DE3) cells and grown in 1L LB medium supplemented with 50 μg/ml kanamycin at 37°C until the absorbance at 600 nm reached 0.5. Protein expression was induced with 0.5 mM IPTG, and the cells were incubated for an additional 5 hours. The harvested cells were resuspended in lysis buffer (20 mM Tris-HCl pH 7.5, 400 mM KCl, 10 μM $ZnSO_4$, and 0.1 mM PMSF), lysed by sonication, and then centrifuged at 20,000g for 30 min at 4°C. The supernatant was applied to a 5 ml HisTrap column (GE Healthcare), washed with lysis buffer supplemented with 10 mM imidazole until the UV absorbance reached the base line, and then eluted with lysis buffer supplemented with 250 mM imidazole. To digest the His-MBP fusion protein, 10 μg of recombinant His-tagged TEV protease was added per milligram of the His-MBP protein and dialyzed overnight at 4°C with cleavage buffer (20 mM Hepes-K pH 7.5, 50 mM KCl, 10 μM $ZnSO_4$, and 1 mM DTT). After cleavage, the protein solution was filtered, supplemented with 25 mM imidazole and applied to a HisTrap 5 ml column to remove the cleaved His-MBP fragment and uncleaved fusion protein. The flow-through fraction containing eIF5 was collected and further applied to a Mono-Q (5/5) column (GE Healthcare) equilibrated with cleavage buffer, and eluted with a linear gradient of buffer containing 50 - 500 mM KCl. The fractions containing eIF5 were pooled, concentrated to 13.7 mg/ml and stored in small aliquots at -80°C. Note that the purified recombinant protein has an additional two amino acid residues (GH) at the N-terminus.

RT-PCR

The gel lane of HeLa cell lysate was horizontally sliced into 2.5 mm pieces, with the slice position determined relative to the in vitro reconstituted MFC run in the adjacent lane. $tRNA_i$ was extracted from each gel slice essentially as above with 200 μl of 89 mM Tris-borate pH 8.3, 2 mM EDTA and 0.1 % SDS, then phenol extracted, ethanol precipitated, and dissolved in 10 μl of 10 mM Tris-HCl pH 8.0 and 1 mM EDTA. The gel extract (1 μl) was annealed with 2 pmol of a primer complementary to the 16 nt 3'-end of $tRNA_i$ by incubating at 70°C for 5 min and slow cooling to room temperature for 10 min. Superscript III reverse transcriptase (50 U, Invitrogen), 1 mM dNTPs, 10 mM DTT, 50 mM Tris-HCl pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$ were then added to the annealing mixture in a total volume of 20 μl, incubated at 25°C for 5 min, then 42°C for 30 min, and inactivated at 70°C for 5 min. The PCR (10 μl) was carried out with the Platinum PCR SuperMix (Invitrogen), 2 pmol each of primers complementary to the 16 nt 3'-end of the antisense and sense $tRNA_i$ sequence, and 0.4 μl of the RT product according to the manufacturer's protocol with an annealing temperature of 50 °C, 45 s elongation time, and 24 reaction cycles. Each PCR product (3 μl) was analyzed by 3 % agarose gel electrophoresis in TAE

buffer, and stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen). Mock reactions were also carried out without reverse transcriptase for each gel extract and showed no discernible signal under this conditions.

Met-tRNA$_i$ filter binding assay

eIF2 was initially incubated with 1 mM GDPNP or GDP at 30°C for 5 min in the atsence of Mg, and then further incubated at 30°C for 10 min with 20 nM [^{35}S]Met-tRNA$_i$ and other factors (as appropriate) in reaction buffer containing 20 mM Hepes-K pH 7.5, 80 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mg/ml creatine phosphokinase, and 5% glycerol in a total volume of 30 μl. Creatine phosphokinase is necessary for reducing non-specific binding of eIF2 to tube walls (21,30). The concentration of eIF2 was varied from 9 nM to 3000 nM, depending on the affinity to be measured. The concentrations of the other factors were kept at 300 nM or 3000 nM in the GDPNP and GDP conditions to ensure protein complex formation. The reaction mixtures (27 μl) were filtered through 25 mm HAWP nitrocellulose membranes (Millipore), and quickly washed twice with 600 μl ice cold reaction buffer without creatine phosphokinase. The membrane was dried and its radioactivity was measured in a liquid scintillation counter. The background was measured in a mixture without eIF2 and was subtracted from the other values. The resulting saturation curves were fitted to the following equation by using KaleidaGraph (Synergy Software): Fraction bound = $B_{max}[S]/(K_d + [S])$, where B_{max} is the maximum fraction bound at 3000 nM eIF2-GDPNP. Interestingly, in the presence of a high concentration of eIF1 (3000 nM), the background was unusually high, while it was greatly reduced when 1mg/ml of poly(U) RNA was added, suggesting a non-specific RNA binding activity of eIF1 (dissociation constant against Met-tRNA$_i$ is estimated to be ~10 μM). However, this RNA binding activity was significantly reduced and almost completely lost when eIF5 or eIF3 are present.

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30. Benne,R., Amesz,H., Hershey,J.W.B. and Voorma,H.O. (1979) The activity of eukaryotic initiation factor eIF-2 in ternary complex formation with GTP and Met-tRNA$_f$. *J. Biol. Chem.*, **254**, 3201-5.

Figure S1

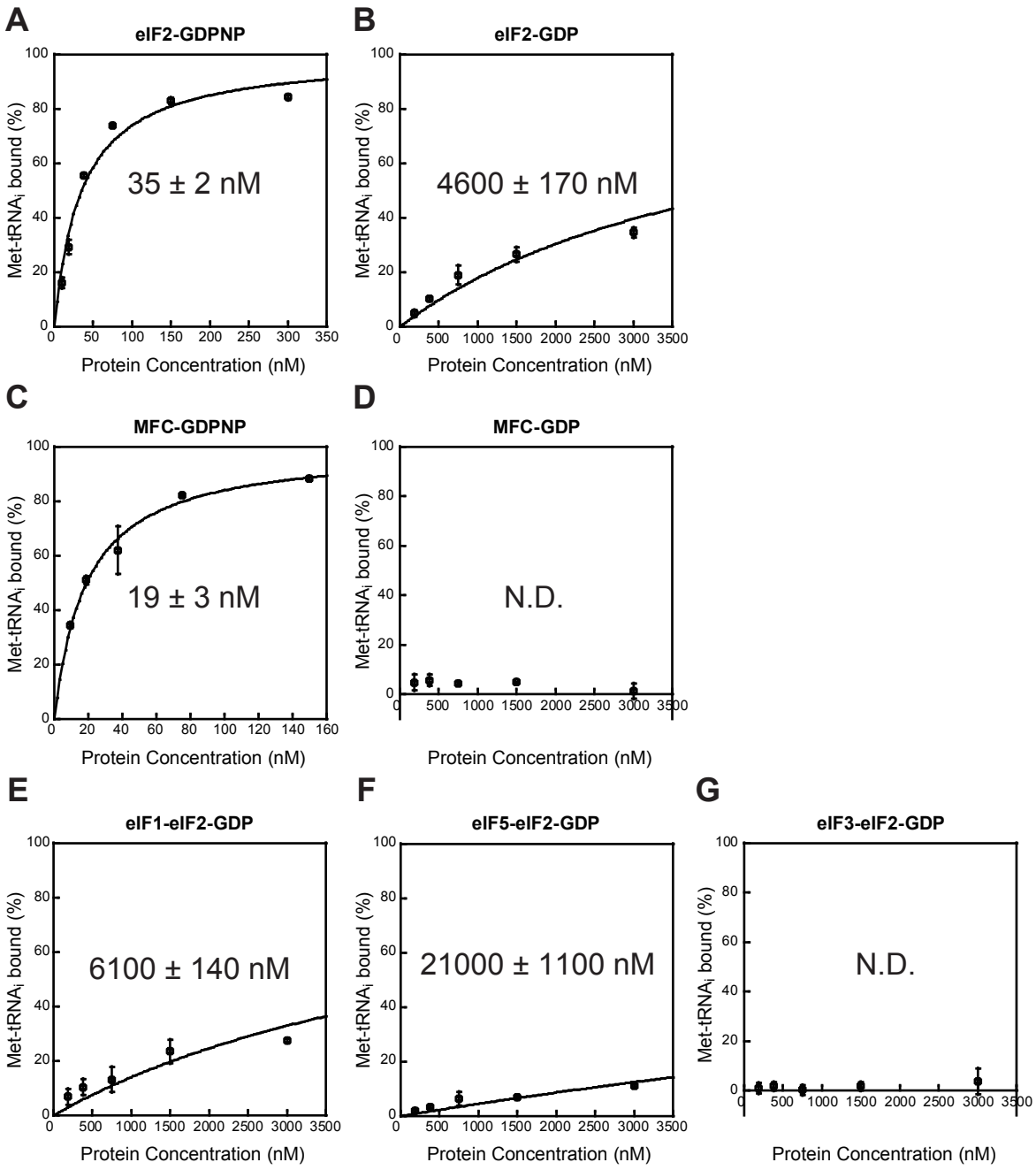

Figure S1. Met-tRNA$_i$ binding curves of **(A)** eIF2-GDPNP, **(B)** eIF2-GDP, **(C)** MFC-GDPNP, **(D)** MFC-GDP, **(E)** eIF1-eIF2-GDP, **(F)** eIF5-eIF2-GDP, and **(G)** eIF3-eIF2-GDP with calculated dissociation constants shown within. Met-tRNA$_i$ binding to the various proteins was measured as described in Materials and Methods. Values are means of two independent experiments, and errors shown are standard errors.