Supplementary Figure Legends

Figure S1. The GTPase center of eIF5B bound to GTP or GTPγS. A-C) F_o-F_c omit maps for GTP and Na^+ (A), GTPγS and Na^+ (B) or GTPγS and K^+ (C) bound to eIF5B (contoured at 3σ). P-loop, switch 1 and switch 2 are colored in cyan. The electron density maps for GTPγS clearly indicate that the sulfur atom of the γ-phosphate is oriented outward and interacts with the respective M^+ ion. **D)** Overview of the active site of eIF5B bound to GTPγS and Na^+ (purple sphere). The pentameric coordination sphere is indicated by purple lines. GTP, Asp^{MC}, Gly^{MC} and His^{cat} are shown as balls and sticks; P-loop, switch 1 and switch 2 are colored in cyan; the Mg^{2+} ion and water molecules are shown as spheres in light brown and grey, respectively. **E)** Superposition of eIF5B·GTP·Na^+ (coloring as in D) and dynamin (brown) bound to GDP, AlF_4 and Na^+ (PDB: 2X2E). The M^+ ions are coordinated by both proteins in nearly identical positions, despite differences in the ligands forming the coordination shell: Asp^{MC} is replaced by a Ser residue and two backbone oxygens from the K-loop replace Gly^{MC} from the MC-loop.

Figure S2. A-C) Titration curves (upper panels) and binding isotherms (lower panels) of eIF5B(517-858) interactions with GDP (A), GDPNP (B) or GTPγS (C) at 30°C.

Figure S3. Analysis of M^+ ion coordination in structures of GTP-bound aEF1A. A) 2mFo-DFc map (blue mesh) and mFo-DFc difference map (green mesh) for GTP and its surrounding in aEF1A molecule E of the aEF1A/Pelota complex (PDB: 3AGJ) (contoured at 3σ). The coloring is the same as in Fig. 4. In the original structure a water molecule is modeled into the pentameric coordination sphere between Asp^{MC} and Gly^{MC} and the phosphates of the GTP molecule with coordination distances of ~2.45 Å. Despite its solvent exposed position, the electron density for this water molecule is significantly stronger than that for the catalytic water (W^{cat}) or the water molecules coordinating the Mg^{2+} ion in the center of the active site. The

positive difference electron density for the supposed water molecule (green mesh) indicates a higher density of electrons in this position than provided by H_2O. **B)** The GTPase center in EF-Tu in its GDPNP conformation is virtually identical to that of aEF1A·GTP. With the exception of the β-γ-bridging oxygen of GTP EF-Tu·GDPNP provides all structural elements that are involved in M^+ coordination in the archaeal ortholog. The M^+ ion is indicated as semi-transparent blue sphere. Some universally conserved residues involved in GTP/GDPNP binding or GTP hydrolysis are shown as sticks. **C and D)** F_o-F_c omit maps for GTP and Na^+ (C), or GTP and NH_4^+ (D) bound to aEF1A (contoured at 3σ). P-loop and switch regions are colored yellow.

Figure S4. GDPNP and GDPCP prevent the coordination of the M^+ ion, which results in their inability to stably induce the conformational switch in trGTPases. A) Detailed view on the nucleotide binding pocket in a superposition of eIF5B·GTP (P-loop and switch regions in cyan) and aIF5B·GDPNP (P-loop and switch regions in yellow). GDPNP provides the γ-phosphate oxygens, the Mg^{2+} ion (light brown sphere) and its water ligands (red sphere; W1) in the identical positions as GTP for interactions with switch 2 and the stable association of switch 1 through Glu552 and Thr557 (*C. thermophilum* numbering). However, in contrast to the GTP-structure switch 1 and switch 2 remain in their GDP-like conformation in GDPNP-bound aIF5B and the position of Thr39 (corresponding to Thr557) is instead occupied by an additional water

molecule (red sphere; W2). The only significant difference between GTP and GDPNP is the inability of the latter to coordinate the M^+ ion (purple sphere), resulting in the loss of its contribution as structural cofactor to stabilize the GTP-conformation of switch 1 through the interaction with Gly^{MC} (purple dashed line). (It is important to note that in the originally downloaded structure file for aIF5B·GDPNP (PDB: 1G7T), W1 (red sphere) is interpreted as the Mg^{2+} ion, whereas a water (number 844) was modeled in the position which is here indicated as Mg^{2+} ion (light brown sphere). Our reinterpretation is based on the coordination geometry as well as the coordination distances for these two positions (coordination distances between 2.1 and 2.4 Å for water 844 and between 2.6 and 3.4 Å for the supposed Mg^{2+} ion). This reinterpretation is consistent with previously reported values for the coordination of Mg^{2+} ions (Harding, 2001). As the structure factors are not deposited in the PDB, we were unable to examine whether our reinterpretation is consistent with the experimental data). **B)** Surface presentation of the G domain in free EF-G·GDPCP (PDB: 2J7K). As for aIF5B·GDPNP, switch 1 (orange) and switch 2 (blue) remain in their 'off' state conformation despite the presence of the GTP analog. Like in the case of GDPNP, the coordination of the M^+ ion is prevented by the β-γ-bridging CH_2 group instead of the required oxygen ligand. **C)** In SelB·GDPNP switch 2 (blue) interacts with the γ-phosphate in the canonical way. Switch 1 as well undergoes a conformational change, resulting in the canonical direct contact between Thr46 (corresponding to Thr557 in *ct*eIF5B) and the Mg^{2+} ion (inset). However, despite the stabilization of Thr46, the preceding regions including the residues corresponding to the MC-loop in eIF5B and aEF1A remain flexible and are not defined in the electron density, most likely due to the loss of the M^+

ion as stable interaction partner. **D)** Surface presentation of GDPNP-bound eRF3. As in the cases of aIF5B and EF-G, both switch regions remain in their 'off' state conformation.

Figure S5. Crystal structures of eIF5B Asp^{MC} (D533) mutants bound to GTP or GTPγS. The coloring is the same as in Figure S1. **A)** Nucleotide binding pocket of eIF5B-D533A bound to GTPγS. Due to the replacement of Asp^{MC} by Ala, one of the key ligands and thus the pentagonal coordination sphere for the M^+ ion is lost. Accordingly, a water molecule (H_2O) is now asymmetrically coordinated by four ligands provided by Gly^{MC} in the P-loop and the GTPγS molecule instead of the M^+ ion. The coordination distances lie between 2.8 Å (to the α-phosphate) and 3.2 Å (to Gly^{MC}) to the oxygen ligands and ~3.3 Å to the sulfur atom. These distances to the oxygen ligands are significantly too large for a Na^+ ion (~2.42 Å (Harding, 2002)). The F_o-F_c omit map is shown for GTPγS and the water molecule, contoured at 3σ. **B)** The nucleotide binding pocket of eIF5B-D533N bound to GTP and Na^+. Unlike the D533A mutant, Asn in lieu of Asp^{MC} still provides the oxygen ligand required for the coordination of the M^+ ion, analogous to the P-loop Asn in MnmE (see Figure 1D). Consistently, the crystal structure of eIF5B-D533N was found to coordinate a Na^+ ion virtually identically to the wild-type protein (see Figure 1A). The coordination distances in the pentameric coordination shell lie between 2.22 Å (to Gly^{MC}) and 2.45 Å (to the α-phosphate), consistent with the expected values for a Na^+ ion (Harding, 2002). The F_o-F_c omit map is shown for GTP and the Na^+ ion, contoured at 3σ. **C)** The nucleotide binding pocket of eIF5B-D533R bound to GTPγS. In both eIF5B molecules of the asymmetric unit, the side chain of Arg533 is oriented toward the nucleotide and forms a hydrogen bond to Gly^{MC} (in one of the eIF5B molecules, Arg533 adopts a second (alternative) conformation, in which the guanidino group is oriented away from the nucleotide (not shown)). As expected from the disruption of the coordination sphere, no M^+ ion is bound in the active site. Moreover, no water molecule seems to be bound in lieu of the M^+ ion as seen in

the D533A mutant (A). Although the guanidino group in this structure does not form a direct contact to the phosphate moieties of GTPγS, it is conceivable that – particularly at low salt concentrations – Arg533 may transiently adopt a conformation similar to that observed for Arg48, the *cis*-acting arginine-finger, in the GTPase hGBP1 (D) (Ghosh et al, 2006), to allow the observed slightly stimulating effect on the intrinsic GTPase activity (see Fig. 2F). The F_o-F_c omit map is shown for GTPγS, contoured at 3σ. **D)** The nucleotide binding pocket of hGBP1 (with P-loop and switch 1 in brown) bound to the transition state mimic GDP/AlF_3 (PDB: 2B92). Several hydrogen bonds to switch 1 stabilize Arg48 in its active conformation, in which the guanidino group forms direct contacts to GDP and AlF_3. **E-H)** Steady-state fluorescence measurements for the affinity of wild-type eIF5B(517-858) and Asp^{MC} mutants to mant-GTP. Increasing amounts of mant-GTP were titrated to 2 µM wild-type eIF5B (E) or the Asp^{MC} mutants D533A (F), D533N (G) and D553R (H) at 20 °C in buffer containing 25 mM Tris/HCl (pH 7.5), 200 mM KCl, 3 mM $MgCl_2$ and 2 mM DTT. Under these conditions, wild-type eIF5B(517-858) bound mant-GTP with a K_d of 1.7 ± 0.09 µM. A similar value was obtained for the D533N mutant (K_d of 1.5 ± 0.05 µM), while D533A and D533R showed slightly lower K_d values of 1.08 ± 0.05 µM and 0.97 ± 0.06 µM, respectively. The K_d value for each construct was calculated from three independent equilibrium titrations.

Figure S6. Comparison of the effect of different species of M^+ ions on the intrinsic GTPase activity of the *E. coli* EF-Tu D21A mutant (black and white) and wild-type EF-Tu (red and blue). The intrinsic GTPase activity was determined in the presence of 200 mM of the indicated salts under single turnover conditions. The order in which the combinations are given on the right corresponds to the relative rates of GTP hydrolysis. Experiments were repeated two to three times; standard deviations are given by error bars (in some cases not visible because they are smaller than the symbol size).