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Multiple prebiotic metals mediate translation

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ABSTRACT

Today, Mg^{2+} is an essential cofactor with diverse structural and functional roles in life's oldest macromolecular machine, the translation system. We tested whether ancient Earth conditions (low O_2, high Fe^{2+}, high Mn^{2+}) can revert the ribosome to a functional ancestral state. First, SHAPE (Selective 2'-Hydroxyl Acylation analyzed by Primer Extension) was used to compare the effect of Mg^{2+} vs. Fe^{2+} on the tertiary structure of rRNA. Then, we used *in vitro* translation reactions to test whether Fe^{2+} or Mn^{2+} could mediate protein production, and quantified ribosomal metal content. We found that: (i) Fe^{2+} and Mg^{2+} had strikingly similar effects on rRNA folding; (ii) Fe^{2+} and Mn^{2+} can replace Mg^{2+} as the dominant divalent cation during translation of mRNA to functional protein; (iii) Fe^{2+} and Mn^{2+} associated extensively with the ribosome. Given that the translation system originated and matured when Fe^{2+} and Mn^{2+} were abundant, these findings suggest that Fe^{2+} and Mn^{2+} played a role in early ribosomal evolution.

SIGNIFICANCE

Ribosomes are found in every living organism where they are responsible for the translation of messenger RNA into protein. The ribosome's centrality to cell function is underscored by its evolutionary conservation; the core structure has changed little since its inception ~4 billion years ago when ecosystems were anoxic and metal-rich. The ribosome is a model system for the study of bioinorganic chemistry, owing to the many highly coordinated divalent metal cations that are essential to its function. We studied the structure, function, and cation content of the ribosome under early Earth conditions (low O_2, high Fe^{2+}, high Mn^{2+}). Our results expand the roles of Fe^{2+} and Mn^{2+} in ancient and extant biochemistry as a cofactor for ribosomal structure and function.

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Life arose around 4 billion years ago on an anoxic Earth with abundant Fe^{2+} and Mn^{2+}, which would have maintained solubility in the absence of O_2 (1-5). Biochemistry had access to these metals for over a billion years before biological O_2 production was sufficient to oxidize and precipitate them. The pervasive use of these "prebiotic" metals in extant biochemistry, despite barriers to their biological acquisition, likely stems from their importance in the evolution of the early biochemical systems.

The translation system, which synthesizes all coded protein (6), originated and matured during the Archean Eon (4-2.5 Ga) in low-O_2, high-Fe^{2+}, and high-Mn^{2+} conditions (7). The common core of the ribosome, and many other aspects of the translation system, have remained essentially frozen since the last universal common ancestor (8). In extant biochemistry, Mg^{2+} ions are essential for both structure and function of the ribosome (9) and other enzymes involved in translation (10). In ribosomes, Mg^{2+} ions are observed in a variety of structural roles (**Table 1**), including in Mg^{2+}-rRNA clamps (11, 12) (**Fig. 1a**), in dinuclear microclusters that frame the peptidyl transferase center (PTC) (12) (**Fig. 1b**), and at the small subunit-large subunit (SSU-LSU) interface (13) (**Fig. 1c)**. Functional Mg^{2+} ions stabilize a critical bend in mRNA between the P-site and A-site codons (14) (**Fig. 1d**), and mediate rRNA-tRNA and rRNA-mRNA interactions (15) (**Fig. 1e, f**). Accessory enzymes needed for translation – aminoacyl tRNA synthetases, methionyl-tRNA transformylase, creatine kinase, myokinase, and nucleoside-diphosphate kinase – also require Mg^{2+} ions as cofactors (**Table 1**).

There is abundant evidence that multiple types of cationic species can interact productively with various RNAs (16-18). Recent results support a model in which Fe^{2+} and Mn^{2+}, along with Mg^{2+}, were critical cofactors for ancient nucleic acid function. As predicted by this model, functional Mg^{2+}-to-Fe^{2+} substitutions under anoxic conditions were experimentally verified to support RNA folding and catalysis by ribozymes (19, 20), a DNA polymerase, a DNA ligase, and an RNA polymerase (21). Functional Mg^{2+}-to-Mn^{2+} substitution has long been known for DNA polymerase (21-23). In contrast to Mg^{2+}, Fe^{2+} has been shown to more effectively 'activate' RNA. The phosphate affinity of Fe^{2+}, because of the lower energies of its d-orbitals, is slightly greater than that of Mg^{2+}. In first shell coordination complexes, the proximal phosphorus shows greater electron depletion and enhanced susceptibility to nucleophilic attack. Therefore, for at least some nucleic acid processing enzymes, optimal activity is observed at lower concentrations of Fe^{2+} than Mg^{2+} (19, 21).

Based on these previous results, we hypothesized that Fe^{2+} and Mn^{2+} could partially or fully replace Mg^{2+} in the ribosome during translation. In this study, we relocated the translation system to the low-O_2, high-Fe^{2+}, and high-Mn^{2+} environment of its ancient roots, and compared its structure, function, and cation content under modern vs. ancient conditions.

Results

Fe^{2+} folds LSU rRNA to a near-native state. To test whether Fe^{2+} can substitute for Mg^{2+} in folding rRNA to a native-like state, we compared LSU rRNA folding in the presence of Fe^{2+} or Mg^{2+} by SHAPE (Selective 2'-Hydroxyl Acylation analyzed by Primer Extension). SHAPE provides quantitative, nucleotide resolution information about flexibility, base pairing and 3D structure, and has previously been used to monitor the influence of cations, small molecules, or proteins on RNA structure (24-28). We previously used SHAPE to show that the LSU rRNA adopts a near-native state in the presence of Mg^{2+}, with the core inter-domain architecture of the assembled ribosome and residues positioned for interactions with rProteins (29). In this study, SHAPE experiments were performed in an anoxic chamber to maintain the oxidation state and solubility of Fe^{2+}. The minimum concentration required to fully fold rRNA, 2.5 mM Fe^{2+} or 10 mM Mg^{2+}, was used for all SHAPE experiments (**Additional Data Table S1**).

Addition of Fe^{2+} or Mg^{2+} induced widespread structural changes in the Na^{+}-form LSU rRNA, reflected in SHAPE reactivity as ΔFe^{2+} or ΔMg^{2+} (see **Materials and Methods**) and displayed as 'heat maps' on the LSU rRNA secondary structure (**Fig. 2**). The ΔFe^{2+} and ΔMg^{2+} heat maps were broadly similar at the nucleotide and regional scales (**Fig. 2d**, **e)**, indicating similar effects of Fe^{2+} or Mg^{2+} on rRNA folding. Helical regions tended to be invariant, whereas rRNA loops and bulges were impacted by addition of Fe^{2+} or Mg^{2+}. Of the sites that exhibited a significant response (>0.3 SHAPE units) to Mg^{2+}, 86% of nucleotides (43/50) exhibited a similar trend with Fe^{2+}. The greatest discrepancy between Fe^{2+} and Mg^{2+} is observed in the L11 binding domain (**Fig. 2d, e**).

Fe^{2+} and Mn^{2+} mediate translation. Translation reactions were performed in an anoxic chamber in the presence of different divalent cations and cation concentrations. Production of the protein dihydrofolate reductase (DHFR) from its mRNA was used to monitor translational activity. Protein synthesis was assayed by measuring the rate of NADPH oxidation by DHFR. These reactions were conducted in a small background of 2.5 mM Mg^{2+} (**Fig. S1a**). This background is below the requirement to support translation, consistent with previous findings that a minimum of ~5 mM Mg^{2+} is needed for assembly of mRNA onto the SSU (30-32). As a control, we recapitulated the previously established Mg^{2+} dependence of the translation system, and then repeated the assay with Fe^{2+}.

The activity profile of the translation system with varied Fe^{2+} closely tracked the profile with varied Mg^{2+} (**Fig. 3**). Below 7.5 mM total divalent cation concentration, minimal translation occurred with either Fe^{2+} or Mg^{2+}, as observed elsewhere (33). Activity peaked at 9.5 mM for both cations and decreased modestly beyond the optimum. At a given divalent cation concentration, Fe^{2+} supported around 50-80% of activity with Mg^{2+}. This result was observed with translation reactions run for 15, 30, 45, 60, 90 and 120 min with optimal divalent cation concentrations (**Fig. 4**). Along with Mg^{2+} and Fe^{2+}, we investigated the abilities of other divalent cations to support translation, including Mn^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}. We found that Mn^{2+} supported translation at levels similar to Fe^{2+}, whereas no activity was detected with Co^{2+}, Cu^{2+}, or Zn^{2+} (**Fig. S2**).

To test whether alternative divalent cations could completely replace Mg^{2+} in translation, we decreased the background Mg^{2+} from 2.5 to 1 mM by thoroughly washing the ribosomes prior to translation reactions with 7-11 mM Fe^{2+} or Mn^{2+} (**Fig. S1b**). With 1 mM background Mg^{2+}, Fe^{2+} supported 12-23% of the activity with Mg^{2+} over the concentrations tested, while Mn^{2+} supported 43-50% activity relative to Mg^{2+} (**Fig. 5a**). Washing the factor mix allowed us to decrease the background Mg^{2+} in translation reactions to ~4-6 μM (**Fig. S1c**). At this level, minimal protein production was observed with Fe^{2+}, while Mn^{2+} supported 29-38% of the activity measured with Mg^{2+} (**Fig. 5b**).

Fe^{2+} and Mn^{2+} associate extensively with the ribosome. To experimentally confirm that Fe^{2+} and Mn^{2+} associate with the assembled ribosome, we analyzed the Fe^{2+} and Mn^{2+} content of ribosomes after incubation in anoxic reaction buffer containing 7 mM Fe^{2+} or 7 mM Mn^{2+}. Under the conditions of our translation reactions, 584 ± 9 Fe^{2+} ions or 507 ± 28 Mn^{2+} ions associate with each ribosome.

Finally, we computationally investigated whether Mg^{2+}, Fe^{2+}, and Mn^{2+} might be interchangeable during translation using quantum mechanical characterization of M^{2+}-rRNA clamps, which are frequent in the ribosome (11, 12) **(Fig. 1a, S3).** The geometries of Fe^{2+}-rRNA clamps and Mg^{2+}-rRNA clamps are nearly identical (**Table S1**). However, due to the accessibility of its d-orbitals, more charge is transferred to Fe^{2+} (**Table S2**), leading to greater stability of Fe^{2+}-rRNA vs. Mg^{2+}-rRNA clamps **(Table S3)**. The effect of the modestly greater radius of Mn^{2+} (**Table S1)** is offset by d-orbital charge transfer **(Table S2)**, leading to comparable stabilities of Mg^{2+}-rRNA and Mn^{2+}-rRNA clamps (**Table S3).**

Discussion

130 In this study, we successfully replaced ribosomal Mg^{2+} with Fe^{2+} or Mn^{2+} under conditions mimicking the
131 anoxic Archean Earth. Previously, the only cation known to mediate rRNA folding and function of the
132 translation system was Mg^{2+}. We found that rRNA folds to the same extent, and to essentially the same
133 global state (34, 35), with Fe^{2+} or Mg^{2+} under anoxia. The minimum concentration of Fe^{2+} required to fold
134 the rRNA is 4-fold less than the requisite Mg^{2+} concentration. We showed that Fe^{2+} or Mn^{2+} can serve as
135 the dominant divalent cation during translation. Background Mg^{2+} as high as 2.5 mM was insufficient to
136 mediate protein synthesis, and at least 5 mM additional Mg^{2+}, Fe^{2+}, or Mn^{2+} was required to restore
137 translational activity. Further, we demonstrated that Mn^{2+} can mediate translation in place of Mg^{2+},
138 suggesting that Mn^{2+} can mediate each of the M^{2+}-RNA interactions in **Fig. 1**. These findings suggest that
139 functional Mg^{2+}-Mn^{2+} and Mg^{2+}-Fe^{2+} substitutions can occur in large ribozymes, similar to previous reports
140 for protein enzymes and small ribozymes (21-23, 36, 37). Near-complete removal of Mg^{2+} prevented Fe^{2+}-
141 mediated translation and partially inhibited Mn^{2+}-mediated translation, suggesting that Mg^{2+} is optimal for
142 some specific roles in the translation system. Regardless, the general effectiveness of Mg^{2+}-Mn^{2+} and Mg^{2+}-
143 Fe^{2+} substitutions in the translation system is astounding considering the enormous number of divalent
144 cations associated with a given ribosome, and the diversity of their structural and functional roles (9, 10)
145 (**Table 1, Fig. 1**).

146 The observation that >500 Fe^{2+} or Mn^{2+} ions can associate with a ribosome is consistent with the extent of
147 Mg^{2+} association observed by x-ray diffraction (100-1000 Mg^{2+} per ribosome (38)), and supports a model
148 in which Fe^{2+} or Mn^{2+} has replaced Mg^{2+} as the dominant divalent cation in our experiments. The high
149 capacity of ribosomes for Fe^{2+} and Mn^{2+} reflects all ribosome-associated ions, including condensed, glassy
150 and chelated divalent cations (39), and possibly also ions associated with ribosomal proteins, such as those
151 previously shown to bind Zn^{2+} (e.g. S2, S15, S16, S17, L2, L13, L31, L36 in *E. coli*) (40).

152 The differences in protein production observed among the three divalent cations in this study arise from a
153 variety of evolutionary, and physiological factors. For instance, *E. coli* may be evolutionarily adapted to
154 use Mg^{2+} instead of Fe^{2+} or Mn^{2+} in ribosomes. The difference in translational activity between Mn^{2+} and
155 Fe^{2+}, particularly when background Mg^{2+} was removed, suggests that Mn^{2+} is more viable upon full
156 substitution. Such Mn^{2+}/Mg^{2+} interchangeability may result from the comparable stabilities of Mn^{2+} and
157 Mg^{2+} in M^{2+}-rRNA clamps (**Fig. S3**). An additional contribution to the lower net effectiveness of Fe^{2+} in
158 translation may be due to its higher redox activity; indeed, Fe^{2+}-mediated oxidative damage to RNA has
159 been observed in ribosomes purified from Alzheimer hippocampus (41).
160
161 Besides the ribosome, our translation reactions utilize many accessory enzymes such as elongation factors
162 and aminoacyl-tRNA synthetases that also have divalent cation requirements. Therefore, both Mn^{2+} and
163 Fe^{2+} may be fully functional ribosomal substitutes with different effectiveness caused by lower activity of
164 one or more of these accessory enzymes. Indeed, the relative activity of arginine t-RNA synthetase and
165 myokinase are both lower with Mn^{2+} or Fe^{2+} than with Mg^{2+} (42, 43).
166
167 While intracellular Mg^{2+} is consistently 10^{-3} M in cellular life (44), specific physiological or environmental
168 conditions can significantly elevate intracellular Fe^{2+} and Mn^{2+}. Under oxidative stress, some microbes
169 accumulate excess Mn^{2+} (e.g. radiation-tolerant *Deinococcus radiodurans* contains ~10 times higher Mn^{2+}
170 than *E. coli* (~10^{-5} M Mn^{2+} (45, 46))). In the absence of oxygen, *E. coli* contains ~10 times higher labile
171 Fe^{2+} (~10^{-4} M) than when grown with oxygen (~10^{-5} M (47)). Thus, it is possible that the absence of Fe^{2+}
172 and Mn^{2+} in experimentally determined ribosomal structures is reflective of culturing and purification
173 conditions (high O_2, high Mg^{2+}, low Fe^{2+}, and low Mn^{2+}), and that other cations may also be present under
174 diverse physiological conditions.

175 We have shown that the translation system functions with mixtures of divalent cations, which are variable
176 during long-term evolutionary processes and during short term changes in bioavailability and oxidative
177 stress. When combined with previous results that DNA replication and transcription can be facilitated by
178 Fe^{2+} and Mn^{2+} (16-23, 36, 37), our findings that Fe^{2+} can mediate rRNA folding and that both Fe^{2+} and Mn^{2+}

179 can support translation of active protein has revealed that these prebiotically relevant metals can facilitate
180 the entire central dogma of molecular biology (DNA→RNA→protein). These findings raise important
181 questions about evolutionary and physiological roles for Fe^{2+} and Mn^{2+} in ancient and extant biological
182 systems. Were Mg^{2+}, Fe^{2+} and Mn^{2+} collaborators as cofactors on the ancient Earth, when Fe^{2+} and Mn^{2+}
183 were more abundant (1-5), and Mg^{2+} was less abundant (2), than today? What was the role of Fe^{2+} and Mn^{2+}
184 in the origin and early evolution of the translational system? Finally, what are the implications for ribosome-
185 bound Fe^{2+} in oxidative damage and disease?

186 **Materials and Methods**
187
188 **rRNA folding via SHAPE.** SHAPE methods were adapted from previously published protocols (29) using
189 the intact ~2900 nt *Thermus thermophilus* 23 rRNA (LSU) in the presence of 250 mM Na^+, which favors
190 formation of secondary structure, and in 250 mM Na^+ plus a divalent cation (10 mM Mg^{2+} or 2.5 mM Fe^{2+},
191 both as chloride salts) to favor formation of tertiary interactions. Adaptations were made to facilitate folding
192 and N-methylisatoic anhydride modification of RNA in an anoxic chamber under a 98% Ar and 2% H_2
193 atmosphere. To keep rRNA samples oxygen-free, rRNA samples in 200 mM NaOAc, 50 mM NaHEPES
194 (pH 8) were lyophilized, transferred into the anoxic chamber, and rehydrated with nuclease-free, degassed
195 water. After rRNA modification, as described in ref. (29), divalent cations were extracted from the rRNA
196 by chelating beads. Samples were then removed from the anoxic chamber for analysis by capillary
197 electrophoresis (29). Nucleotides were classified as exhibiting a significant change in SHAPE reactivity if
198 either the original reactivity (in Na^+) or final reactivity (in Na^+/Mg^{2+} or Na^+/Fe^{2+}) was >0.3 SHAPE units.
199 To compare the Fe^{2+}- and Mg^{2+}-responsiveness of specific nucleotides, we binned nucleotides into three
200 categories (increased, decreased, or little/no change) based on their general SHAPE reactivity response to
201 each divalent cation (SHAPE data are found in **Additional Data Table S1**).

202 ***In vitro* translation.** Each 30 μL reaction contained 2 μM (4.5 μL of 13.3 μM stock) *E. coli* ribosomes in
203 10 mM Mg^{2+} (New England Biolabs, Ipswich MA, USA; catalog # P0763S), 3 μL factor mix (with RNA
204 polymerase, and transcription/translation factors in 10 mM Mg^{2+}) from the PURExpress® Δ Ribosome Kit
205 (New England Biolabs E3313S), 0.1 mM amino acid mix (Promega, Madison WI, USA; catalog # L4461),
206 and 0.2 mM tRNAs from *E. coli* MRE 600 (Sigma-Aldrich, St. Louis MO, USA; product # TRNAMRE-
207 RO). Thus, a total of 2.5 mM "background" Mg^{2+} was present in each reaction (**Fig. S1a**). To remove the
208 background Mg^{2+}, we exchanged the buffer of the ribosome and factor mix using centrifugal filter units.
209 Thirty microliters of either ribosome solution or factor mix was added to an Amicon Ultra 0.5 mL
210 centrifugal filter (Millipore-Sigma), followed by 450 μL of divalent-free buffer (20 mM HEPES pH 7.6, 30
211 mM KCl, and 7 mM β-mercaptoethanol). Samples where spun at 14,000 x g at 4°C until the minimum
212 sample volume (~15 μL) was reached. The samples were resuspended in 450 μL of divalent-free buffer and
213 centrifugation was repeated. The samples were then transferred to new tubes and 15 μL of divalent-free
214 buffer was added to bring the volume to 30 μL. This process decreased Mg^{2+} concentrations in the ribosome
215 and factor mix from 10 mM to 10-30 μM Mg^{2+}, resulting in 4-6 μM Mg^{2+} in each reaction (**Fig. S1b, S1c)**.
216
217 **Translation reaction buffer.** The reaction buffer was based on Shimizu et al. (48), with HEPES-OH
218 instead of phosphate buffer to avoid precipitation of metal phosphates. We found that rates of translation
219 were consistently lower with Tris-HCl than HEPES-OH, and therefore HEPES-OH was used as the buffer
220 for all experiments. Buffer consisted of 20 mM HEPES-OH (pH 7.3), 95 mM potassium glutamate, 5 mM
221 NH_4Cl, 0.5 mM $CaCl_2$, 1 mM spermidine, 8 mM putrescine, 1 mM dithiothreitol (DTT), 2 mM adenosine
222 triphosphate (ATP), 2 mM guanosine triphosphate (GTP), 1 mM uridine triphosphate (UTP), 1 mM cytidine
223 triphosphate (CTP), 10 mM creatine phosphate (CP), and 53 μM 10-formyltetrahydrofolate. The reaction
224 buffer was lyophilized and stored at -80°C until resuspension in anoxic nuclease-free water immediately
225 before experiments in an anoxic chamber.
226

Translation experimental conditions. All reactions (30 μL total volume) were assembled and incubated in the anoxic chamber. All divalent cation salts used in experiments ($MgCl_2$, $FeCl_2$, $MnCl_2$, $Zn(OAc)_2$, $CoCl_2$, $CuSO_4$) were added to 7 mM final concentration, with the exception of $MgCl_2$ and $FeCl_2$, which were tested over a range of final concentrations (1, 3, 4, 5, 6, 7, 8, 9, and 11 mM; **Fig. S1**). Solutions were clear, with no indication of metal precipitate, suggesting that reduced, divalent metals cations were the primary chemical species. All experiments were assembled in the following order: dihydrofolate reductase (DHFR) mRNA (~5 μg per 30 μL reaction; see **Supplemental Information** for more details), factor mix, ribosomes, amino acids, tRNA, nuclease-free H_2O, reaction buffer. Changing the order of reactant addition did not affect translational activity. Reactions were run in triplicate on a 37°C heat block for up to 120 minutes. Reactions were quenched on ice and stored on ice until they were assayed for protein synthesis.

Protein activity assay. Protein synthesis was measured using a DHFR assay kit (Sigma-Aldrich product # CS0340), which measures the oxidation of NADPH (60 mM) to $NADP^+$ by dihydrofolic acid (51 μM). Assays were performed by adding 5 μL of protein synthesis reaction to 995 μL of 1x assay buffer. The NADPH absorbance peak at 340 nm (Abs_{340}) was measured in 15 s intervals over 2.5 min. The slope of the linear regression of Abs_{340} vs. time was used to determine protein activity (Abs_{340} min^{-1}). Different counter ions (Cl^-, CH_3COO^-, SO_4^{2-}) had no effect on protein synthesis from mRNA. To our knowledge, no dependence on, nor inhibitory effect of Mg^{2+} or Fe^{2+} exists for DHFR. We confirmed this by varying the amounts of metals in our assay reaction, which had no effect on DHFR activity (data not shown).

Ribosome metal content. The Fe and Mn content of *E. coli* ribosomes was measured by total reflection x-ray fluorescence (TRXF) spectroscopy after the ribosomes were incubation in 7 mM $FeCl_2$ or 7 mM $MnCl_2$. Detailed methods are provided in the **Supplemental Information**.

Quantum mechanical calculations. The atomic coordinates of a Mg^{2+}-rRNA clamp were initially extracted from the X-ray structure of the *Haloarcula marismortui* large ribosomal subunit (PDB 1JJ2) (49). The free 5′ and 3′ termini of the phosphate groups were capped with methyl groups in lieu of the remainder of the RNA polymer, and hydrogen atoms were added, where appropriate (**Fig. S3**). The details of calculations were adapted from previous publications (11, 19) and are provided in the **Supplemental Information**.

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Author contributions: MSB, TKL, JCB, ARR, NVH, LDW, and JBG conceived and designed the experiments. MSB and TKL collected data and performed analysis. ASP contributed analysis. MSB, TKL, LDW, and JBG wrote the paper with editorial input from all authors.

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Table 1. Structural and functional roles for select divalent cations in the translation system. All biomolecules in the table have been shown to require Mg^{2+} and may also be active with Fe^{2+} or Mn^{2+}. "n.a." indicates that data are not available.

Translation system component(s)	Location of divalent ion	Role of divalent cation	Optimal $[Mg^{2+}]$ (mM)
	Ribosome		
LSU/SSU	M^{2+}-rRNA clamps (11)	Mediates and maintains folding/structure of rRNAs	~10 (31)
LSU	Dinuclear microclusters (12)	Frames peptidyl transferase center (PTC)	~10 (31)
LSU/SSU	LSU/SSU interface (24)	Mediates docking of mRNA to SSU and association of SSU with LSU	~10 (31)
SSU/mRNA	Critical bend in mRNA between the P-site and A-site codons (15, 50)	Maintains correct reading frame mRNA	~10 (31)
A-site tRNA/ P-site tRNA	tRNA-tRNA interface (24)	Stabilize tRNAs in the PTC	~10 (31)
LSU/tRNA	rRNA-tRNA interface (24)	Stabilize rRNA-tRNA in the PTC	~10 (31)
	Auxiliary		
EF-Tu	GTP binding site (51)	Stabilizes the transition state	5-15 (52)
EF-G	GTP binding site (53)	Stabilizes the transition state	n.a.
Aminoacyl-tRNA synthetases	ATP binding site (54)	Stabilizes the transition state	>1 (55)
Methionyl-tRNA transformylase	ATP binding site (56)	Stabilizes the transition state	7 (56)
Creatine kinase	NTP binding site (57)	Stabilizes the transition state	~5 (57)
Myokinase	Acceptor NDP binding site (58)	Stabilizes the transition state	~3 (43)
Nucleoside-diphosphate kinase	NTP binding site (59)	Stabilizes the transition state	>1
Pyrophosphatase	Active site (60)	Stabilizes the transition state	>7 (61)

396

397 **Figure Legends**
398
399 **Fig. 1.** Divalent cations serve many structural and functional roles in the ribosome. Mg^{2+} ions: **a)** form
400 bidentate clamps with adjacent phosphate groups of rRNA (beige carbon atoms), **b)** form dinuclear
401 microclusters that frame the rRNA of the peptidyl transferase center, **c)** stabilize the LSU-SSU interface,
402 **d)** stabilize a functional kink in mRNA (green), **e)** stabilize association of tRNA (teal) with 23S rRNA, and
403 **f)** stabilize association of mRNA with 16S rRNA. Thick dashed lines are first shell RNA interactions of
404 Mg^{2+}. Dotted lines indicate second shell interactions. Images are of the *Thermus thermophilus* ribosome
405 (PDB ID: 1VY4) because the structural information available for *T. thermophilus* ribosomes is more
406 accurate than for *E. coli*. This figure was generated with the program RiboVision (62).

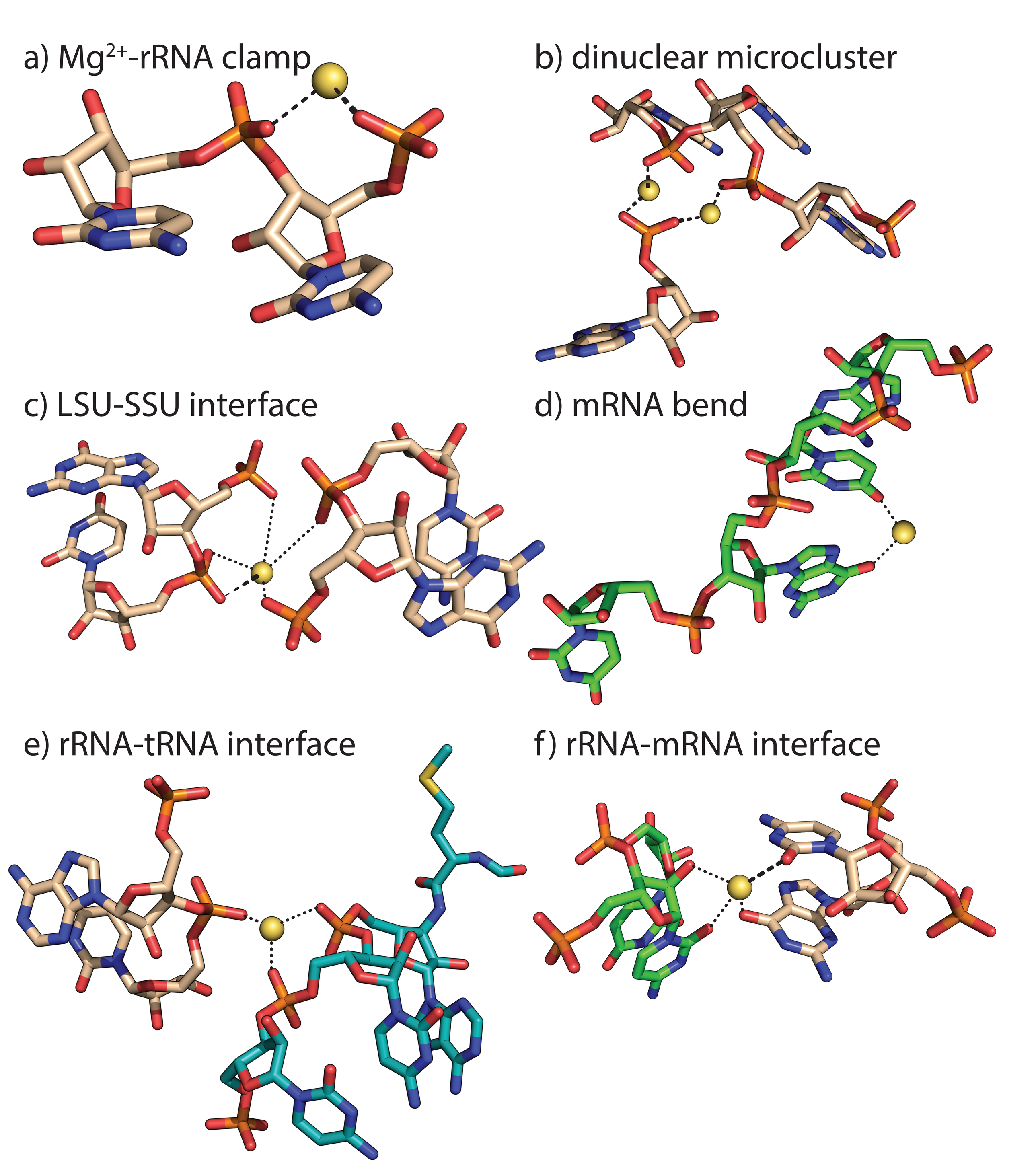
407 **Fig. 2.** SHAPE reactivities mapped onto the LSU rRNA secondary structure in **a)** Na^+, **b)** Na^+/Fe^{2+}, or **c)**
408 Na^+/Mg^{2+}. Key functional elements are labeled in panel a, and the scale in panel (a) applies to panels b and
409 c. **d)** Fe^{2+}-induced changes (ΔFe^{2+}) in SHAPE reactivity calculated by subtracting Na^+ data from Na^+/Fe^{2+}
410 data for each nucleotide, and **e)** Mg^{2+}-induced changes (ΔMg^{2+}) in SHAPE reactivity calculated by
411 subtracting Na^+ data from Na^+/Mg^{2+} data for each nucleotide. The scale shown for panel d also applies to
412 panel e. Positive values indicate increased SHAPE reactivity in presence of the divalent cation, while
413 negative values denote decreased reactivity. Regions where data are not available (5' and 3' ends) are grey.
414 These figures were generated with the program RiboVision (62). *Thermus thermophilus* rRNA was used as
415 the analytical model for SHAPE experiments because the structural information is more accurate than for
416 *E. coli*. The L11 binding region, where the greatest discrepancy between Fe^{2+} and Mg^{2+} is observed, is
417 indicated with an arrow.

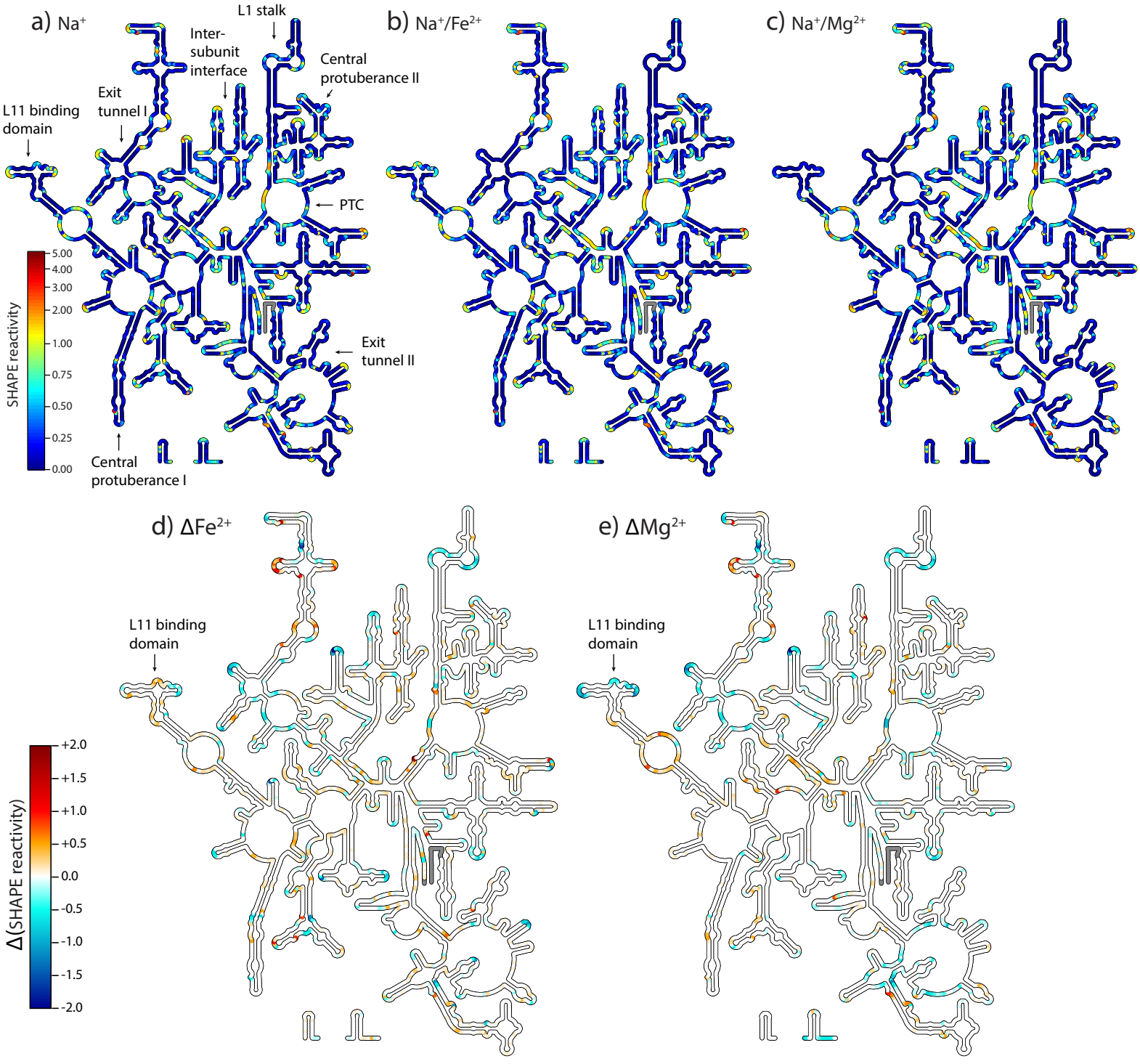
418 **Fig. 3**. Mg^{2+} and Fe^{2+} stimulate translational activity over a range of concentrations. The activity of the
419 translation product (dihydrofolate reductase, which catalyzes the oxidation of NADPH, with a maximum
420 absorbance at 340 nm) was used as a proxy for protein production. Translation reactions were run for 120
421 minutes. All translation reactions contained 2.5 mM background Mg^{2+}, to which varying amounts of
422 additional Mg^{2+} or Fe^{2+} were added. The error bars for triplicate experiments (N=3) are plotted as the
423 standard error of the mean.

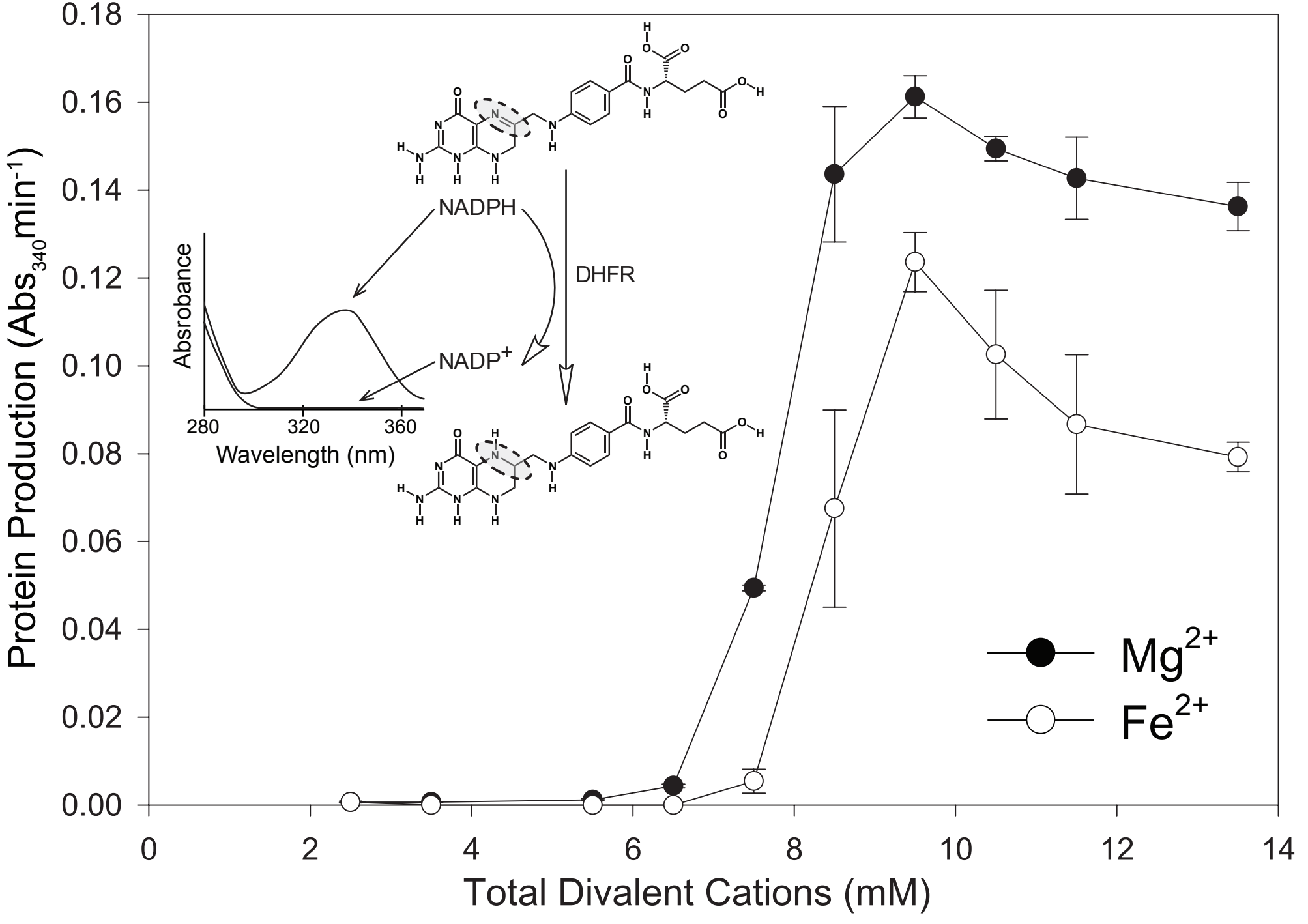
424 **Fig. 4.** Fe^{2+} consistently supports 50-80% of the translational activity as Mg^{2+} when the translation
425 experiments are run for 15-120 minutes. The activity of the translation product (dihydrofolate reductase,
426 which catalyzes the oxidation of NADPH, with a maximum absorbance at 340 nm) was used as a proxy for
427 protein production. All translation reactions contained 2.5 mM background Mg^{2+}, to which 7 mM additional
428 Mg^{2+} or Fe^{2+} were added, totaling to 9.5 mM divalent cation. The error bars for triplicate experiments (N=3)
429 are plotted as the standard error of the mean.

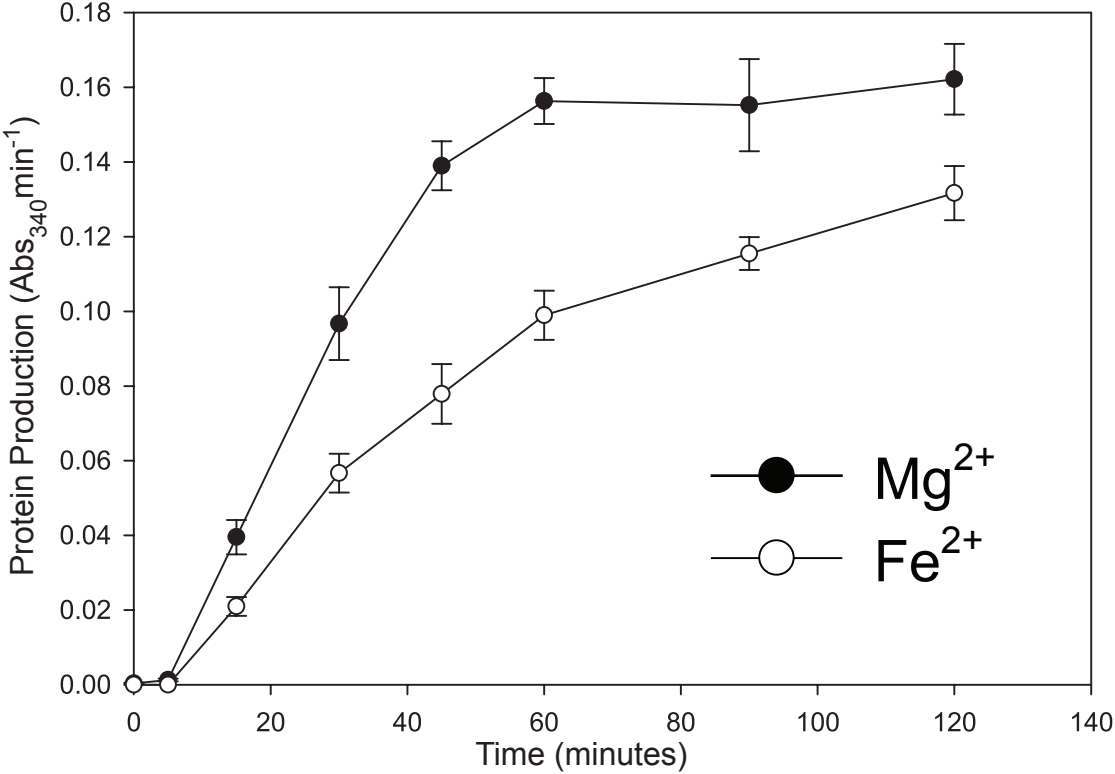
430 **Fig. 5.** Mn^{2+} can support translation after removal of background Mg^{2+}. **a)** Reactions prepared with washed
431 *E. coli* ribosomes, reducing the background Mg^{2+} to 1 mM, to which 7, 9, or 11 mM additional Mg^{2+}, Fe^{2+},
432 or Mn^{2+} were added, totaling 8, 10, or 12 mM divalent cation (M^{2+}). **b)** Reactions prepared using washed
433 *E. coli* ribosomes and washed factor mix, which reduced the background Mg^{2+} to the low µM level, to
434 which 8, 10, or 12 mM additional, Mg^{2+}, Fe^{2+}, or Mn^{2+} were added. The activity of the translation product
435 (dihydrofolate reductase, which catalyzes the oxidation of NADPH, with a maximum absorbance at 340
436 nm) was used as a proxy for protein production. The error bars for triplicate experiments (N=3) are plotted
437 as the standard error of the mean.

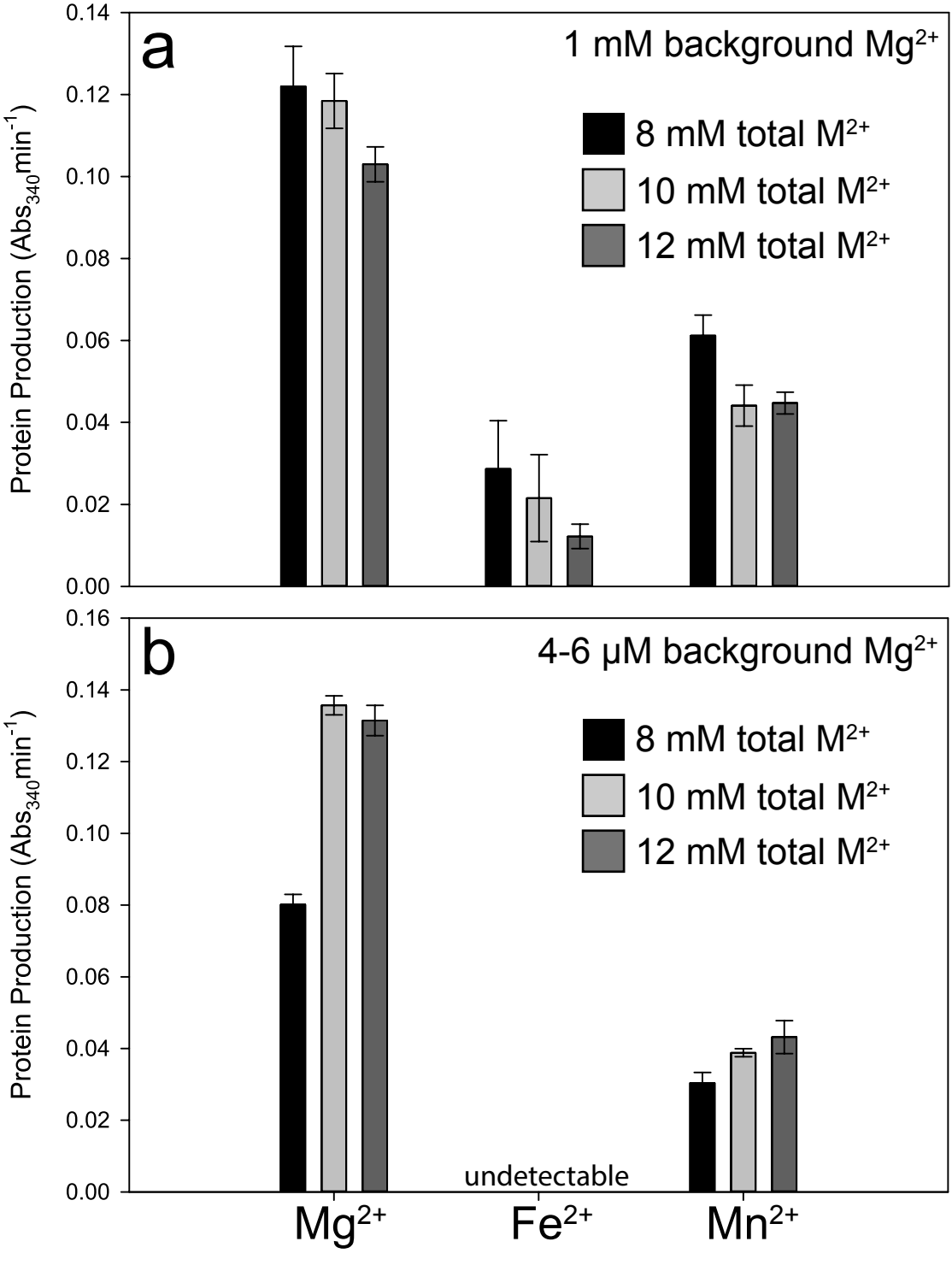
438


a) Mg^{2+}-rRNA clamp
b) dinuclear microcluster
c) LSU-SSU interface
d) mRNA bend
e) rRNA-tRNA interface
f) rRNA-mRNA interface


a) Na+
L1 stalk
Inter-
subunit
interface
Central
protuberance II
Exit
tunnel I
L11 binding
domain
PTC
Exit
tunnel II
Central
protuberance I
SHAPE reactivity
5.00
4.00
3.00
2.00
1.00
0.75
0.50
0.25
0.00
b) Na+/Fe2+
c) Na+/Mg2+
d) ΔFe2+
L11 binding
domain
e) ΔMg2+
L11 binding
domain
Δ(SHAPE reactivity)
+2.0
+1.5
+1.0
+0.5
0.0
-0.5
-1.0
-1.5
-2.0


Protein Production (Abs340min-1)
Total Divalent Cations (mM)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0
2
4
6
8
10
12
14
Mg2+
Fe2+
NADPH
DHFR
NADP+
Absrobance
Wavelength (nm)
280
320
360


0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Protein Production (Abs340min-1)
0
20
40
60
80
100
120
140
Time (minutes)
Mg2+
Fe2+


a
1 mM background Mg2+
8 mM total M2+
10 mM total M2+
12 mM total M2+
Protein Production (Abs340min-1)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
b
4-6 μM background Mg2+
8 mM total M2+
10 mM total M2+
12 mM total M2+
Protein Production (Abs340min-1)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
undetectable
Mg2+
Fe2+
Mn2+