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4 **Cutting in-line with iron: ribosomal function and non-oxidative RNA cleavage**

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24

25 **Abstract**

26 Divalent metal cations are essential to the structure and function of the ribosome. Previous  
27 characterizations of ribosome structure and function performed under standard laboratory  
28 conditions have implicated  $Mg^{2+}$  as the primary mediator of ribosomal structure and function.  
29 The contribution of  $Fe^{2+}$  as a ribosomal cofactor has been largely overlooked, despite the  
30 ribosome's evolution in a high  $Fe^{2+}$  environment, and its continued use by obligate anaerobes  
31 inhabiting high  $Fe^{2+}$  niches. Here we show that (i) iron readily cleaves RNA by a non-oxidative  
32 mechanism that has not been detected previously, (ii) functional ribosomes purified from cells  
33 grown under low  $O_2$ , high  $Fe^{2+}$  conditions are associated with  $Fe^{2+}$ , (iii) a small subset of  $Fe^{2+}$   
34 that is associated with the ribosome is not exchangeable with surrounding cations, presumably  
35 because they are highly coordinated by rRNA. In total, these results expand the ancient role of  
36 iron in biochemistry, suggest a novel method for regulation of translation by iron, and highlight a  
37 possible new mechanism of iron toxicity.

38 **Key Points:**

39 1) iron readily cleaves RNA by a non-oxidative mechanism that has not been detected  
40 previously;  
41 2) functional ribosomes purified from cells grown under low  $O_2$ , high  $Fe^{2+}$  conditions are  
42 associated with  $Fe^{2+}$ ;  
43 3) a small subset  $Fe^{2+}$  that is associated with the ribosome is not exchangeable.

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## 51 Introduction

52 The ribosome is responsible for the synthesis of all coded proteins and contains life's most  
53 conserved ribonucleic acids. The common core of the ribosome is universal to all life (1,2) and  
54 has been essentially invariant since the last universal common ancestor (3-5). Thus, ribosomes  
55 can be interrogated as molecular fossils (6-8). Because ribosomal structure and function are  
56 strongly dependent on divalent cations ( $M^{2+}$ ) (9), and because ribosomes originated long before  
57 the Great Oxidation Event (GOE), understanding ribosomal origins and evolution requires  
58 characterization of ribosomal interactions with  $M^{2+}$  ions under pre-GOE conditions (10-14).

59 In extant aerobic life,  $Mg^{2+}$  appears to be the dominant  $M^{2+}$  ion in the translation system.  
60 Hundreds of  $Mg^{2+}$  ions mediate ribosomal RNA (rRNA) folding and ribosomal assembly, in  
61 some instances binding to specific sites in the universal rRNA common core by direct  
62 coordination (9,15-17).  $Mg^{2+}$  ions facilitate association of the large ribosomal subunit (LSU) and  
63 small ribosomal subunit (SSU) (18), stabilize folded tRNA (19), maintain the reading frame  
64 during translation (20), and link ribosomal proteins (rProteins) to rRNA (21).

65 Before the GOE, anoxia would have stabilized abundant  $Fe^{2+}$  in the biosphere and hydrosphere.  
66 Under pre-GOE conditions,  $Fe^{2+}$  would not have caused the oxidative damage to biomolecules  
67 that occurs today in the presence of  $O_2$ , via Fenton chemistry (22). We recently reported that  $Fe^{2+}$   
68 can mediate *in vitro* translation under "pre-GOE" conditions: in the presence of abundant  $Fe^{2+}$   
69 and in the absence of  $O_2$  (23,24). Based on these findings, we proposed that early ribosomal  
70 folding and catalysis used  $Fe^{2+}$  instead of, or in combination with  $Mg^{2+}$  and other  $M^{2+}$  ions.  
71 However, our observation of lower translation rates with anoxic  $Fe^{2+}$  than with  $Mg^{2+}$  (23)  
72 suggests that  $Fe^{2+}$  might mediate non-oxidative damage of RNA at faster rates than  $Mg^{2+}$ .

73 Here we demonstrate that  $Fe^{2+}$  can damage RNA, and the ribosome, by two separate and distinct  
74 mechanisms. The first mechanism is the well-known Fenton reaction (22) whereby the reaction  
75 of  $Fe^{2+}$  with  $O_2$  or  $H_2O_2$  generates hydroxyl radicals, causing oxidative damage of nucleic acids  
76 (25-29). A second, non-oxidative mechanism of  $Fe^{2+}$ -mediated RNA damage can be more  
77 extensive in some conditions than oxidative damage. We have discovered that in-line cleavage of  
78 RNA is catalyzed by  $Fe^{2+}$  by promoting the attack of a ribose 2'-hydroxyl group on the proximal  
79 phosphorous. The catalysis of in-line cleavage by  $Mg^{2+}$  is well established (30,31).  $Mg^{2+}$ -  
80 mediated in-line cleavage has been used, for example, to detect changes in RNA conformation  
81 upon binding of target compounds to riboswitches (32,33). Other metals are known to cleave  
82 RNA by non-oxidative processes. Europium, lead, or terbium have been used to monitor RNA  
83 folding or to identify metal binding sites (34-37). Here we show that anoxic  $Fe^{2+}$  is efficient in  
84 catalyzing in-line cleavage, cleaving RNA far more rapidly and extensively than  $Mg^{2+}$ .

85 We have also investigated whether ribosomes in *E. coli* grown in pre-GOE conditions associate  
86 functionally with  $Fe^{2+}$  *in vivo*. We have grown *E. coli* in anoxic conditions with ample  $Fe^{2+}$  in the  
87 growth media. We have purified ribosomes from these bacteria and have probed their  
88 interactions with metals. We have identified tightly bound  $M^{2+}$ , which survive ribosomal  
89 purification. A small subset of  $Fe^{2+}$  ions are not exchangeable with  $Mg^{2+}$  in solution and are  
90 detectable after purification involving repeated washes in high  $[Mg^{2+}]$  buffers. We use these  
91 tightly bound ions as reporters for more general  $M^{2+}$  association *in vivo*. The data are consistent  
92 with a model in which certain  $M^{2+}$  ions are deeply buried and highly coordinated within the  
93 ribosome (16). Indeed, our results suggest that ribosomes grown in pre-GOE conditions contain

94 ~10 tightly bound  $\text{Fe}^{2+}$  ions compared to ~1  $\text{Fe}^{2+}$  ion in ribosomes from standard growth  
95 conditions. Ribosomes washed with  $\text{Fe}^{2+}$  contained significantly higher  $\text{Fe}^{2+}$  and showed more  
96 rRNA degradation than ribosomes washed with  $\text{Mg}^{2+}$ . Our combined results show the capacity  
97 for  $\text{Fe}^{2+}$  to (i) associate with functional ribosomes *in vivo* and *in vitro* and (ii) mediate significant  
98 non-oxidative damage.

## 99 Materials and Methods

100 *Cell culture and harvesting.* Culturing media consisted of LB broth (10 g L<sup>-1</sup> NaCl, 10 g L<sup>-1</sup>  
101 tryptone, 5 g L<sup>-1</sup> yeast extract) amended with 4 mM tricine, 50 mM sodium fumarate, and 80 mM  
102 3-(N-morpholino)propanesulfonic acid (MOPS; pH 7.8). Fifty mL cultures containing all of  
103 these ingredients plus 0.25% v/v glycerol were inoculated from glycerol stocks of *Escherichia*  
104 *coli* MRE600 cells and shaken overnight at 37°C with or without O<sub>2</sub> and with either 1 mM  
105 FeCl<sub>2</sub> or ambient Fe<sup>2+</sup>[6-9 μM, measured by the ferrozine assay (38)]. Two mL of each overnight  
106 culture was used to inoculate 1-L cultures in the same conditions. These cultures were then  
107 orbitally shaken at 37°C to OD<sub>600</sub> 0.6-0.7. Aerobic cultures were grown in foil-covered  
108 Erlenmeyer flasks. Anaerobic fumarate-respiring cultures were inoculated into stoppered glass  
109 bottles containing medium that had been degassed with N<sub>2</sub> for one hour to remove O<sub>2</sub>. Cells were  
110 then harvested by centrifugation at 4,415 x g for 10 minutes, washed in 20 mL buffer containing  
111 10 mM Tris pH 7.4, 30 mM NaCl, and 1 mM EDTA, and pelleted at 10,000 x g for 10 minutes.  
112 Cell pellets were stored at -80°C until ribosome purification.

113 *Ribosome purification.* The ribosome purification procedure was modified from Maguire et. al  
114 (39). All purification steps were performed in a Coy anoxic chamber (97% Ar, 3% H<sub>2</sub>  
115 headspace) unless otherwise noted. Buffers varied in their metal cation content. The typical wash  
116 buffer contained 100 mM NH<sub>4</sub>Cl, 0.5 mM EDTA, 3 mM β-mercaptoethanol, 20 mM Tris pH 7.5,  
117 3 mM MgCl<sub>2</sub>, and 22 mM NaCl. For “Fe purification” experiments, buffer was composed of 100  
118 mM NH<sub>4</sub>Cl, 0.5 mM EDTA, 3 mM β-mercaptoethanol, 20 mM Tris pH 7.5, 1 mM FeCl<sub>2</sub> and 28  
119 mM NaCl. Sodium chloride concentrations were increased here to maintain the ionic strength of  
120 the buffer (131 mM). Elution buffers contained the same composition as the wash buffer except  
121 for NH<sub>4</sub>Cl (300 mM). Frozen cell pellets were resuspended in ribosome wash buffer and lysed in  
122 a BeadBug microtube compact homogenizer using 0.5 mm diameter zirconium beads  
123 (Benchmark Scientific). Cell lysate was transferred into centrifuge bottles inside the anoxic  
124 chamber which were tightly sealed to prevent O<sub>2</sub> contamination. Cell debris were removed by  
125 centrifuging outside of the anoxic chamber at 30,000 x g for 30 minutes at 4°C. The soluble  
126 lysate was then transferred back into the chamber and loaded onto a column containing pre-  
127 equilibrated, cysteine-linked, SulfoLink™ Coupling Resin (Thermo Fisher Scientific). The resin  
128 was washed with 10 column volumes of wash buffer. Ribosomes were eluted into three 10 mL  
129 fractions with elution buffer. Eluted fractions were pooled inside the anoxic chamber into  
130 ultracentrifuge bottles which were tightly sealed. Ribosomes were pelleted outside the chamber  
131 by centrifuging at 302,000 x g for 3 hours at 4°C under vacuum in a Beckman Optima XPN-100  
132 Ultracentrifuge using a Type 70 Ti rotor. Tubes containing ribosome pellets were brought back  
133 into the chamber and suspended in buffer containing 20 mM N-(2-hydroxyethyl)piperazine-N'-  
134 2-ethanesulfonic acid (HEPES; pH 7.6), 30 mM KCl, and 7 mM β-mercaptoethanol, heat-sealed  
135 in mylar bags, and stored at -80°C. Ribosome concentrations were calculated with a NanoDrop  
136 spectrophotometer assuming 1A<sub>260</sub> = 60 μg ribosome mL<sup>-1</sup> (conversion factor provided by New  
137 England Biolabs). This conversion factor was used to estimate the molecular mass of bacterial

138 ribosomes, from which molarity was calculated. Biological triplicates of each  
139 growth/purification method were taken for downstream analyses.

140 *Ribosomal Fe content.* Purified ribosomes were analyzed for iron content by total reflection X-  
141 ray fluorescence spectroscopy (TRXF) as described in Bray and Lenz et al (23).

142 *Ribosomal RNA purification.* rRNA was isolated from purified ribosomes by phenol chloroform  
143 extraction and suspended in 0.1 mM EDTA. RNA concentrations were quantified by  $A_{260}$  ( $1A_{260}$   
144 = 40  $\mu$ g rRNA  $mL^{-1}$ ).

145 *rProtein electrophoresis.* For SDS-PAGE, purified ribosomes were normalized to 3.33 mg  $mL^{-1}$   
146 in 2X SDS-PAGE dye, heated at 95°C for 5 minutes, and then incubated on ice for 2 minutes.  
147 Samples were loaded onto a 12% SDS acrylamide gel with a 4% stacking gel and run at 180 V  
148 for 60 minutes.

149 *In vitro translation.* Translation reactions were based on the methods of Bray and Lenz et al. (23)  
150 with minor modifications. All 15  $\mu$ L reactions contained 2.25  $\mu$ L of purified ribosome samples  
151 normalized to 9  $\mu$ g  $\mu$ L $^{-1}$  (so that the final concentration of ribosomes in our reactions was 1.35  
152  $\mu$ g  $\mu$ L $^{-1}$ ), 0.1 mM amino acid mix, 0.2 mM tRNAs, ~0.2  $\mu$ g  $\mu$ L $^{-1}$  of dihydrofolate reductase  
153 mRNA, and 3  $\mu$ L of factor mix (with RNA polymerase, and transcription/translation factors in  
154 10 mM Mg $^{2+}$ ) from the PURExpress®  $\Delta$  Ribosome Kit (New England Biolabs). The reaction  
155 buffer was based on Shimizu et al. (40), with HEPES-OH instead of phosphate buffer to avoid  
156 precipitation of metal phosphates. Buffer consisted of 20 mM HEPES-OH (pH 7.3), 95 mM  
157 potassium glutamate, 5 mM NH<sub>4</sub>Cl, 0.5 mM CaCl<sub>2</sub>, 1 mM spermidine, 8 mM putrescine, 1 mM  
158 dithiothreitol (DTT), 2 mM adenosine triphosphate (ATP), 2 mM guanosine triphosphate (GTP),  
159 1 mM uridine triphosphate (UTP), 1 mM cytidine triphosphate (CTP), 10 mM creatine phosphate  
160 (CP), and 53  $\mu$ M 10-formyltetrahydrofolate. Divalent cation salts (MgCl<sub>2</sub> or FeCl<sub>2</sub>) were added  
161 to 9 mM final concentration. The reaction buffer was lyophilized and stored at -80°C until  
162 resuspension in anoxic nuclease-free water immediately before experiments in the anoxic  
163 chamber. Reaction mixtures were assembled in the anoxic chamber and run at 37°C in a heat  
164 block for 120 minutes. Reactions were quenched on ice and stored on ice until they were assayed  
165 for the extent of protein synthesis. Protein synthesis was measured using a DHFR assay kit  
166 (Sigma-Aldrich), which measures the oxidation of NADPH (60 mM) to NADP $^{+}$  by dihydrofolic  
167 acid (51  $\mu$ M). Assays were performed by adding 5  $\mu$ L of protein synthesis reaction to 995  $\mu$ L of  
168 1X assay buffer. The NADPH absorbance peak at 340 nm (Abs<sub>340</sub>) was measured in 15 s  
169 intervals over 2.5 minutes. The slope of the linear regression of Abs<sub>340</sub> vs. time was used to  
170 estimate protein activity (Abs<sub>340</sub>  $min^{-1}$ ).

171 *In-line cleavage reaction rates.* Nuclease free water (IDT) was used in all experiments involving  
172 purified or transcribed RNA. rRNA for in-line cleavage experiments was purified by phenol-  
173 chloroform extraction followed by ethanol precipitation of commercial *E. coli* ribosomes (New  
174 England Biolabs, Ipswich MA, USA; catalog # P0763S). All in-line cleavage reaction solutions  
175 were prepared and incubated in the anoxic chamber Fe and Mg solutions were prepared by  
176 dissolving a known mass of FeCl<sub>2</sub>-4H<sub>2</sub>O or MgCl<sub>2</sub> salt in degassed water inside the chamber. 0.5  
177  $\mu$ g  $\mu$ L $^{-1}$  of rRNA was suspended in degassed 20 mM HEPES pH 7.6, 30 mM KCl, 5% v/v  
178 glycerol [Invitrogen (UltraPure)], and either 25 mM of MgCl<sub>2</sub> or 1 mM of FeCl<sub>2</sub> both with and  
179 without 100 mM EDTA. Reactions were placed on a 37°C heat block and incubated for 4 days  
180 for the MgCl<sub>2</sub> and no M $^{2+}$  conditions and for 8 hours for the FeCl<sub>2</sub> conditions. At each time point

181 (0, 1.5, 3, 6, 12, 24, 48, and 96 hours for the MgCl<sub>2</sub> and no M<sup>2+</sup> conditions and 0, 7.5, 15, 30, 60,  
182 120, 240, and 480 minutes for the FeCl<sub>2</sub> conditions) 4.5  $\mu$ L aliquots were combined with 0.5  $\mu$ L  
183 of 1 M sodium phosphate buffer pH 7.6 to precipitate the Fe<sup>2+</sup> or Mg<sup>2+</sup> from solution and stored  
184 at -80°C. Aliquots were defrosted on ice and combined with 2X Gel Loading Buffer II  
185 (Amicon) then loaded onto a 1% Tris/Borate/EDTA agarose gel and run at 120V for 1.25 hours.  
186 The gel was stained with GelStar™ and imaged with an Azure 6000 Imaging System (Azure  
187 Biosystems). Azurespot software was used as a pixel counter to create lane profiles. rRNA peaks  
188 were integrated by fitting to an Exponentially Modified Gaussian distribution using Igor Pro (v  
189 7.08), which calculated discrepancies between fits and observed peaks. Observed rate constants  
190 ( $k_{obs}$ ) were found by taking the negative of the slope from the natural logarithm of the  
191 normalized peak area vs. time plot. Uncertainties reported on the plots as error bars are  
192 discrepancies between fits and observed peaks. The uncertainties of  $k_{obs}$  values were estimated  
193 with the LINEST function in Excel. Rate constants (k) were calculated by  $k = k_{obs}/[M^{2+}]$ . The  
194 uncertainties of k's were estimated using  $k_{obs}$ , the uncertainties of  $k_{obs}$ ,  $[M^{2+}]$  and the  
195 uncertainties of  $[M^{2+}]$  through following the equation (41).

$$\sigma_k = \sqrt{\frac{(\sigma_{k_{obs}})^2}{[M^{2+}]^2} + \frac{(k_{obs})^2(\sigma_{[M^{2+}]})^2}{[M^{2+}]^4}} \quad \# \text{Equation 1}$$

196 Empirical error analysis confirms the assumption of equation 1 that systematic errors in [rRNA],  
197 caused for example by the approximate nature of the rRNA extinction coefficient, do not cause  
198 errors in k.

200 *In-line cleavage banding patterns.* a-rRNA (42), which is composed of the core of the LSU  
201 rRNA, was synthesized and purified as previously described. Lyophilized a-rRNA was  
202 resuspended in degassed nuclease free water (IDT) inside the anoxic chamber. Fe and Mg  
203 solutions were prepared by dissolving known amounts of FeSO<sub>4</sub>·7H<sub>2</sub>O or MgSO<sub>4</sub> in degassed  
204 nuclease free water inside the anoxic chamber. To initiate the reaction, 1 mM (final  
205 concentration) of Mg or Fe was added to 0.02  $\mu$ g  $\mu$ L<sup>-1</sup> a-rRNA in 20 mM HEPES-TRIS (pH 7.2)  
206 in a 37°C heating block. Samples were removed at 0, 0.25, 0.5, and 1 hrs for added Fe<sup>2+</sup> and 1 hr  
207 for added Mg<sup>2+</sup>, and divalent chelation beads (Hampton Research) were added to quench the  
208 reactions. Chelation beads were removed using spin columns. The RNA cleavage products were  
209 visualized using denaturing PAGE (6%, 8M urea) run at 120 V for ~1.3 hours stained with  
210 SYBR Green II.

211 *Fenton chemistry reactions.* Purified rRNA from *E. coli* ribosomes (New England Biolabs) was  
212 obtained by phenol-chloroform extraction and ethanol precipitation. A stock solution of  
213 Fe/EDTA was prepared inside the anoxic chamber by dissolving a known amount of FeCl<sub>2</sub>·4H<sub>2</sub>O  
214 salt in degassed water then mixing with EDTA in degassed water. The Fe/EDTA was removed  
215 from the chamber for the Fenton reactions. Ribosomal RNA was suspended to 0.5  $\mu$ g  $\mu$ L<sup>-1</sup> in 20  
216 mM HEPES pH 7.6, and 30 mM KCl, with 0% or 5% v/v glycerol and either 1 mM Fe/10 mM  
217 EDTA/10 mM ascorbate plus 0.3% v/v H<sub>2</sub>O<sub>2</sub> or 10 mM EDTA as the reaction initiators wherein  
218 the initiators were separately dispensed onto the tube wall and vortexed with the other  
219 components. For the zero time points, reaction components were mixed in tubes containing the  
220 thiourea quenching agent at a final concentration of 100 mM. For non-zero time points the  
221 reaction mixtures were prepared as bulk solutions and incubated at 37°C on a heat block, after  
222 which aliquots were removed at 0, 10, and 60 minutes and mixed with the thiourea quenching  
223 agent at a final concentration of 100 mM. The stopped solutions were immediately frozen and

224 stored at -80°C. For analysis, samples were defrosted on ice, combined with 2X Gel Loading  
225 Buffer II (Amicon), loaded onto a 1% Tris/Borate/EDTA agarose gel and run at 120V for 1.25  
226 hours.

227 *Protein characterization by LC-MS/MS.* Protein solutions were reduced with  $\beta$ -mercaptoethanol,  
228 and then alkylated with 14 mM iodoacetamide for 30 minutes at room temperature in the dark.  
229 Alkylation was quenched with 5 mM dithiothreitol for 15 minutes at room temperature in the  
230 dark. Proteins were purified by the methanol/chloroform purification method and digested with  
231 trypsin in a buffer containing 5% acetonitrile, 1.6 M urea, and 50 mM HEPES pH 8.8 at 37°C  
232 with shaking overnight. The digestion was quenched with 1% formic acid. Peptides were  
233 purified by Stage-Tip (43) prior to LC-MS/MS analysis.

234 Peptides were dissolved in 5% acetonitrile and 4% formic acid and loaded onto a C18-packed  
235 microcapillary column (Magic C18AQ, 3  $\mu$ m, 200 Å, 75  $\mu$ m x 16 cm, Michrom Bioresources) by  
236 a Dionex WPS-3000TPL RS autosampler (Thermostatted Pulled Loop Rapid Separation  
237 Nano/Capillary Autosampler). Peptides were separated by a Dionex UltiMate 3000 UHPLC  
238 system (Thermo Scientific) using a 112-minute gradient of 4-17% acetonitrile containing 0.125%  
239 formic acid. The LC was coupled to an LTQ Orbitrap Elite Hybrid Mass Spectrometer (Thermo  
240 Scientific) with Xcalibur software (version 3.0.63). MS analysis was performed with the data  
241 dependent Top15 method; for each cycle, a full MS scan with 60,000 resolution and  $1*10^6$  AGC  
242 (automatic gain control) target in the Orbitrap cell was followed by up to 15 MS/MS scans in the  
243 Orbitrap cell for the most intense ions. Selected ions were excluded from further sequencing for  
244 90 seconds. Ions with single or unassigned charge were not sequenced. Maximum ion  
245 accumulation time was 1,000 ms for each full MS scan, and 50 ms for each MS/MS scan.

246 Raw MS files were analyzed by MaxQuant (version 1.6.2.3; 44). MS spectra were searched  
247 against the *E. coli* database from UniProt containing common contaminants using the integrated  
248 Andromeda search engine (45). Due to the unavailability of the proteome database for *E. coli*  
249 strain MRE-600, the database for strain K12 was used. It has been shown that the two strains  
250 have nearly identical ribosome associated proteins (46). All samples were searched separately  
251 and set as individual experiments. Default parameters in MaxQuant were used, except the  
252 maximum number of missed cleavages was set at 3. Label-free quantification was enabled with  
253 the LFQ minimum ratio count of 1. The match-between-runs option was enabled. The false  
254 discovery rates (FDR) were kept at 0.01 at the peptide and protein levels.

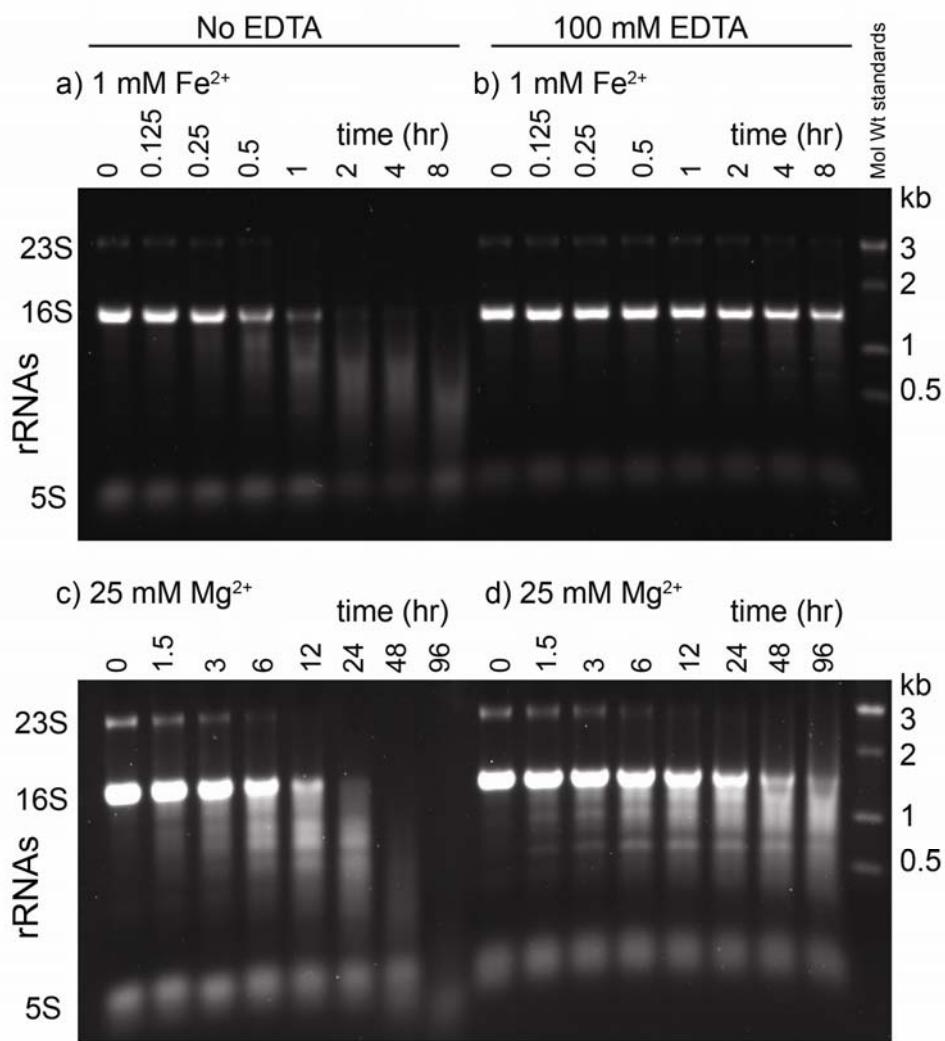
255 The results were processed using Perseus software (47). In the final dataset, the reverse hits and  
256 contaminants were removed. The LFQ intensity of each protein from the proteinGroups table  
257 was extracted and reported. For the volcano plots showing differential regulation of proteins,  
258 ratios used were from the LFQ intensities of samples from each of the three experiments. The  
259 cutoff for differential expression was set at 2-fold. P-values were calculated using a two-sided T-  
260 test on biological triplicate measurements with the threshold p-value of 0.05 for significant  
261 regulation. The raw files are publicly available at <http://www.peptideatlas.org/PASS/PASS01418>  
262 (username: PASS01418 and password: ZW2939nnw).

263 **Results**

264 *In-line cleavage of rRNA: Mg<sup>2+</sup> and anoxic Fe<sup>2+</sup>.* By manipulating reaction conditions, we could  
265 switch the mode of rRNA cleavage between Fenton and in-line mechanisms. In-line is the only

266 possible mechanism of cleavage by  $Mg^{2+}$  due to its fixed oxidation state and inability to generate  
267 hydroxyl radicals. We confirm the expectation that  $Mg^{2+}$ -mediated in-line cleavage reactions are  
268 not inhibited by anoxia or hydroxyl radical quenchers.  $Mg^{2+}$ -mediated in-line cleavage reactions  
269 are inhibited by chelators, as expected for a mechanism that requires direct metal-RNA  
270 interaction.

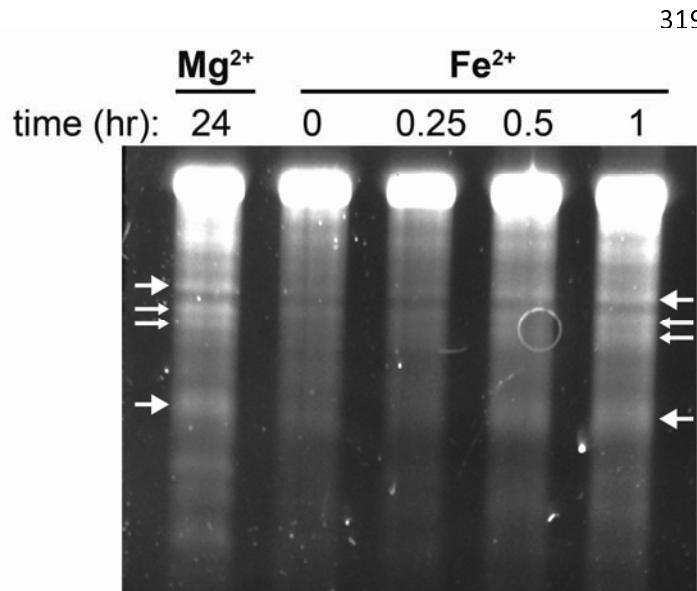
271 We confirm here in a variety of experiments that RNA is degraded by in-line cleavage when  
272 incubated with  $Fe^{2+}$  under anoxic conditions (Fig. 1a). Most of the experiments employed the  
273 16S rRNA of *E. coli* as substrate. A shorter RNA [a-RNA (42)] showed on a higher size  
274 resolution gel that RNA banding patterns and reaction products were nearly identical for  $Mg^{2+}$   
275 and anoxic  $Fe^{2+}$  reactions (Fig. 2), indicating that preferred sites of cleavage are the same for  
276 both metals. Common sites of cleavage are indications of common mechanisms of cleavage (31).  
277 In the absence of  $O_2$ , cleavage by either  $Mg^{2+}$  or  $Fe^{2+}$  was inhibited by EDTA (Fig. 1b,d) as  
278 expected for a mechanism that requires direct metal-RNA interaction but not for a mechanism  
279 with a diffusible intermediate. Neither  $Mg^{2+}$  nor anoxic  $Fe^{2+}$  cleavage was inhibited by glycerol  
280 (5%), which is known to quench hydroxyl radical and to inhibit hydroxyl radical cleavage (48).  
281 By contrast, glycerol inhibited cleavage by  $Fe^{2+}$  under conditions that favor Fenton type  
282 cleavage. Glycerol did not inhibit  $Mg^{2+}$  in-line cleavage under any conditions ( $Fe^{2+}$ : Fig. S1;  
283  $Mg^{2+}$ : Fig. S2).



**Figure 1. In-line cleavage of rRNA in anoxia.** In-line cleavage of purified rRNAs with a) 1 mM  $Fe^{2+}$  (0-8 hr), b) 1 mM  $Fe^{2+}$  plus 100 mM EDTA (0-8 hr), c) 25 mM  $Mg^{2+}$  (0-96 hr), and d) 25 mM  $Mg^{2+}$  plus 100 mM EDTA (0-96 hr).. Reactions were conducted in an anoxic chamber at 37°C in the presence of the hydroxyl radical quencher glycerol (5% v/v) and were analyzed by 1% agarose gels.

In the absence of  $O_2$ , cleavage rates

310 are significantly greater for  $\text{Fe}^{2+}$  than for  $\text{Mg}^{2+}$ . For 16S and 23S rRNAs, 1 mM  $\text{Fe}^{2+}$  caused  
311 significant in-line cleavage of rRNA after 30 minutes at 37°C. Both rRNAs were completely  
312 degraded after 2 hours in anoxic  $\text{Fe}^{2+}$  (Fig. 1a). By contrast, when the  $\text{M}^{2+}$  ion was switched  
313 from 1 mM  $\text{Fe}^{2+}$  to 25 mM  $\text{Mg}^{2+}$ , only a modest amount of in-line cleavage was observed after 6  
314 hours (Fig. 1c). Fitting of the data to a first order rate model (Figure S3) and converting  $k_{\text{obs}}$  to  $k$   
315 using  $k = k_{\text{obs}}[\text{M}^{2+}]$  reveals that the apparent rate constant for in-line cleavage of the full-length  
316 16S rRNA is  $0.45 \pm 0.03 \text{ s}^{-1}$  for  $\text{Fe}^{2+}$  and  $0.00095 \pm 0.00008 \text{ s}^{-1}$  for  $\text{Mg}^{2+}$ . Addition of EDTA  
317 inhibited cleavage, with  $k$  dropping to  $0.012 \pm 0.002 \text{ s}^{-1}$  for  $\text{Fe}^{2+}$  and  $0.00016 \pm 0.00003 \text{ s}^{-1}$  for  
318  $\text{Mg}^{2+}$ .



335 Several primary cleavage bands of a-rRNA (42) are indicated by arrows. This gel is 6%  
336 polyacrylamide, 8 M urea showing cleavage with in-line cleavage mediated by 1 mM  $\text{Mg}^{2+}$  or 1  
337 mM anoxic  $\text{Fe}^{2+}$  at 37°C for varying amounts of time. Reactions were run in 20 mM Tris-  
338 HEPES, pH 7.2.

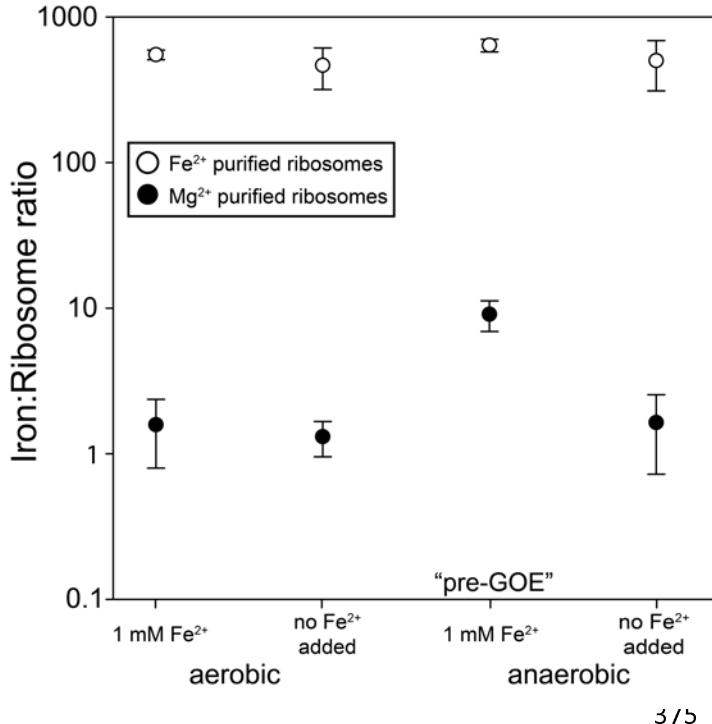
339  $\text{M}^{2+}$  exchange during ribosomal purification. The vast majority of ribosomal  $\text{M}^{2+}$  ions are  
340 exchangeable.  $\text{M}^{2+}$  exchange takes place during purification. The  $\text{Fe}^{2+}$  content of purified  
341 ribosomes depended on the type of  $\text{M}^{2+}$  in the purification buffer. Ribosomes purified in  
342 solutions with 1 mM  $\text{Fe}^{2+}$  contained significantly higher  $\text{Fe}^{2+}$  than those purified in 3 mM  $\text{Mg}^{2+}$   
343 (Fig. 3). All ribosome samples purified in 1 mM  $\text{Fe}^{2+}$  contained similar  $\text{Fe}^{2+}$  (~400-600 mol Fe  
344 mol<sup>-1</sup> ribosome).

345  $\text{Tight ribosomal binding of a subset of } \text{M}^{2+}$ . A small subset of ribosomal  $\text{M}^{2+}$  ions are not  
346 exchangeable during purification. Ribosomes retain this subset of *in vivo* divalent cations after  
347 purification. Ribosomes from *E. coli* grown in pre-GOE conditions (anoxic, high  $\text{Fe}^{2+}$ ) contained  
348 quantitatively reproducible elevated levels of  $\text{Fe}^{2+}$  after purification in solutions containing  $\text{Mg}^{2+}$ .  
349 We detect around 9 mol Fe mol<sup>-1</sup> ribosome from cells grown in pre-GOE conditions purified in  
350 solutions with high  $\text{Mg}^{2+}$  (Fig. 3). Ribosome-associated  $\text{Fe}^{2+}$  was quantified with TXRF, as  
351 described previously (23). Three non-pre-GOE growth conditions yielded ribosomes containing  
352 near background levels of  $\text{Fe}^{2+}$  (< 2 mol Fe mol<sup>-1</sup> ribosome). To make these comparisons, *E. coli*  
353 were harvested in log phase from each of four growth conditions: oxic or anoxic with high  $\text{Fe}^{2+}$  in

In sum, reactions with  $\text{Mg}^{2+}$  and anoxic  $\text{Fe}^{2+}$  showed the same responses to potential inhibitors. Reactions with  $\text{Fe}^{2+}$  in the absence of  $\text{O}_2$  were not inhibited by a hydroxyl radical quencher but were inhibited by a chelator. By contrast, reactions with  $\text{Fe}^{2+}$  in the presence of  $\text{O}_2$  were inhibited by a hydroxyl radical quencher but not by a chelator. The apparent rate constant for inline cleavage is ~475-fold greater for  $\text{Fe}^{2+}$  than for  $\text{Mg}^{2+}$ .

**Figure 2. In-line cleavage banding patterns are the same for rRNA cleavage with  $\text{Mg}^{2+}$  and anoxic  $\text{Fe}^{2+}$ .**

354 the medium (1 mM  $\text{Fe}^{2+}$ ), and oxic or anoxic without added  $\text{Fe}^{2+}$  in the growth medium (6-9  $\mu\text{M}$   
 355  $\text{Fe}^{2+}$ ).

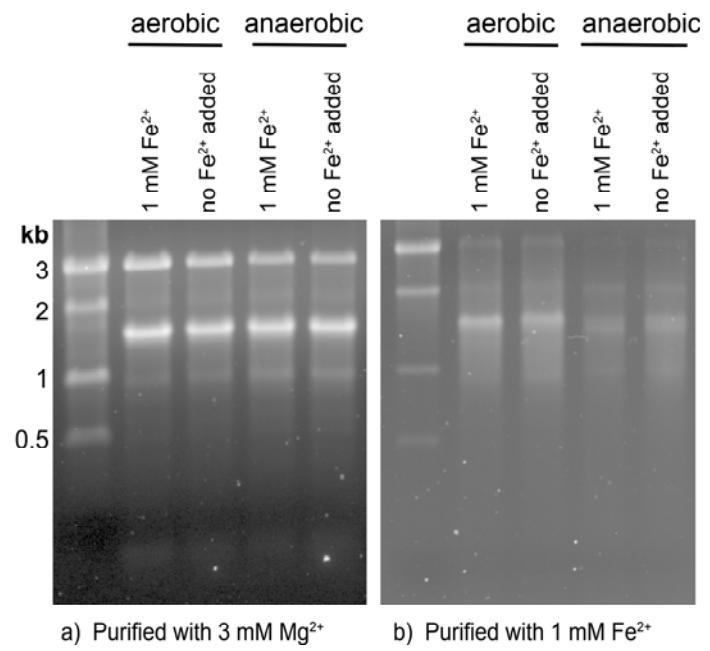


**Figure 3. Iron content (mol Fe mol<sup>-1</sup> ribosome) of purified ribosomes.** *E. coli* were grown aerobically or anaerobically at 1 mM  $\text{Fe}^{2+}$  or ambient  $\text{Fe}^{2+}$  (6-9  $\mu\text{M}$ , no Fe added), and purified in buffers containing either 3 mM  $\text{Mg}^{2+}$  (black circles) or 1 mM  $\text{Fe}^{2+}$  (white circles). Error bars represent standard error of the mean (n=3).

*Quantitating translation.* Ribosomes from all four growth conditions produced active protein in translation assays. Ribosomes were functional *in vitro* under standard conditions (with 10 mM  $\text{Mg}^{2+}$ ) and also in 8 mM  $\text{Fe}^{2+}$  plus 2 mM  $\text{Mg}^{2+}$  under anoxia (Table 1). The rate of translation was slower in the presence of  $\text{Fe}^{2+}$  than in  $\text{Mg}^{2+}$ , consistent with our previous work (23). The translational activity of ribosomes harvested from

376 anaerobic cells was slightly less than from those from aerobic cells. Ribosomes from all four  
 377 growth conditions contained intact 23S, 16S, and 5S rRNAs with purification in 3 mM  $\text{Mg}^{2+}$   
 378 (Fig. 4a) resulting in a higher proportion of intact rRNA relative to purification in 1 mM  $\text{Fe}^{2+}$   
 379 (Fig. 4b). Each purification also contained a full suite of rProteins as indicated by mass  
 380 spectrometric analysis and by gel electrophoresis (Fig. S4). The protein composition of  
 381 ribosomes from 1 mM  $\text{Fe}^{2+}$  growth conditions (Fig. S4b) was similar to that from  $\text{Mg}^{2+}$  growth  
 382 conditions (Fig. S4a).

383 **Figure 4. 1% agarose gels showing rRNA from**  
 384 **ribosomes purified in (a) 3 mM  $\text{Mg}^{2+}$  and (b) 1**  
 385 **mM  $\text{Fe}^{2+}$ .** The banding pattern suggests that rRNA  
 386 is relatively more intact in ribosomes purified with  
 387 3 mM  $\text{Mg}^{2+}$  than in ribosomes purified with 1 mM  
 388  $\text{Fe}^{2+}$ .



a) Purified with 3 mM  $\text{Mg}^{2+}$

b) Purified with 1 mM  $\text{Fe}^{2+}$

390

Growth conditions	Translation activity (Abs <sub>340</sub> min <sup>-1</sup> )		391
	Translation reaction [M <sup>2+</sup> ]		392
	10 mM Mg <sup>2+</sup>	8 mM Fe <sup>2+</sup> + 2 mM Mg <sup>2+</sup>	393
Aerobic 1 mM Fe <sup>2+</sup>	0.112 ± 0.005	0.027 ± 0.006	394
Aerobic No Fe <sup>2+</sup> added	0.100 ± 0.010	0.028 ± 0.005	395
Anaerobic 1 mM Fe <sup>2+</sup>	0.074 ± 0.004	0.021 ± 0.005	396
Anaerobic No Fe <sup>2+</sup> added	0.066 ± 0.016	0.013 ± 0.005	397

398

**Table 1. *In vitro* translation activity of purified ribosomes<sup>a</sup>.**

399

400

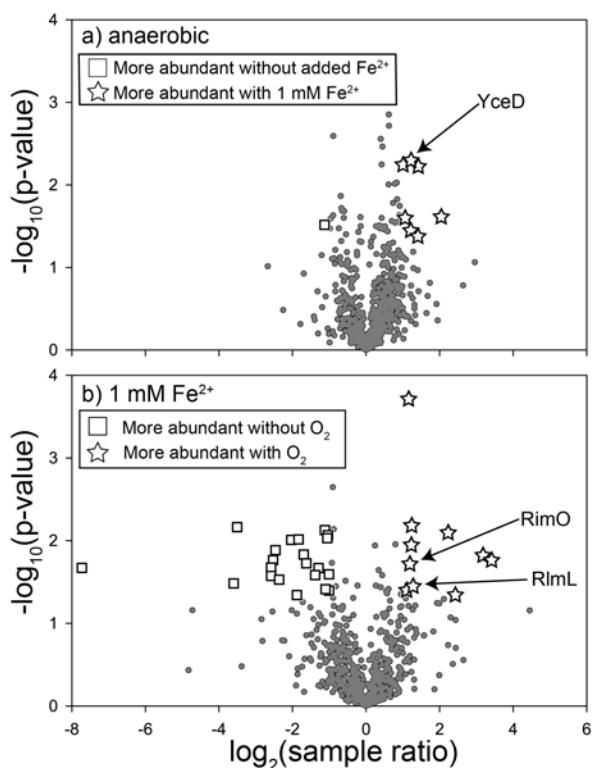
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402

403 <sup>a</sup>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 at Abs<sub>340</sub> by DHFR. Average values are reported ± standard error of the mean (n=4). All ribosomes were normalized to 9 mg mL<sup>-1</sup> before adding to translation reactions.

407 **rProtein characterization.** Ribosomes under all four growth conditions contained a full repertoire 408 of rProteins, and were associated with additional proteins, as determined by mass spectrometry. 409 These non-ribosomal proteins ranged in function from translation to central metabolism. Proteins 410 from anaerobic pathways were generally more abundant in ribosomes from anaerobic cells while 411 proteins from aerobic pathways were more abundant in ribosomes from aerobic cells (**Tables S1, S2**). Proteins for synthesis of enterobactin, an Fe<sup>3+</sup>-binding siderophore, were more abundant in 412 ribosomes from aerobic cells and from those grown without added Fe, while the bacterial non- 413 heme ferritin subunit was more abundant in ribosomes from anaerobic cells regardless of the 414 Fe<sup>2+</sup> content in the media (**Table S2**). Several proteins were differentially expressed in pre-GOE 415 ribosomes relative to other growth conditions (**Fig. 5**). Notably, pre-GOE ribosomes had five 416 times the abundance of the protein YceD than ribosomes grown anaerobically without added 417 Fe<sup>2+</sup>.

Fe<sup>2+</sup>. Pre-GOE ribosomes had one third the abundance of the rProtein S12 methylthiotransferase protein RimO and rRNA LSU methyltransferase K/L protein RlmL than ribosomes from aerobically grown cells with 1 mM Fe<sup>2+</sup>.



**Figure 5. Differential protein abundance between ribosomes purified from cells grown under four growth conditions.** Graphs display relative protein abundance in ribosome samples between two growth conditions. Black circles represent proteins not significantly more abundant in either sample. Gray rectangle and white stars represent proteins significantly more abundant in one of the samples. Proteins with a 2-fold or greater abundance in one sample versus another and a p-value less than or equal

435 to 0.05 (n=3), were classified as significantly more abundant.

436

437

## 438 Discussion

439 *Iron promotes rapid in-line cleavage of rRNA.*  $Mg^{2+}$  is known to cleave the RNA phosphodiester  
440 backbone via an in-line mechanism (30,31). An oxidative cleavage mechanism for  $Mg^{2+}$ , with a  
441 fixed oxidation state, is not accessible. We have shown here that  $Fe^{2+}$ , like  $Mg^{2+}$ , can cleave  
442 RNA by a non-oxidative in-line mechanism. The apparent first-order rate constant for in-line  
443 cleavage by  $Fe^{2+}$  is around 475-fold greater than for  $Mg^{2+}$ . We have used cleavage of 16S rRNA  
444 to determine the apparent rate constants of both  $Mg^{2+}$ - and  $Fe^{2+}$ -mediated cleavage. For  $Mg^{2+}$ ,  
445 the apparent rate constant, normalized for the number of phosphodiester bonds, is comparable to  
446 previous reports (49), lending support to our results and highlighting the rapidity of cleavage by  
447  $Fe^{2+}$ . The in-line cleavage rate of  $Fe^{2+}$  relative to  $Mg^{2+}$  appears to place the rate constant of  $Fe^{2+}$ -  
448 mediated cleavage above that of other metals that cause in-line cleavage, including  $Zn^{2+}$ ,  $Pb^{2+}$ ,  
449  $Eu^{3+}$ , and  $Yb^{3+}$  (49).

450 Support for a non-oxidative in-line mechanism of cleavage of RNA by anoxic  $Fe^{2+}$  is provided  
451 by observations that the rate of the reaction is not attenuated by anoxia and that the sites of  
452 cleavage are conserved for  $Mg^{2+}$  and anoxic  $Fe^{2+}$ . The absence of hydroxyl radical intermediates  
453 in the anoxic cleavage reaction is confirmed by the lack of inhibition by a radical quencher (25).  
454 Direct  $Fe^{2+}$ -RNA interactions, as required for in-line cleavage (30,31) but not for Fenton  
455 chemistry, are indicated by inhibition by the chelator EDTA. In-line cleavage is the dominant  
456 mechanism of  $Fe^{2+}$  cleavage when contributions from Fenton-mediated processes are minimized  
457 and is the only mechanism of  $Mg^{2+}$  cleavage, which is considerably slower. By contrast, in oxic  
458 environments, transient  $Fe^{2+}$  oxidation generates hydroxyl radicals that cleave nucleic acids  
459 (22,25-28).

460  $Fe^{2+}$  appears to be a potent all-around cofactor for nucleic acids. The combined results indicate  
461 that:

- 462 a) rRNA folds at lower concentration of  $Fe^{2+}$  than  $Mg^{2+}$  (23),
- 463 b) at least a subset of ribozymes and DNAzymes are more active in  $Fe^{2+}$  than in  $Mg^{2+}$   
(50,51),
- 464 c) the translation system is functional when  $Fe^{2+}$  is the dominant divalent cation (23),
- 465 d) at low concentrations of  $M^{2+}$ , T7 RNA polymerase is more active with  $Fe^{2+}$  than with  
 $Mg^{2+}$  (52),
- 466 e) a broad variety of nucleic acid processing enzymes are active with  $Fe^{2+}$  instead of  $Mg^{2+}$   
(52),
- 467 f) rates of in-line cleavage are significantly greater for  $Fe^{2+}$  than for  $Mg^{2+}$  (here), and
- 468 g)  $Fe^{2+}$  but not  $Mg^{2+}$  confers oxidoreductase functionality to some RNAs (17,53).

472 *Why so fast?* Our previous DFT computations (52) help explain why  $Fe^{2+}$  is such a potent  
473 cofactor for RNA. Conformations and geometries of coordination complexes with water and/or  
474 phosphate are nearly identical for  $Fe^{2+}$  or  $Mg^{2+}$ . However, differences between  $Mg^{2+}$  and  $Fe^{2+}$  are  
475 seen in the electronic structures of coordination complexes.

476 Firstly, because of low lying d orbitals,  $\text{Fe}^{2+}$  has greater electron withdrawing power than  $\text{Mg}^{2+}$   
477 from first shell phosphate ligands. In coordination complexes with phosphate groups, the  
478 phosphorus atom is a better electrophile when  $\text{M}^{2+} = \text{Fe}^{2+}$  than when  $\text{M}^{2+} = \text{Mg}^{2+}$ . This difference  
479 between  $\text{Mg}^{2+}$  and  $\text{Fe}^{2+}$  is apparent in ribozyme reactions and in-line cleavage reactions.

480 Secondly,  $\text{Fe}^{2+}(\text{H}_2\text{O})_6$  is a stronger acid than  $\text{Mg}^{2+}(\text{H}_2\text{O})_6$ ; depletion of electrons is greater from  
481 water molecules that coordinate  $\text{Fe}^{2+}$  than from those that coordinate  $\text{Mg}^{2+}$ . The lower pKa of  
482  $\text{Fe}^{2+}(\text{H}_2\text{O})_6$  may promote protonation of the 5'OH leaving group during cleavage. Additionally,  
483 the superior electron-depleting power of  $\text{Fe}^{2+}$  may better promote activation of the 2'-OH  
484 nucleophile. Metal hydrates with low pKa's have been reported to induce RNA cleavage better  
485 than less acidic metal hydrates (30).

486 The mechanisms of in-line cleavage suggest that direct  $\text{M}^{2+}$ -RNA coordination is required  
487 (30,31). Indeed, studies of the in-line fragment patterns have previously been used to probe  
488 structural information on RNA molecules, such as determination of metal-binding sites (34,35).

489 *Ribosomal iron content is elevated in vivo by pre-GOE conditions.* Our data show for the first  
490 time that environmental conditions can affect the *in vivo* iron content of bacterial ribosomes.  
491 Ribosomal Fe content in cells is impacted by the availability and reactivity of  $\text{Fe}^{2+}$  and  $\text{O}_2$ . In  
492 oxic conditions, extracellular Fe is insoluble, and is difficult for cells to assimilate (54), requiring  
493 siderophores like enterobactin for  $\text{Fe}^{3+}$  uptake (55,56). Once in the cell, byproducts of aerobic  
494 metabolism, such as  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ , or  $\text{O}_2$  can react with  $\text{Fe}^{2+}$  to form hydroxyl radicals  
495 (22,28,57). Thus, aerobic cells tightly regulate intracellular Fe to maintain low  $\text{Fe}^{2+}$  levels in the  
496 cytosol (58), minimizing  $\text{Fe}^{2+}$  availability for incorporation into ribosomes. We detect only  
497 around 1-2  $\text{Fe}^{2+}$  per ribosome in aerobic growth conditions, consistent to what was previously  
498 seen for yeast ribosomes (59). In anoxic conditions,  $\text{Fe}^{2+}$  is more bioavailable and is less harmful  
499 because anaerobic growth generates fewer reactive oxygen species and there is no threat from  $\text{O}_2$   
500 diffusion into cells. Thus, anaerobic cells do not sequester Fe, and labile  $\text{Fe}^{2+}$  accumulates in the  
501 cytoplasm (55). Under pre-GOE conditions,  $\text{Fe}^{2+}$  is abundant and bioavailable, allowing cellular  
502 assimilation (60). We detect around 9  $\text{Fe}^{2+}$  per ribosome in pre-GOE conditions.

503  *$\text{Fe}^{2+}$  associates with rRNA in vivo.* Exchange of non-native metals for native metals is well-  
504 known during purification of proteins (61). We observe analogous phenomena with rRNA.  $\text{Fe}^{2+}$   
505 can exchange with  $\text{Mg}^{2+}$  (and vice versa) during purification of ribosomes. Ribosomes purified  
506 in either  $\text{Fe}^{2+}$  or  $\text{Mg}^{2+}$  associate with 500-1000  $\text{M}^{2+}$  ions that match the type of ion in the  
507 purification buffers.

508 However, our data support the tight association and lack of exchange of around 9  $\text{M}^{2+}$  per  
509 ribosome. This subset of  $\text{M}^{2+}$  do not exchange during purification. The number of non-  
510 exchangeable  $\text{M}^{2+}$  closely matches the number of  $\text{M}^{2+}$  identified previously as a special class of  
511 deeply buried and highly coordinated  $\text{M}^{2+}$  in dinuclear microclusters ( $\text{M}^{2+}$ - $\mu\text{c}$ 's) (16).  $\text{Mg}^{2+}$  ions  
512 in  $\text{M}^{2+}$ - $\mu\text{c}$ 's are directly chelated by multiple phosphate oxygens of the rRNA backbone and are  
513 substantially dehydrated.  $\text{M}^{2+}$ - $\mu\text{c}$ 's within the LSU provide a framework for the ribosome's  
514 peptidyl transferase center, the site of protein synthesis in the ribosome, suggesting an essential  
515 and ancient role for  $\text{M}^{2+}$ - $\mu\text{c}$ 's in the ribosome. There are four dinuclear  $\text{M}^{2+}$ - $\mu\text{c}$ 's in the LSU and  
516 one in the SSU, accounting for 10  $\text{M}^{2+}$  (16). Displacement of these  $\text{M}^{2+}$  would require large-scale  
517 changes in ribosomal conformation. In sum, there are ten  $\text{M}^{2+}$  per ribosome that are expected to

518 be refractory to exchange. We hypothesize that this subset  $M^{2+}$  are contained in  $M^{2+}$ - $\mu$ c's, which  
519 can be occupied by either  $Mg^{2+}$  or  $Fe^{2+}$  (17), depending on growth conditions.

520 We also hypothesize that ribosomes harvested from aerobic cells have low  $Fe^{2+}/Mg^{2+}$  ratios  
521 because of low intracellular  $Fe^{2+}$  availability and lability. This hypothesis is supported by our  
522 observation that the number of slow exchanging  $Fe^{2+}$  per ribosome from aerobic cells is near the  
523 baseline of our measurements. It appears that ribosomes harvested from pre-GOE conditions  
524 have high  $Fe^{2+}/Mg^{2+}$  ratios because of high intracellular  $Fe^{2+}$  availability and lability, as  
525 indicated by the close match in the number of slowly exchanging  $Fe^{2+}$  per ribosome and the  
526 number of available  $M^{2+}$  sites in ribosomal  $M^{2+}$ - $\mu$ c's. In these experiments we detect only the  
527  $Fe^{2+}$  ions that do not exchange during purification.

528 *Anoxic  $Fe^{2+}$  degrades rRNA within ribosomes.* rRNA from all four growth conditions showed  
529 partial hydrolysis when ribosomes were purified in anoxic  $Fe^{2+}$ . It appears that  $Fe^{2+}$  can mediate  
530 rRNA degradation by an in-line mechanism during ribosomal purification in anoxic  $Fe^{2+}$ . Less  
531 rRNA cleavage was observed in ribosomes purified with  $Mg^{2+}$ , which contain orders of  
532 magnitude lower  $Fe^{2+}$ .

533 *What about proteins?* While eukaryotic iron binding rProteins have been reported (62), to our  
534 knowledge, there are currently no reports of prokaryotic iron binding rProteins. However, our  
535 MS analysis indicates a variety of non-rProteins co-purified with the ribosome and presumably  
536 associate with the ribosome *in vivo*. Some of these may represent nascent polypeptide being  
537 translated at the time of cell harvesting. Several of the ribosome-associated proteins are known to  
538 bind to iron. A notable example is the bacterial non-heme ferritin subunit protein, which is  
539 associated with the ribosome in each of our growth conditions. Bacterial non-heme ferritin is an  
540 iron storage protein that can hold as many as 3,000  $Fe^{3+}$  atoms as the mineral ferrihydrite (56) in  
541 a 24-mer of identical subunits that self-assemble into the mature protein (63). There is previous  
542 evidence for ferritin copurifying with ribosomes in sucrose gradients. The use of column  
543 purification in our study makes coincidental copurification unlikely, and supports direct  
544 association of ferritin with ribosomes (64). Non-heme ferritin is upregulated under high  
545 intracellular iron so it is perhaps unsurprising that this protein is most abundant in ribosomes  
546 from pre-GOE conditions (65,66). We cannot discount the contribution of iron loaded ferritin  
547 towards the elevated ribosomal iron content. However, recent evidence suggests that ferritin-  
548 bound iron makes up a very small portion of the total iron pool in exponentially growing *E. coli*  
549 (67).

550 *Differential expression of YceD under pre-GOE conditions.* We used mass spectrometry to  
551 determine if pre-GOE growth conditions had any significant effect on the rProtein or ribosome-  
552 associated protein content of our samples. The only protein predicted to be involved in ribosome  
553 function or assembly that was significantly more abundant under pre-GOE conditions was the  
554 large rRNA subunit accumulation protein YceD. YceD is a 173 amino acid protein with a single  
555 C-X<sub>(2)</sub>-C cysteine motif suggesting a potential metal binding site. The function of YceD remains  
556 unclear. The *yceD* gene is co-transcribed with the rProtein L32 gene *rpmF*.  $\Delta yceD$  mutants had  
557 decreased 23S rRNA content compared to the wild type, suggesting that YceD is involved in 23S  
558 rRNA synthesis and/or processing (68). The higher abundance of YceD associated with pre-GOE  
559 ribosomes suggests that YceD may play a role in incorporating  $Fe^{2+}$  into the ribosome *in vivo*.  
560 Proteins that were less abundant in pre-GOE ribosomes included rProtein S12  
561 methylthiotransferase protein RimO and the rRNA LSU methyltransferase K/L. While the

562 functions of these protein and rRNA modifications in the ribosome are not totally clear, some  
563 evidence points to structural roles (67). Whatever their utility, their reduced abundance in pre-  
564 GOE ribosomes suggests that the increased  $\text{Fe}^{2+}$  association in these ribosomes may render the  
565 function of these proteins less important.

566 *Cellular significance of the newfound  $\text{Fe}^{2+}$ -rRNA and ribosome relationship.* Given the presence  
567 of  $\text{M}^{2+}$ -μc's in the universal common core of ribosomes (17), and our finding that  $\text{Fe}^{2+}$  may  
568 occupy  $\text{M}^{2+}$ -μc's *in vivo*, a diversity of ribosomes, including those of humans, may specifically  
569 incorporate  $\text{Fe}^{2+}$ . Iron in the ribosome correlates with a modified abundance of select rProteins,  
570 possibly causing altered translation and gene expression. Moreover, iron likely decreases  
571 ribosome longevity within the cell, as we have illustrated the potency of  $\text{Fe}^{2+}$  in inducing rRNA  
572 cleavage. In fact, rRNA cleavage events commonly linked to  $\text{Fe}^{2+}$  oxidation, such as in the  
573 human ribosome factoring in Alzheimer's disease (69), or in *Saccharomyces cerevisiae* rRNA  
574 where cleavage is tied to downstream oxidative stress response (59) could be in some measure  
575 attributable to  $\text{Fe}^{2+}$  in-line cleavage. The rapid  $\text{Fe}^{2+}$  in-line cleavage phenomena can be expected  
576 to hold to any RNAs regardless of the presence of an oxidant, given they do not specifically  
577 coordinate or shield themselves from the iron to prevent cleavage. We have uncovered a new  
578 avenue for RNA to act as a response molecule in Fe regulation or stress pathways and  
579 highlighted a potential mechanism by which  $\text{Fe}^{2+}$  induces cellular toxicity particularly relevant to  
580 anoxic environments. Involvement of  $\text{Fe}^{2+}$  in RNA offers fine-tuning within cellular systems, in  
581 that in one capacity it is a cofactor and in another it causes in-line cleavage.

582 *Summary.* Here we have shown for the first time that bacteria grown in pre-GOE conditions  
583 contain functional ribosomes with tightly bound Fe atoms. The ~10 ribosomal Fe ions in pre-  
584 GOE ribosomes are likely deeply buried and specifically bound to rRNA. Depending on  
585 intracellular Fe lability, ribosomes may have higher Fe content *in vivo* given the high capacity  
586 for the ribosome to substitute ~600 loosely bound  $\text{Mg}^{2+}$  ions for  $\text{Fe}^{2+}$ . Furthermore, direct  
587 association of the naked rRNA with Fe atoms results in a fast rate of in-line cleavage. This  
588 highlights a potential role of protection from in-line cleavage for rProteins, and also suggests that  
589 iron may drive ribosomes through a rapid life cycle. Our results support a model in which  
590 alternate  $\text{M}^{2+}$  ions, namely  $\text{Fe}^{2+}$ , participated in the origin and early evolution of life: first in  
591 abiotic proto-biochemical systems, through potentially rapid rounds of formation and breakdown  
592 of RNA structures, and then within early cellular life up until the GOE (70). Our study also  
593 expands the role of  $\text{Fe}^{2+}$  in modern biochemistry by showing that extant life retains the ability to  
594 incorporate Fe into ribosomes. We surmise that extant organisms under certain environmental  
595 and cellular states may use  $\text{Fe}^{2+}$  as a ribosomal cofactor. In addition, obligate anaerobic  
596 organisms that have spent the entirety of their evolutionary history in permanently anoxic  
597 environments may still use abundant  $\text{Fe}^{2+}$  in their ribosomes *in vivo*.

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603  
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607

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608

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