

1 **Calculating metalation in cells reveals CobW acquires Co<sup>II</sup> for vitamin B<sub>12</sub>**  
2 **biosynthesis upon binding nucleotide**

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16 **Protein metal-occupancy (metalation) *in vivo* has been elusive. Here we develop a**  
17 **metalation-calculator which accounts for inter-metal competition and changing metal-**  
18 **availabilities inside cells. The calculations are based on available free-energies of**  
19 **metals determined from the responses of metal sensors. We use the calculator to**  
20 **understand the function and mechanism of CobW, a predicted Co<sup>II</sup>-chaperone for**  
21 **vitamin B<sub>12</sub>. CobW is calculated to acquire negligible metal alone: But, upon binding**  
22 **nucleotide (GTP) and Mg<sup>II</sup>, CobW assembles a high-affinity site that can obtain Co<sup>II</sup> or**  
23 **Zn<sup>II</sup> from the intracellular milieu. In idealised cells with sensors at the mid-points of**  
24 **their responses, competition within the cytosol enables Co<sup>II</sup> to outcompete Zn<sup>II</sup> for**  
25 **binding CobW. Thus, Co<sup>II</sup> is the cognate metal. However, after growth in different**  
26 **[Co<sup>II</sup>], Co<sup>II</sup>-occupancy ranges from 10 to 97% which matches CobW-dependent B<sub>12</sub>**  
27 **synthesis. The calculator reveals how CobW acquires its metal and is made available**  
28 **for use with other proteins.**

29

30 Paradoxically, *in vitro*, most metalloproteins prefer to bind incorrect metals<sup>1,2</sup>. A non-cognate  
31 metal may bind more tightly to the native site or bind by using a subset of the native ligands,  
32 by recruiting additional ligand(s) and/or by distorting the geometry of a binding site. Some  
33 enzymes are cambialistic and can function with alternative metals<sup>3</sup>, but more commonly a  
34 non-cognate metal will inactivate an enzyme<sup>4,5</sup>. Correct metalation occurs *in vivo* because  
35 cells carefully control the availability of metals to nascent proteins<sup>1,6-8</sup>. For example,  
36 specialised delivery proteins support metal acquisition by about a third of metalloproteins,  
37 (which in turn represent about a third of all proteins and about a half of all enzymes)<sup>1,8</sup>. A

1 substantial fraction (>80%) of these delivery systems initially supply metal to cofactors such  
2 as heme, chlorophyll, iron sulphur clusters and vitamin B<sub>12</sub><sup>1</sup>. Subsequent acquisition of a  
3 preassembled cofactor is then less of a challenge since a binding pocket is more readily  
4 selective for a complex molecule as opposed to a single metal atom. However, metal  
5 delivery proteins do not ultimately solve the challenge of metalation because now the correct  
6 metal must somehow partition onto the delivery protein. Here we discover how the correct  
7 metal is acquired by a metal delivery protein.

8

9 The G3E GTPase superfamily contains three branches of delivery proteins involved  
10 in the assembly of metal centres, two for Ni<sup>II</sup> (HypB, UreG), one for handling the cobalamin  
11 cofactor (MeaB), plus a fourth family, COG0523<sup>9,10</sup>. Though ubiquitous, from bacteria to  
12 plants and humans, members of COG0523 have been persistently enigmatic<sup>10</sup>. Gene  
13 context and informatics have linked subgroups of this family to at least three different metals:  
14 These include Nha3 associated with Fe<sup>III</sup>-requiring nitrile hydratases<sup>11-13</sup>, various subgroups  
15 (including YeiR, YjiA, ZigA and ZagA) implicated in Zn<sup>II</sup> metallostasis<sup>10,14-18</sup>, and CobW  
16 associated with the aerobic biosynthesis of cobalamin (vitamin B<sub>12</sub>) and hence Co<sup>II</sup> (ref.<sup>19</sup>).  
17 Metal insertion into the preformed corrin ring in the aerobic pathway for vitamin B<sub>12</sub>  
18 biosynthesis appears to be irreversible<sup>20,21</sup>, highlighting the importance of Co<sup>II</sup> specificity at  
19 this step. Disruption of *cobW* impairs B<sub>12</sub> biosynthesis<sup>19</sup>, and a role in Co<sup>II</sup> delivery has been  
20 suggested<sup>22</sup>, but not established.

21

22 Nucleotide hydrolysis is critical for the metallochaperone activities of HypB<sup>23</sup>, UreG<sup>24</sup>  
23 and MeaB<sup>25</sup>, and evidence is emerging that this is also the case for other COG0523  
24 proteins. Recently, the putative Zn<sup>II</sup> chaperone ZagA was shown to interact with a Zn<sup>II</sup>-  
25 requiring enzyme of folate biosynthesis (FolE)<sup>18</sup>. Rather than the anticipated GTP, this  
26 interaction is stimulated by the purine intermediate ZTP (5-amino 4-imidazole carboxamide  
27 riboside 5'-triphosphate), an alarmone that accumulates during low Zn<sup>II</sup> (ref.<sup>18,26</sup>). A Zn<sup>II</sup>-  
28 requiring histidine lyase (HutH) together with ZigA enables depletion of histidine in cells  
29 cultured in low Zn<sup>II</sup> and this may serve to liberate histidine-bound Zn<sup>II</sup> (ref.<sup>16</sup>). Zn<sup>II</sup> binding to  
30 ZigA enhances GTP hydrolysis and weakens GDP binding<sup>17</sup>. The impact of triphospho-  
31 nucleotide binding on metal binding by COG0523 proteins remains to be tested.

32

33 For metalloproteins generally, there is a need to relate metal binding to the  
34 intracellular availability of metals. Our recent work provides the basis for such  
35 contextualisation<sup>27</sup>. Cells are thought to assist protein metalation by maintaining availabilities  
36 to the opposite of the Irving-Williams series with weaker binding metals such as Mg<sup>II</sup>, Mn<sup>II</sup>  
37 and Fe<sup>II</sup> highly available and tighter binding metals such as Ni<sup>II</sup>, Zn<sup>II</sup> and Cu<sup>I</sup> at low

1 availabilities<sup>28-30</sup>. We have demonstrated this to be correct by determining the sensitivities of  
2 the DNA-binding metal-sensing transcriptional regulators of *Salmonella enterica* serovar  
3 Typhimurium (hereafter *Salmonella*)<sup>27</sup>. The sensors trigger expression of genes whose  
4 products, for example, import metals that are deficient or export those in excess<sup>6,31</sup>. A  
5 collection of thermodynamic parameters were measured for each sensor and used to  
6 calculate the (dynamic range of) buffered intracellular metal concentrations to which each  
7 sensor is finely tuned to switch gene expression<sup>27,32</sup>. For the more competitive metals,  
8 detection is so sensitive as to suggest that there is no hydrated metal in the cell<sup>27,28</sup>. Instead,  
9 rapid associative metal-exchange can occur between labile ligands in the crowded cytosol  
10 and the binding sites of metalloproteins, making it unhelpful to express metal availabilities as  
11 concentrations of the (largely irrelevant and negligible) hydrated species: Thus, the chemical  
12 potentials of the bound available metals were expressed as free energies  $\Delta G$ <sup>27</sup>. It is  
13 hypothesised that metal-delivery proteins acquire their metals from these exchangeable,  
14 buffered pools. By reference to available  $\Delta G$  values and by assuming an idealised cell in  
15 which the sensors are at the mid-points of their dynamic ranges, the correct metal (Co<sup>II</sup>) was  
16 previously predicted to partition to the known chelatase of the anaerobic cobalamin  
17 biosynthetic pathway, CbiK<sup>27</sup>. Here, we build upon this approach to account for (1) multiple  
18 competing metals and (2) non-idealised (conditional) cell cultures, in order to understand the  
19 actions of the putative metal delivery protein CobW. With so many enzymes requiring  
20 metals, an ability to calculate and optimise *in vivo* metalation has far-reaching applications,  
21 for example in industrial biotechnology.

22  
23 Vitamin B<sub>12</sub> is an essential nutrient that is neither made nor required by plants<sup>33</sup>.  
24 Prokaryotes present in the ruminant microbiome produce B<sub>12</sub> and hence dairy products  
25 provide a dietary source<sup>34</sup>. Vitamin B<sub>12</sub> supplements are recommended for those on a vegan  
26 diet and its biomanufacture (the only feasible production method for such a complex  
27 molecule) is increasingly in demand<sup>35</sup>. *E. coli* has significant advantages (namely, it is fast-  
28 growing and genetically tractable) over currently employed production strains<sup>36</sup>. Native *E.*  
29 *coli* does not make vitamin B<sub>12</sub> but strains containing functional B<sub>12</sub> pathways have been  
30 created, initially utilising genes of the anaerobic pathway from *Salmonella*<sup>37</sup> and more  
31 recently using those of the aerobic pathway primarily from *Rhodobacter capsulatus*<sup>38-40</sup>. The  
32 latter has enabled the production of previously difficult to isolate intermediates, including the  
33 metal-free corrinoids hydrogenobyric acid and its diamide<sup>38-40</sup>. In *R. capsulatus* Co<sup>II</sup> is  
34 inserted into the corrin ring of hydrogenobyric acid *a,c*-diamide by a cobalt chelatase  
35 ATPase (CobNST)<sup>41</sup>, putatively via CobW<sup>22</sup>. However, a better understanding of Co<sup>II</sup>-  
36 availability inside engineered *E. coli* strains (referred to hereafter as *E. coli*\*) is required in  
37 order to optimise Co<sup>II</sup> supply for the B<sub>12</sub> pathway within the heterologous host.

1           A purpose of this work was to determine whether CobW can acquire Co<sup>II</sup> and supply  
2 the metal to the aerobic B<sub>12</sub> biosynthetic pathway. *E. coli*\* strains have been used as the  
3 model because this has direct relevance to biomanufacturing, but also because high B<sub>12</sub>  
4 production in these cells coupled with the close similarity between the DNA-binding metal  
5 sensors of *E. coli* and *Salmonella* both serve to make this system experimentally tractable:  
6 The metal sensors of *Salmonella* having been thermodynamically characterised<sup>27</sup>. Here we  
7 determine the metal affinities of CobW and discover that a high-affinity metal-binding site is  
8 assembled only upon association with Mg<sup>II</sup> and GTP. We calculate the metal-occupancy of  
9 CobW *in vivo* using metal-availabilities in an idealised cell determined from the sensitivities  
10 of metal sensors. This establishes Co<sup>II</sup> as the cognate metal, despite CobW also having a  
11 tight (sub-picomolar) Zn<sup>II</sup>-affinity. By calculating the Co<sup>II</sup> availabilities in *E. coli*\* from the  
12 response of the Co<sup>II</sup>-sensor RcnR, we show that Mg<sup>II</sup>GTP-CobW can be mis-metalated by  
13 Zn<sup>II</sup> *in vivo*, but this is precluded when Co<sup>II</sup> availability increases. These predictions are  
14 reflected in the CobW-dependent production of vitamin B<sub>12</sub> in *E. coli*\*, establishing a role for  
15 CobW in Co<sup>II</sup>-supply for B<sub>12</sub>. Together, these data reveal a mechanism for Co<sup>II</sup>-acquisition  
16 and Co<sup>II</sup>-supply by CobW, with significance for understanding the actions of other COG0523  
17 proteins. These data will also allow optimisation of B<sub>12</sub> manufacture in *E. coli*\* strains.

18

19           An easy-to-use metalation calculator has been developed which accounts for  
20 competition between metals at a protein metal-binding site, for competition from the  
21 intracellular milieu, and for variable metal availabilities in bacterial cells. The calculator can  
22 be readily applied by others to a diversity of metalloproteins across bioscience and  
23 biotechnology.

24

## 25 **Results**

26

### 27 **Guanine nucleotides create two metal-sites in CobW**

28

29 The first objective was to measure the Co<sup>II</sup> affinities of the form of CobW that acquires metal  
30 inside a cell. A modelled structure of CobW (Fig. 1a) showed hypothetical nucleotide-binding  
31 sequences adjacent to a putative metal-binding motif, CxCC, and both of these features are  
32 conserved in the COG0523 subfamily<sup>9,10</sup>. To assess the effect of nucleotides on metal-  
33 binding, CobW was overexpressed and purified (Fig. 1b and Supplementary Fig. 1). The  
34 protein mass determined by ESI-MS (37,071 Da; Fig. 1c) is consistent with that expected for  
35 CobW after cleavage of the N-terminal methionine (37,072.6 Da).

36

1           Co<sup>II</sup>-titration of CobW alone (26.1 μM) produced a non-linear increase in absorbance  
2 at 315 nm (Fig. 1d) but gel-filtration of a mixture of CobW (10 μM) and Co<sup>II</sup> (30 μM) resulted  
3 in their complete separation (Fig. 1e). Taken together, these results suggest only weak  
4 interactions between Co<sup>II</sup> and CobW in the absence of cofactors. In the presence of excess  
5 GMPPNP (60 μM), a less readily hydrolysed analogue of GTP (Fig. 1f), Co<sup>II</sup>-titration of  
6 CobW (24 μM) produced an absorbance feature at 339 nm characteristic of ligand-to-metal  
7 charge transfer with an extinction coefficient ( $\epsilon \sim 2,800 \text{ cm}^{-1} \text{ M}^{-1}$ ) indicative of coordination by  
8 three cysteine side-chains<sup>42</sup> (Fig. 1g). Visible absorbance features (500 – 700 nm,  $\epsilon \sim 300 -$   
9  $700 \text{ cm}^{-1} \text{ M}^{-1}$ ) are characteristic of *d-d* transitions, diagnostic of tetrahedral Co<sup>II</sup>-coordination  
10 geometry (Fig. 1g and Supplementary Fig. 2). Equivalent experiments performed with GTP  
11 and an alternate stable analogue, GTPγS, generated indistinguishable spectra  
12 (Supplementary Fig. 3a,b). These absorbance features increased linearly saturating at 2:1  
13 ratio Co<sup>II</sup>:CobW, and gel-filtration of a mixture of CobW (10 μM) and Co<sup>II</sup> (30 μM) pre-  
14 incubated with GMPPNP (30 μM) resulted in co-migration of ~ 2 equivalents Co<sup>II</sup> per protein  
15 monomer (Fig. 1h). These data show that binding of guanine nucleotides to CobW promotes  
16 tight coordination of two metals ions.

17

### 18 **Addition of cellular [Mg<sup>II</sup>] reveals one distinct Co<sup>II</sup> site**

19

20 The uniform absorbance increase observed across both metal-binding events in Fig. 1g,  
21 could be explained by either the presence of two sequentially filled sites with identical  
22 spectroscopic features, or two spectrally distinct sites being filled in a pairwise manner.  
23 Competition between GMPPNP-CobW and ethylene glycol tetraacetic acid (EGTA) for Co<sup>II</sup>  
24 produced a sigmoidal binding isotherm indicating positive cooperativity ( $K_{D2} < K_{D1}$ ) between  
25 the two metal-sites (Fig. 2a). Such cooperativity will result in pairwise filling of the two metal-  
26 sites. Given that GTPases typically bind nucleotides in complex with Mg<sup>II</sup>, we hypothesised  
27 that the cognate metal for the first (weak-affinity) site is Mg<sup>II</sup>, and that Mg<sup>II</sup> binding triggers  
28 assembly of the second (tight-affinity) metal-site in GMPPNP-CobW. Co<sup>II</sup>-titration of CobW  
29 (20 μM) with GMPPNP (60 μM) and Mg<sup>II</sup> (2.7 mM, *ie* available idealised intracellular  
30 concentration, [Mg<sup>II</sup>]<sub>cell</sub><sup>27,30</sup>) produced identical spectra to that observed without Mg<sup>II</sup> but  
31 saturating at 1:1 ratio Co<sup>II</sup>:CobW (Fig. 2b). Equivalent experiments performed with GTP and  
32 GTPγS also revealed 1:1 Co<sup>II</sup>:CobW stoichiometry in the presence of [Mg<sup>II</sup>]<sub>cell</sub>  
33 (Supplementary Fig. 3c,d). Thus, binding of Mg<sup>II</sup> and guanine nucleotides preassembles one  
34 distinct Co<sup>II</sup> site in CobW. Occupancy of the first site by Mg<sup>II</sup> was spectroscopically silent in  
35 these experiments. The features at 339 nm and at 500 – 700 nm therefore correspond  
36 exclusively to a distinct tetrahedral Co<sup>II</sup> site and the coordinating sulfhydryl side-chains likely  
37 derive (at least in part) from the CxCC motif adjacent to the nucleotide-binding site.

1  
2 Mg<sup>II</sup> had negligible impact on the conditional affinity of EGTA for Co<sup>II</sup> at the  
3 concentrations used here (Supplementary Table 1): For this reason Mg<sup>II</sup> was not  
4 incorporated into curve-fitting models. Competition between Mg<sup>II</sup>GMPPNP-CobW and EGTA  
5 for Co<sup>II</sup> yielded a binding isotherm consistent with 1:1 stoichiometry for both Co<sup>II</sup>:protein and  
6 Co<sup>II</sup>:EGTA, and enabled  $K_{Co(II)}$  of  $2.7 (\pm 0.4) \times 10^{-9}$  M for Mg<sup>II</sup>GMPPNP-CobW to be  
7 determined (Fig. 2a, Supplementary Fig. 4a,b and Supplementary Tables 2,3). Competition  
8 with EGTA revealed a Co<sup>II</sup> affinity for Mg<sup>II</sup>GTPγS-CobW ( $K_{Co(II)} = 1.7 (\pm 0.8) \times 10^{-10}$  M;  
9 Supplementary Fig. 4c-e), that was more than 10-fold tighter than Mg<sup>II</sup>GMPPNP-CobW,  
10 establishing that the nature of the bound nucleotide exerts an effect on metal-binding to  
11 CobW.

### 12 13 **Co<sup>II</sup> binds a thousand-fold tighter with GTP than GDP**

14  
15 Observed variation in Co<sup>II</sup> affinities of CobW in association with Mg<sup>II</sup>GTPγS versus  
16 Mg<sup>II</sup>GMPPNP, prompted us to assess the Co<sup>II</sup> affinities of all three anticipated biological  
17 species: nucleotide-free CobW, Mg<sup>II</sup>GTP-CobW and Mg<sup>II</sup>GDP-CobW. Co<sup>II</sup> affinities of CobW  
18 and Mg<sup>II</sup>GDP-CobW were determined via competition with fura-2 (Fig. 3a,b and  
19 Supplementary Fig. 4f-i). Fura-2 is too weak to compete effectively with Mg<sup>II</sup>GTP-CobW  
20 (Supplementary Fig. 4j), but high concentrations of EGTA or nitrilotriacetic acid (NTA)  
21 imposed sufficient competition to enable  $K_{Co(II)}$  of  $3.0 (\pm 0.8) \times 10^{-11}$  M to be determined (Fig.  
22 3c and Supplementary Fig. 4k-m). GTP concentration was not a limiting factor in these  
23 affinity measurements (Supplementary Fig. 5). Under identical conditions used for affinity  
24 measurements, we confirmed that CobW-catalysed GTP hydrolysis is sufficiently slow such  
25 that nucleotides remain predominantly unhydrolysed over the duration of metal-binding  
26 experiments (Fig. 3d,e and Supplementary Fig. 6). Mg<sup>II</sup>GDP-CobW, despite displaying  
27 identical absorbance features indicating the persistence of the Cys-rich tetrahedral site  
28 (Supplementary Fig. 7), has a Co<sup>II</sup> affinity more than one thousand-fold weaker than  
29 Mg<sup>II</sup>GTP-CobW and only marginally tighter than unbound CobW which lacks this site  
30 altogether (Supplementary Table 3). GTP also confers higher Co<sup>II</sup> affinity than either of the  
31 tested non-hydrolysable analogues in which the γ-phosphates have been modified (Fig. 1f  
32 and Supplementary Table 3). Thus, the presence of an intact nucleotide γ-phosphate is a  
33 prerequisite for high-affinity Co<sup>II</sup> binding.

### 34 35 **Cu<sup>I</sup> and Zn<sup>II</sup> bind Mg<sup>II</sup>GTP-CobW more tightly than Co<sup>II</sup>**

36

1 In view of the challenges associated with correct metal-protein speciation, we sought to  
2 determine Mg<sup>II</sup>GTP-CobW affinities for other first-row transition metals (Fe<sup>II</sup>, Ni<sup>II</sup>, Cu<sup>I</sup>, Zn<sup>II</sup>).  
3 Fe<sup>II</sup>-titration into a mixture of Mg<sup>II</sup>GTP-CobW (50 μM) and probe ligand 4-(2-thiazolylazo)-  
4 resorcinol (Tar) (16 μM) showed Fe<sup>II</sup> being withheld by Tar which revealed a limiting affinity  
5 ( $K_{\text{Fe(II)}} > 10^{-6}$  M) for Mg<sup>II</sup>GTP-CobW (Fig. 4a and Supplementary Fig. 8). Competition  
6 between Mg<sup>II</sup>GTP-CobW (10 μM) and mag-fura-2 (Mf2; 20 μM) for Ni<sup>II</sup> showed that Mg<sup>II</sup>GTP-  
7 CobW has one Ni<sup>II</sup>-site which outcompetes Mf2 ( $K_{\text{Ni(II)}} < 10^{-8}$  M) in addition to two weaker  
8 sites which compete with Mf2 for Ni<sup>II</sup> ( $K_{\text{Ni(II)}} \sim 10^{-7}$  M) and are also present in CobW alone  
9 (Supplementary Fig. 9a). Competition with Tar allowed the affinity of the tight Ni<sup>II</sup>-site in  
10 Mg<sup>II</sup>GTP-CobW to be determined ( $K_{\text{Ni(II)}} = 9.8 (\pm 6.5) \times 10^{-10}$  M; Fig. 4b and Supplementary  
11 Fig. 9b,c). The conditional  $\beta_2$  value ( $4.3 (\pm 0.6) \times 10^{15}$  M<sup>-2</sup>) for Ni(Tar)<sub>2</sub> formation under  
12 experimental conditions (pH 7.0, 100 mM NaCl, 400 mM KCl) was independently established  
13 by competition with EGTA (Supplementary Fig. 10). Titration of Mg<sup>II</sup>GTP-CobW (15 μM) and  
14 bathocuproine disulfonate (Bcs; 30 μM) with Cu<sup>I</sup> did not reach the expected intensity at  
15 saturating metal concentrations (Supplementary Fig. 11a) suggesting the presence of a  
16 stable ternary complex, which would preclude accurate affinity determinations<sup>43</sup>. An  
17 equivalent experiment with alternative Cu<sup>I</sup>-probe bicinchoninic acid (Bca) showed that  
18 Mg<sup>II</sup>GTP-CobW has two Cu<sup>I</sup>-sites which outcompete Bca and at least three additional  
19 weaker Cu<sup>I</sup> sites which effectively compete with the probe (Supplementary Fig. 11b).  
20 Effective competition imposed by excess Bca enabled  $K_{\text{Cu(I)}}$  of  $2.4 (\pm 0.9) \times 10^{-16}$  M to be  
21 determined (Fig. 4c, Supplementary Fig. 11c,d and Supplementary Fig. 12), assuming only  
22 the tightest Cu<sup>I</sup>-site can acquire metal at the limiting Cu<sup>I</sup> availabilities employed (*eg*  $[\text{Cu}^{\text{I}}_{\text{aq}}] <$   
23  $3 \times 10^{-16}$  M in Fig. 4c). Zn<sup>II</sup>-titration into a mixture of quin-2 (20 μM) and Mg<sup>II</sup>GTP-CobW (10  
24 μM) revealed one high-affinity Zn<sup>II</sup>-site in the protein which was too tight to be quantified by  
25 using quin-2 thus showing  $K_{\text{Zn(II)}} < 10^{-12}$  M (Fig. 4d).

26  
27 Because of the limiting affinity of quin-2 we employed inter-metal competition, which  
28 presumably also occurs within the buffered intracellular milieu, to determine  $K_{\text{Zn(II)}}$  for  
29 Mg<sup>II</sup>GTP-CobW.  $K_{\text{Zn(II)}}$  was determined, relative to the known  $K_{\text{Co(II)}}$ , via competition between  
30 the two metals. This approach required an excess of metal ions competing for a limited  
31 number of protein metal-sites (*ie*  $[\text{Co}^{\text{II}}]_{\text{tot}} + [\text{Zn}^{\text{II}}]_{\text{tot}} > [\text{CobW}]_{\text{tot}}$ ) thus it was essential to include  
32 a buffering ligand, in this case NTA, to control the speciation of all Co<sup>II</sup> and Zn<sup>II</sup> in solution (*ie*  
33  $[\text{NTA}]_{\text{tot}} > [\text{Co}^{\text{II}}]_{\text{tot}} + [\text{Zn}^{\text{II}}]_{\text{tot}}$ ). The measured equilibrium ( $K_{\text{ex}}$  in Fig. 5a) was the exchange  
34 constant for Co<sup>II</sup>/Zn<sup>II</sup> exchange between the protein (Mg<sup>II</sup>GTP-CobW) and buffering ligand  
35 (NTA). Equilibrium ratios of  $[\text{Co}^{\text{II}}\text{Mg}^{\text{II}}\text{GTP-CobW}]/[\text{Zn}^{\text{II}}\text{Mg}^{\text{II}}\text{GTP-CobW}]$  were determined (Fig.  
36 5b-e and Supplementary Table 4): absorbance intensity at  $A_{339 \text{ nm}}$  reported specifically on the  
37 Co<sup>II</sup>-protein complex and all remaining protein was Zn<sup>II</sup>-bound (since Mg<sup>II</sup>GTP-CobW was

1 metal-saturated under experimental conditions; Supplementary Fig. 13). The concentrations  
2 of NTA-bound metals were determined from mass balance relationships (equations (6-8) in  
3 Methods). Experiments were conducted at multiple relative availabilities of Co<sup>II</sup> and Zn<sup>II</sup> and  
4 reciprocally (Fig. 5b-e), with consistent results (Supplementary Table 4), to confirm reliability  
5 of measured affinities. We thus determined a tight  $K_{Zn(II)}$  of  $1.9 (\pm 0.6) \times 10^{-13}$  M for Mg<sup>II</sup>GTP-  
6 CobW (Supplementary Table 3).

7

## 8 **GTP not GDP will enable Co<sup>II</sup> acquisition in cells**

9

10 In the same manner that Fig. 4 considered competition between a ligand (Tar, Bca or quin-2)  
11 and a protein (Mg<sup>II</sup>GTP-CobW) for metal-binding *in vitro*, metal acquisition by proteins *in vivo*  
12 likewise involves competition with a surplus of cytosolic ligands that buffer metals to different  
13 availabilities<sup>8,27,32,44,45</sup>. Recent work has estimated the buffered availabilities of metals M  
14 (where M = Mg<sup>II</sup>, Mn<sup>II</sup>, Fe<sup>II</sup>, Co<sup>II</sup>, Ni<sup>II</sup>, Cu<sup>I</sup>, Zn<sup>II</sup>) in a reference bacterium (*Salmonella*<sup>27</sup>)  
15 expressed here as free energies ( $\Delta G$ ; Fig. 6). The intracellular available  $\Delta G$  for each metal,  
16  $\Delta G_M$ , is defined as the free energy required for a ligand to become 50% metalated from  
17 available and exchangeable intracellular metal (see Supplementary Note 1). Fig. 6 and  
18 Supplementary Fig. 14 show the intracellular available  $\Delta G_M$  values in an 'idealised cell' (*ie*  
19 neither metal-deficiency nor -excess) defined as the metal availabilities at which each  
20 cognate sensor undergoes half of its transcriptional response. Bars show the changes in  
21 available intracellular  $\Delta G_M$  as sensors shift from 10 – 90% (Fig. 6) or 1 – 99%  
22 (Supplementary Fig. 14) of their respective responses. The percentage occupancy of a  
23 protein, P, with metal, M, *in vivo* is governed by the difference between the free energy for  
24 protein metalation,  $\Delta G_{MP}$ , and the intracellular available  $\Delta G_M$  (equation (1)) and can be  
25 calculated via equation (2) (see Supplementary Note 1):

26

$$27 \quad \Delta \Delta G_M = \Delta G_{MP} - \Delta G_M \quad (1)$$

28

$$29 \quad \text{Fractional occupancy (\%)} = 100 \times \frac{[MP]}{[P]_{\text{tot}}} = 100 \times \frac{e^{-\frac{\Delta \Delta G_M}{RT}}}{1 + e^{-\frac{\Delta \Delta G_M}{RT}}} \quad (2)$$

30

31 In an idealised cell, the  $\Delta G_{Co(II)}$  for CobW and Mg<sup>II</sup>GDP-CobW were both significantly  
32 more positive than intracellular available  $\Delta G_{Co(II)}$  ( $\Delta \Delta G_{Co(II)} \gg 0$ ; Fig. 6) resulting in negligible  
33 Co<sup>II</sup>-occupancies of 1.0% and 2.5% for these two protein forms, respectively. Conversely,  
34  $\Delta G_{Co(II)}$  for Mg<sup>II</sup>GTP-CobW was significantly more negative than intracellular available  $\Delta G_{Co(II)}$



1 ( $\Delta\Delta G_{\text{Co(II)}} \ll 0$ ), resulting in almost complete protein metalation (99%). Thus, CobW needs  
2  $\text{Mg}^{\text{II}}\text{GTP}$  to acquire  $\text{Co}^{\text{II}}$  in a cell.

3

#### 4 **$\text{Mg}^{\text{II}}\text{GTP-CobW}$ may also acquire $\text{Zn}^{\text{II}}$**

5

6 In addition to  $\text{Co}^{\text{II}}$  other metals also bound to  $\text{Mg}^{\text{II}}\text{GTP-CobW}$  (Figs. 4 and 5). However,  $\Delta\Delta G$   
7 for  $\text{Fe}^{\text{II}}$ ,  $\text{Ni}^{\text{II}}$  and  $\text{Cu}^{\text{I}}$ , was significantly greater than zero (equation (1) and Fig. 6), thus  
8 preventing acquisition of these metals (equation (2) and Table 1). In contrast,  $\Delta\Delta G_{\text{Zn(II)}}$  was <  
9 0 with *in vivo*  $\text{Zn}^{\text{II}}$  occupancy predicted to be 86% (Fig. 6 and Table 1). However, based on  
10 equation (2) the sum of metal occupancies of  $\text{Mg}^{\text{II}}\text{GTP-CobW}$  gave an impossible total  
11 metalation > 100% (Table 1). Since  $\Delta\Delta G$  was < 0 for both  $\text{Co}^{\text{II}}$  and  $\text{Zn}^{\text{II}}$ , a more sophisticated  
12 approach needs to account for competition between multiple buffered metals in order to  
13 predict how much  $\text{Zn}^{\text{II}}$  binds  $\text{Mg}^{\text{II}}\text{GTP-CobW}$  *in vivo*.

14

#### 15 **Calculating inter-metal competition in a cell**

16

17 Figure 5 considered competition between  $\text{Co}^{\text{II}}$  and  $\text{Zn}^{\text{II}}$  for a single metal-binding site in a  
18 protein ( $\text{Mg}^{\text{II}}\text{GTP-CobW}$ ) when the metals were buffered to different availabilities *in vitro* by  
19 an excess of NTA. This can be represented as an available  $\Delta G_{\text{M}}$  (Supplementary Table 4).  
20 The observed  $\text{Co}^{\text{II}}$  occupancy was a function of the protein's affinities for both  $\text{Co}^{\text{II}}$  and  $\text{Zn}^{\text{II}}$   
21 relative to their buffered availabilities in solution (*ie*  $\Delta\Delta G$  values), as described by equation  
22 (3) (see Supplementary Note 1).

23

$$24 \quad \text{Fractional (\%)} \text{Co}^{\text{II}} \text{ occupancy} = 100 \times \frac{e^{-\frac{\Delta\Delta G_{\text{Co(II)}}}{RT}}}{1 + e^{-\frac{\Delta\Delta G_{\text{Co(II)}}}{RT}} + e^{-\frac{\Delta\Delta G_{\text{Zn(II)}}}{RT}}} \quad (3)$$

25

26 By analogy in a cytoplasm multiple metals, each buffered to different intracellular  
27 available  $\Delta G_{\text{M}}$ , compete for a single protein-binding site. We generalised equation (3) to  
28 account for n different metals (equation (4) and Supplementary Note 1).

29

$$30 \quad \text{Fractional (\%)} \text{ occupancy (with metal } M_1 \text{ of interest)} = 100 \times \frac{e^{-\frac{\Delta\Delta G_{M_1}}{RT}}}{1 + \sum_{k=1}^{k=n} e^{-\frac{\Delta\Delta G_{M_k}}{RT}}} \quad (4)$$

31

32 Thus, we developed a metalation calculator (Supplementary Data 1) for determining  
33 *in vivo* metal occupancies of proteins, accounting for multiple inter-metal competitions plus  
34 competition from components of the intracellular milieu.

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## Co<sup>II</sup> specificity under idealised conditions

Since  $\Delta\Delta G$  was  $< 0$  for binding of both Co<sup>II</sup> and Zn<sup>II</sup> to Mg<sup>II</sup>GTP-CobW (Fig. 6), equation (4) was next used to predict *in vivo* metalation in an idealised cell. Between the five metals considered (Fe<sup>II</sup>, Co<sup>II</sup>, Ni<sup>II</sup>, Cu<sup>I</sup> and Zn<sup>II</sup>), Mg<sup>II</sup>GTP-CobW will favour Co<sup>II</sup>-binding in a cell and calculations via equation (4) predicted occupancies of 92% and 7%, for Co<sup>II</sup> and Zn<sup>II</sup>, respectively (Table 1). Thus, although Mg<sup>II</sup>GTP-CobW affinities for both Co<sup>II</sup> and Zn<sup>II</sup> are tight enough to extract either metal from the cytosolic buffer, Co<sup>II</sup> will outcompete Zn<sup>II</sup>, rationalising specificity but only in an intracellular context where there is competition from other cellular components.

## Fine tuning $\Delta G$ for metalation in a cell

Calculated free energies for intracellular metalation ( $\Delta G_M$ ) in Fig. 6 are based on an assumption that cellular metal availabilities are fixed at 'ideal' buffered concentrations where every metal sensor undergoes half of its transcriptional response (*ie* normalised fractional DNA occupancy ' $\theta_D$ ' = 0.5, see ref.<sup>27</sup>). In reality cellular metal availabilities, and consequently  $\theta_D$  of sensors, fluctuate conditionally (*eg* in response to addition of metals or chelators to the growth media). For example, the dynamic response range (defined as  $\theta_D = 0.99 - 0.01$ ) of RcnR, the Co<sup>II</sup> sensor from *Salmonella*, coincides with an increase in the intracellular available [Co<sup>II</sup>] from  $2.4 \times 10^{-11}$  to  $2.7 \times 10^{-7}$  M, corresponding to an increase in intracellular available  $\Delta G_{Co(II)}$  from -60.6 to -37.5 kJ mol<sup>-1</sup> (Fig. 7a and Supplementary Table 5).

In order to account for this variation, we developed a method to fine-tune the free energy calculations for Co<sup>II</sup> under bespoke culture conditions using qPCR analysis of the RcnR-regulated gene *rcnA*. Fine-tuning was performed in *E. coli*\* which has been engineered to synthesise vitamin B<sub>12</sub> (*E. coli* and *Salmonella* RcnR share 93% sequence identity and equivalent responses to available Co<sup>II</sup> were assumed). *E. coli*\* cells were cultured in standard medium with increasing Co<sup>II</sup> supplementation. The *rcnA* transcript abundance (Fig. 7b) was used to calculate  $\theta_D$  of RcnR for each condition (via equation (9) in Methods) following calibration of the maximum and minimum responses (defined as  $\theta_D = 0.99$  and 0.01 at low and high [Co<sup>II</sup>] respectively; Supplementary Fig. 15). This enabled the intracellular Co<sup>II</sup> availabilities, as conditional free energies, to be calculated from the RcnR  $\theta_D$  for each condition (Fig. 7a, Supplementary Table 5).

## Co<sup>II</sup>-acquisition by Mg<sup>II</sup>GTP-CobW predicts B<sub>12</sub> (corrinoid) synthesis

1  
2 Does the amount of Co<sup>II</sup> inserted into B<sub>12</sub> follow the predicted metalation of Mg<sup>II</sup>GTP-CobW?  
3 Metal occupancies of Mg<sup>II</sup>GTP-CobW in *E. coli*\* samples were recalculated (via equation (4))  
4 using bespoke intracellular available free energies,  $\Delta G_{\text{Co(II)}}$ , for each growth condition (Fig. 7  
5 and Supplementary Table 5). This predicted that in unsupplemented LB media the protein  
6 would be predominantly Zn<sup>II</sup>-bound (10% Co<sup>II</sup> and 77% Zn<sup>II</sup>) and that Co<sup>II</sup> occupancies would  
7 increase from 10% to 97% as added [Co<sup>II</sup>] increased from 0 – 30  $\mu\text{M}$  (Fig 8a). Since  
8 intracellular Zn<sup>II</sup> availability was also significant in our predictions, we confirmed that our  
9 previous estimation of  $\Delta G_{\text{Zn(II)}}$  was valid in LB media (Supplementary Fig. 16). Corrin  
10 concentrations (presumed to be predominantly B<sub>12</sub>) were measured in *E. coli*\* strains  
11 containing or missing *cobW* (Fig. 8b and Supplementary Fig. 17), under the growth  
12 conditions for which intracellular available  $\Delta G_{\text{Co(II)}}$  was defined (Supplementary Table 5). As  
13 the added [Co<sup>II</sup>] increased so did B<sub>12</sub> production in *cobW*(+), consistent with the predicted  
14 loading of Mg<sup>II</sup>GTP-CobW with Co<sup>II</sup> (Fig 8). At higher [Co<sup>II</sup>], CobW-independent B<sub>12</sub> synthesis  
15 became evident. Notably, the synthesis of B<sub>12</sub> which is dependent on CobW (Fig. 8b,  
16 compare *cobW*(+) with *cobW*(-)) closely matches the predicted metalation of Mg<sup>II</sup>GTP-CobW  
17 (Fig 8a).

18

## 19 Discussion

20

21 CobW belongs to a ubiquitous family of putative metallochaperones (COG0523) but its  
22 cognate metal, target protein(s) and mechanism of action were undefined. Here we establish  
23 the connection between CobW and Co<sup>II</sup> (Figs. 1-8). We show how CobW can acquire Co<sup>II</sup> in  
24 a cell (Figs. 1-3, Fig. 6 and Table 1). Free-energy calculations reveal that in an idealised cell  
25 Co<sup>II</sup> ions will not flow from the cellular milieu to nucleotide-free CobW ( $\Delta\Delta G_{\text{Co(II)}} > 0$ ).  
26 Crucially, Co<sup>II</sup> will flow from the cellular milieu to the Mg<sup>II</sup>GTP form of CobW ( $\Delta\Delta G_{\text{Co(II)}} < 0$ )  
27 (Fig. 6, Fig. 9a, Table 1 and Supplementary Table 3). Thus, CobW must first bind Mg<sup>II</sup>GTP in  
28 order to acquire Co<sup>II</sup> inside a cell. In contrast, the product of GTP hydrolysis, Mg<sup>II</sup>GDP-  
29 CobW, will release Co<sup>II</sup> to the cellular milieu ( $\Delta\Delta G_{\text{Co(II)}} > 0$ ) (Fig. 6, Fig. 9b, Table 1 and  
30 Supplementary Table 3). Thus, the GTPase activity of CobW will facilitate Co<sup>II</sup> release for  
31 example to CobNST for insertion into the corrin ring of B<sub>12</sub> (Fig. 3d,e and Supplementary Fig.  
32 6). We establish that CobW enhances B<sub>12</sub> production when Co<sup>II</sup> is limiting (Fig. 8b), and Fig.  
33 9 illustrates the proposed mechanism.

34

35 The intrinsic GTPase activity of CobW is slow (Fig. 3d,e and Supplementary Fig. 6),  
36 as observed for other COG0523 proteins<sup>13-15,17</sup>. Giedroc and co-workers hypothesised that  
37 interactions with partner proteins may stimulate GTP hydrolysis in similar proteins<sup>17</sup>.

1 Likewise, we speculate that CobNST could act as a guanine nucleotide activating protein  
2 (GAP) enabling Co<sup>II</sup> release to be targeted to the cobaltocheletase. Release of Co<sup>II</sup> mediated  
3 by CobNST acting as a guanine nucleotide exchange-factor (GEF) is also formally  
4 possible<sup>46</sup>. By analogy to ZTP-ZagA<sup>18</sup>, GTP-binding (and subsequent metal-acquisition) by  
5 CobW could promote interaction with CobNST and contribute to the reaction cycle (Fig. 9).  
6 Dissociation of Mg<sup>II</sup>GDP (or nucleotide exchange), resets the reaction cycle with GTPases  
7 thought to be saturated with nucleotide (either GTP or GDP) inside cells<sup>47</sup>.

8  
9 Initial calculations here, and in previous work<sup>27</sup>, assume an idealised cell in which the  
10 metal sensors are at the mid-points of their dynamic ranges ( $\theta_D = 0.5$ ). Therefore, we have  
11 calculated the available  $\Delta G_{Co(II)}$  in real (conditional) cells from the responses of RcnR ( $\theta_D$ )  
12 estimated experimentally by qPCR of *rcnA* (Fig. 7 and Supplementary Fig. 15). The  
13 observation that *R. capsulatus* CobW functions in *E. coli* cells suggests overlap in the  
14 dynamic ranges for  $\Delta G_{Co(II)}$  in these two bacteria, although evidence here of limited  
15 metalation in LB without additional Co<sup>II</sup> could be a function of the heterologous host (Fig. 8).  
16 Notably, a dedicated Co<sup>II</sup> import system found in *R. capsulatus* (CbiMNQO) is not present in  
17 *E. coli*<sup>48</sup>. As with other metallochaperones<sup>28,49</sup>, CobW is crucial when the cognate metal is  
18 limiting but at elevated Co<sup>II</sup>, CobW-independent synthesis of B<sub>12</sub> occurs (Fig. 8b). CobNST  
19 must acquire Co<sup>II</sup> directly from the cytosol at the higher available  $\Delta G_{Co(II)}$ . Importantly, CobW-  
20 dependent B<sub>12</sub> synthesis tracked with the calculated Co<sup>II</sup> occupancy of Mg<sup>II</sup>GTP-CobW in  
21 cells supplemented with different amounts of Co<sup>II</sup> (Fig. 8). This is an encouraging first test of  
22 this approach, and of the easy-to-use spreadsheet (Supplementary Data 1), to calculate  
23 changes in the metalation state of a protein inside cells.

24  
25 Mg<sup>II</sup>GTP-CobW binds Zn<sup>II</sup> and Cu<sup>I</sup> more tightly than Co<sup>II</sup> (Fig. 3c, Fig. 4, Fig. 5,  
26 Supplementary Table 3). Notably, by taking into account intracellular metal availability,  $\Delta\Delta G$   
27 for Cu<sup>I</sup> was shown to be greater than zero in an idealised cell (Fig. 6), and also in conditional  
28 cells at either 90% or 99% of the dynamic range of the Cu<sup>I</sup> sensor CueR (Fig. 6 and  
29 Supplementary Fig. 14). Thus Mg<sup>II</sup>GTP-CobW will not acquire Cu<sup>I</sup>. However,  $\Delta\Delta G$  for Zn<sup>II</sup>  
30 was below zero in an idealised cell suggesting that Mg<sup>II</sup>GTP-CobW is at risk of mis-  
31 metalation with Zn<sup>II</sup> (Fig. 6). Indeed, given that CobW binds Zn<sup>II</sup> more tightly than many  
32 known Zn<sup>II</sup>-proteins<sup>32,50</sup>, it is remarkable that Zn<sup>II</sup> is not the cognate metal. The data in Figure  
33 5, plus Supplementary Table 4, illustrate how occupancies of Mg<sup>II</sup>GTP-CobW with Co<sup>II</sup>  
34 versus Zn<sup>II</sup> change as a function of change in relative buffered metal availabilities. By  
35 reference to intracellular available free energies, the metal with the most negative  $\Delta\Delta G$  will  
36 have the highest occupancy *in vivo* (equation (4)). In an idealised cell,  $\Delta\Delta G$  for Co<sup>II</sup> is more  
37 negative than  $\Delta\Delta G$  for Zn<sup>II</sup> and so the weaker binding metal dominates (Fig. 6,

1 Supplementary Table 3). In conditional cells without added  $\text{Co}^{\text{II}}$ ,  $\Delta\Delta G$  for  $\text{Zn}^{\text{II}}$  becomes more  
2 negative than  $\Delta\Delta G$  for  $\text{Co}^{\text{II}}$  and the calculations show binding of  $\text{Zn}^{\text{II}}$  dominating (Fig. 8a).  
3 The previously intractable challenge to understand inter-metal competition in a cell now  
4 becomes tractable (Supplementary Data 1). Metallochaperones and chelatases may  
5 introduce kinetic contributions to the partitioning of metals and these can now become  
6 evident in departures from the thermodynamic predictions of the metalation calculator  
7 spreadsheet (Supplementary Data 1).

8  
9 Future structural studies are necessary to understand how  $\text{Mg}^{\text{II}}$ GTP-binding  
10 facilitates high affinity  $\text{Co}^{\text{II}}$  binding to CobW. Spectral features indicate that the  $\text{Co}^{\text{II}}$  site in  
11  $\text{Mg}^{\text{II}}$ GTP-CobW involves thiols, likely derived from the CxCC motif in the GTPase domain,  
12 and a tetrahedral geometry (Figs. 1, 2 and Supplementary Fig. 3). All COG0523 proteins  
13 contain the CxCC motif<sup>10</sup>, including those that putatively handle  $\text{Fe}^{\text{II}}$  (Nha3)<sup>12,13,51</sup> and  $\text{Zn}^{\text{II}}$   
14 (YeiR, YjiA, ZigA, ZagA)<sup>14-16,18</sup>. Differences in coordination spheres may alter the  $\Delta\Delta G$   
15 values sufficiently to adjust the specificities of these proteins with respect to available  
16 intracellular  $\text{Fe}^{\text{II}}$ ,  $\text{Co}^{\text{II}}$  and  $\text{Zn}^{\text{II}}$ . Intriguingly,  $\text{Ni}^{\text{II}}$ -binding to  $\text{Mg}^{\text{II}}$ GTP-CobW does not follow the  
17 order of stabilities of metal-binding predicted by the Irving-Williams series (Fig. 6). An  
18 appealing explanation is that the allosteric coupling of GTP- and metal-binding imposes a  
19 (tetrahedral) geometry on the metal site that would disfavour  $\text{Ni}^{\text{II}}$ -coordination (the Irving-  
20 Williams series applies where there is no steric selection): Notably, related G3E GTPases  
21 involved in  $\text{Ni}^{\text{II}}$  homeostasis (HypB and UreG) display square planar  $\text{Ni}^{\text{II}}$ -coordination  
22 geometry<sup>52,53</sup>.

23  
24 In conclusion, CobW is calculated to be selective for acquiring  $\text{Co}^{\text{II}}$  in its  $\text{Mg}^{\text{II}}$ GTP  
25 form under conditions of ideal metallostasis, but at risk of erroneously binding  $\text{Zn}^{\text{II}}$  when  
26 intracellular  $\text{Co}^{\text{II}}$  is low or  $\text{Zn}^{\text{II}}$  is high (Figs. 6, 7 and 8a). The lack of a dedicated  $\text{Co}^{\text{II}}$  import  
27 system could make under-metalation with  $\text{Co}^{\text{II}}$  (and resultant mis-metalation with  $\text{Zn}^{\text{II}}$ )  
28 especially problematic in *E. coli*<sup>48</sup>. This has tantalising implications for engineering bacterial  
29 strains suited to the manufacture of vitamin B<sub>12</sub>, either via enhanced  $\text{Co}^{\text{II}}$  uptake or impaired  
30  $\text{Zn}^{\text{II}}$  accumulation. More generally, with so many enzymes requiring metals, an ability to  
31 calculate *in vivo* metalation should have widespread utility in industrial biotechnology  
32 (Supplementary Data 1).

## 1 **Methods**

2

### 3 **CobW expression and purification**

4

5 The DNA sequence coding CobW was amplified by PCR using primers 1 and 2  
6 (Supplementary Table 6) with genomic DNA from *Rhodobacter capsulatus* SB1003 as  
7 template. The amplified fragment contained an NdeI restriction site at the 5' end and a SpeI  
8 site at the 3' end, allowing it to be cloned into a modified pET-3a vector as previously  
9 described<sup>39</sup>. *E. coli* pLysS, transformed with this pET-3a-CobW plasmid, were cultured in LB  
10 medium with antibiotics carbenicillin (100 mg L<sup>-1</sup>) and chloramphenicol (34 mg L<sup>-1</sup>). At mid-  
11 log phase, protein expression was induced with IPTG (0.4 mM) at 37°C (3-4h). Cells were  
12 resuspended in 20 mM sodium phosphate pH 7.4, 500 mM NaCl, 5 mM imidazole, 5 mM  
13 DTT and 1 mM PMSF for lysis (sonication). Lysate was loaded to a 5 mL HisTrap HP  
14 column (GE Healthcare) pre-equilibrated in suspension buffer. CobW binds to the HisTrap  
15 column courtesy of a natural His-rich region within the protein. The column was washed with  
16 suspension buffer (10 CVs), then eluted with suspension buffer containing 100 mM  
17 imidazole. Protein-containing fractions were incubated with excess (≥10-fold) EDTA for ≥ 1h  
18 before being loaded to a HiLoad 26/600 Superdex 75 size exclusion column equilibrated in  
19 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM DTT and eluted in the same buffer. Peak CobW-  
20 containing fractions (determined from SDS-PAGE) were pooled, concentrated to ~0.5 mL  
21 (using a Vivaspin® 15 Turbo centrifugal concentrator) then transferred to an anaerobic  
22 chamber. The sample was applied to a PD-10 Sephadex G-25 gel-filtration column (GE  
23 Healthcare) equilibrated in deoxygenated chelex-treated buffer (10 mM HEPES pH 7.0, 100  
24 mM NaCl, 400 mM KCl) and eluted in the same buffer. Purified CobW samples were  
25 quantified by  $A_{280\text{ nm}}$  using extinction coefficient  $\epsilon = 15,300\text{ cm}^{-1}\text{ M}^{-1}$  determined by  
26 quantitative amino acid analysis (performed by Alta Bioscience Ltd). Samples were  
27 confirmed to be of high purity (by SDS-PAGE) and ≥95% metal-free (by inductively coupled  
28 plasma-mass spectrometry; ICP-MS). ICP-MS was conducted using Durham University Bio-  
29 ICP-MS Facility. Protein cysteines were ≥ 90% reduced, determined by reaction with ~10-  
30 fold excess of Ellman's reagent 5,5'-dithio-bis-[2-nitrobenzoic acid] (produces one equivalent  
31 of chromophore TNB<sup>2-</sup> per protein thiol,  $A_{412\text{ nm}} = 14,150\text{ cm}^{-1}\text{ M}^{-1}$ )<sup>54,55</sup>.

32

33 Protein identity was confirmed using electrospray ionisation mass spectrometry (ESI-  
34 MS) by Durham University Department of Chemistry Mass Spectrometry Service. ESI-MS  
35 data were recorded on a QToF Premier mass spectrometer coupled to an Acuity UPLC  
36 system (Waters). Protein samples were desalted prior to injection using a Waters MassPrep  
37 desalting cartridge (2.1 ×10 mm) and eluted with a linear acetonitrile gradient (20–80% v/v;

1 0.1% formic acid). Spectra were processed using Masslynx 4.1 and deconvoluted using  
2 MaxEnt 1.

3

#### 4 **Preparation of metal stocks**

5

6 All metal stocks were prepared in ultrapure water from appropriate salts ( $\text{MgCl}_2$ ,  
7  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ ,  $\text{CoCl}_2$ ,  $\text{NiSO}_4$ ,  $\text{CuSO}_4$ ,  $\text{ZnCl}_2$ ) and quantified by ICP-MS analysis.  $\text{Fe}^{\text{II}}$  stocks  
8 were prepared by dissolving  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  in deoxygenated 0.1% (v/v) HCl in an  
9 anaerobic chamber. Reaction with excess ferrozine (~ 50-fold) confirmed that iron was  $\geq$   
10 95% reduced ( $\text{Fe}^{\text{II}}\text{Fz}_3 \epsilon_{562 \text{ nm}} = 27,900 \text{ cm}^{-1} \text{ M}^{-1}$ )<sup>56</sup>. Concentrated stocks were diluted daily in  
11 deoxygenated ultrapure water to prepare working solutions and confirmed to be  $\geq 90\%$   $\text{Fe}^{\text{II}}$ .  
12 Other metal stocks were prepared aerobically as concentrated stocks and diluted to working  
13 solutions with deoxygenated ultrapure water in an anaerobic chamber.

14

#### 15 **Determination of $\text{Co}^{\text{II}}$ -binding stoichiometries**

16

17 Metal-binding experiments were conducted in an anaerobic chamber in deoxygenated,  
18 chelex-treated 10 mM HEPES pH 7.0, 100 mM NaCl, 400 mM KCl. For stoichiometry  
19 determinations,  $\text{Co}^{\text{II}}$  was titrated into a solution of CobW (15 – 30  $\mu\text{M}$ ) together with relevant  
20 nucleotides (supplied in ~10-fold excess of protein concentration for GTP and GDP and ~3-  
21 fold excess for GMPPNP and  $\text{GTP}\gamma\text{S}$ , as specified in figure legends) in the absence or  
22 presence of  $\text{Mg}^{\text{II}}$  (2.7 mM). Absorbance was recorded using a Lambda 35 UV-visible  
23 spectrophotometer (Perkin Elmer). The extinction coefficient of  $\text{Co}^{\text{II}}\text{Mg}^{\text{II}}\text{GTP-CobW}$  ( $\epsilon_{339 \text{ nm}} =$   
24  $2,800 \pm 100 \text{ cm}^{-1} \text{ M}^{-1}$ , average  $\pm$  s.d of  $n=3$  independent titrations) was determined from  
25 absorbance at saturating metal concentrations (Supplementary Fig. 3d). Extinction  
26 coefficients of related complexes  $\text{Co}^{\text{II}}\text{Mg}^{\text{II}}\text{GMPPNP-CobW}$ ,  $\text{Co}^{\text{II}}\text{Mg}^{\text{II}}\text{GTP}\gamma\text{S-CobW}$ ,  $\text{Co}^{\text{II}}_2\text{GTP-}$   
27  $\text{CobW}$ ,  $\text{Co}^{\text{II}}_2\text{GMPPNP-CobW}$  and  $\text{Co}^{\text{II}}_2\text{GTP}\gamma\text{S-CobW}$  were similarly determined (Figs. 1-2,  
28 Supplementary Fig. 3): within experimental error, all produced the same extinction coefficient  
29 as for  $\text{Co}^{\text{II}}\text{Mg}^{\text{II}}\text{GTP-CobW}$  thus  $\epsilon_{339 \text{ nm}} = 2,800 \text{ cm}^{-1} \text{ M}^{-1}$  was assigned to all species.

30 Gel-filtration chromatography experiments were performed by incubating CobW (10  $\mu\text{M}$ ) and  
31  $\text{Co}^{\text{II}}$  (30  $\mu\text{M}$ ) for 30 minutes with or without cofactor GMPPNP (30  $\mu\text{M}$ ) then applying 0.5 mL  
32 to a PD-10 Sephadex G-25 gel-filtration column (GE Healthcare). Eluted fractions (0.5 mL)  
33 were analysed for cobalt by ICP-MS and for protein by Bradford assay.

34

#### 35 **Determination of metal affinities via ligand competition**

36

1 Ligand competition experiments were conducted in an anaerobic chamber in deoxygenated,  
2 chelex-treated 10 mM HEPES pH 7.0, 100 mM NaCl, 400 mM KCl, except where high  
3 concentrations ( $\geq 1$  mM) of competing ligand were employed, where 50 mM HEPES was  
4 used to maintain buffered pH 7.0. Absorbance was recorded using a Lambda 35 UV-visible  
5 spectrophotometer (Perkin Elmer). Fluorescence spectra were recorded using a Cary  
6 Eclipse fluorescence spectrophotometer (Agilent). Affinities were determined at a range of  
7 different competing conditions (by varying the competing ligand and/or the protein:ligand  
8 ratio) to ensure reliability: details are documented in Supplementary Table 2. Scripts used for  
9 data fitting (using Dynafit<sup>57</sup>) are provided in Supplementary Note 3. The effect of  $Mg^{II}$  (2.7  
10 mM) on apparent dissociation constants of ligand standards (EGTA, NTA, Fura-2, Mf2 and  
11 quin-2) was calculated to be insignificant under the conditions of competition experiments  
12 (Supplementary Table 1). For probes with undefined  $Mg^{II}$  affinities (Tar, Bca) control  
13 experiments confirmed that addition of  $Mg^{II}$  (2.7 mM) had negligible effect on competition  
14 experiments (Supplementary Figs. 10d and 12). Thus,  $Mg^{II}$  was not incorporated into the  
15 curve-fitting models.

16

17 For determination of weaker ( $K_D > 10$  nM)  $Co^{II}$  binding affinities (CobW and CobW-  
18  $Mg^{II}GDP$ ),  $Co^{II}$  was titrated into a solution of fura-2 (quantified by  $\epsilon_{363\text{ nm}} = 28,000\text{ cm}^{-1}\text{ M}^{-1}$ )<sup>58</sup>  
19 and CobW in the presence or absence of cofactors ( $Mg^{II}$  and GDP) and fluorescence  
20 emission ( $\lambda_{\text{ex}} = 360$  nm;  $\lambda_{\text{max}} \sim 505$  nm) was recorded at equilibrium.  $Co^{II}$ -dependent  
21 fluorescence quenching of fura-2 was used to determine  $Co^{II}$  speciation. For determination  
22 of  $Co^{II}$  binding affinities tighter than 10 nM (CobW- $Mg^{II}GMPPNP$ , CobW- $Mg^{II}GTP\gamma S$  and  
23 CobW- $Mg^{II}GTP$ ),  $Co^{II}$  was titrated into a solution containing CobW, competing ligand (EGTA  
24 or NTA),  $Mg^{II}$  and nucleotide (GMPPNP, GTP $\gamma S$  or GTP). UV-visible absorbance (relative to  
25 metal-free solution) was recorded at equilibrium to determine  $Co^{II}$  speciation ( $\epsilon_{339\text{ nm}} = 2,800$   
26  $\text{cm}^{-1}\text{ M}^{-1}$  for  $Co^{II}$ -bound proteins). Data were fit using Dynafit<sup>57</sup> to models describing 1:1  
27 binding stoichiometry for  $Co^{II}$ :protein and 1:1 binding stoichiometry for  $Co^{II}$ :ligand (ligand =  
28 Fura-2, EGTA or NTA). Ligand dissociation constants at pH 7.0: Fura-2  $K_{Co(II)} = 8.6 \times 10^{-9}$  M  
29 (ref.<sup>59</sup>); EGTA  $K_{Co(II)} = 7.9 \times 10^{-9}$  M (ref.<sup>60</sup>); NTA  $K_{Co(II)} = 2.2 \times 10^{-8}$  M (ref.<sup>60</sup>).

30

31  $Fe^{II}$  was titrated into a solution of Tar (16  $\mu M$ ),  $Mg^{II}$  (2.7 mM) and GTP (500  $\mu M$ ) in  
32 the absence or presence of CobW (50  $\mu M$ ) and UV-visible absorbance recorded at  
33 equilibrium to define  $Fe^{II}$  speciation ( $Fe^{II}Tar_2$   $\epsilon_{720} = 19,560\text{ cm}^{-1}\text{ M}^{-1}$  under experimental  
34 conditions, Supplementary Fig 8a). Data were fit in Dynafit<sup>57</sup> to a model describing 1:1  
35 binding stoichiometry for  $Fe^{II}$ :protein and 1:2 binding stoichiometry for  $Fe^{II}$ :Tar using  $\beta_{2,Fe(II)} =$   
36  $4.0 \times 10^{13}\text{ M}^{-2}$  for Tar at pH 7.0 (ref.<sup>61</sup>). Experimental data were compared to simulated fits  
37 with defined protein  $K_{Fe(II)} = 10^{-6}$  M,  $10^{-7}$  M, allowing limiting  $K_D \geq 10^{-6}$  M for CobW- $Mg^{II}GTP$  to



1 be determined. Tar stock concentrations were quantified using  $\epsilon_{470\text{ nm}} = 24,800\text{ cm}^{-1}\text{ M}^{-1}$   
2 (reported value at pH 7.0<sup>61</sup>) and verified by titration with metal stocks ( $\text{Fe}^{\text{II}}$  or  $\text{Ni}^{\text{II}}$ , quantified  
3 by ICP-MS).

4  
5  $\text{Ni}^{\text{II}}$  was titrated into a solution of Tar (20  $\mu\text{M}$ ), CobW (10 – 30  $\mu\text{M}$ ),  $\text{Mg}^{\text{II}}$  (2.7 mM) and  
6 GTP (100 – 300  $\mu\text{M}$ ) and UV-visible absorbance recorded at equilibrium to determine  $\text{Ni}^{\text{II}}$   
7 speciation ( $\text{Ni}^{\text{II}}\text{Tar}_2 \Delta\epsilon_{535\text{ nm}} = 3.8 (\pm 0.1) \times 10^4\text{ cm}^{-1}\text{ M}^{-1}$  relative to ligand only solution;  
8 Supplementary Fig. 10a). Tar stock concentrations were quantified as above. Data were fit  
9 using Dynafit<sup>57</sup> to a model describing 1:1 stoichiometry  $\text{Ni}^{\text{II}}$ :protein and 1:2 stoichiometry  
10  $\text{Ni}^{\text{II}}$ :Tar.  $\beta_{2,\text{Ni}(\text{II})} = 4.3 (\pm 0.6) \times 10^{15}\text{ M}^{-2}$  for Tar at pH 7.0 was independently determined by  
11 preparing a series of solutions of  $\text{NiTar}_2$  ( $[\text{Ni}^{\text{II}}] = 15\text{ }\mu\text{M}$ ,  $[\text{Tar}] = 36\text{ }\mu\text{M}$ ) with varying EGTA  
12 concentrations (0 – 400  $\mu\text{M}$ ) and measuring UV-visible absorbance at equilibrium (following  
13 1-2h incubation). EGTA  $K_{\text{Ni}(\text{II})} = 5.0 \times 10^{-10}\text{ M}$  at pH 7.0 (ref.<sup>60</sup>). Data were fit to equation (5)<sup>62</sup>  
14 using Kaleidagraph (Synergy Software).

15  
16 
$$\frac{[\text{EGTA}]_{\text{tot}}}{[\text{Ni}^{\text{II}}]_{\text{tot}}} = 1 - \frac{[\text{Ni}^{\text{II}}\text{Tar}_2]}{[\text{Ni}^{\text{II}}]_{\text{tot}}} + K_{\text{D}}(\text{EGTA})\beta_2(\text{Tar}) \left( \frac{[\text{Tar}]_{\text{tot}}}{[\text{Ni}^{\text{II}}\text{Tar}_2]} - 2 \right)^2 [\text{Ni}^{\text{II}}\text{Tar}_2] \left( 1 - \frac{[\text{Ni}^{\text{II}}\text{Tar}_2]}{[\text{Ni}^{\text{II}}]_{\text{tot}}} \right) \quad (5)$$

17  
18  $\text{CuSO}_4$  was titrated into a solution of Bca (1.0 mM), CobW (10 – 30  $\mu\text{M}$ ),  $\text{Mg}^{\text{II}}$  (2.7  
19 mM), GTP (100 – 300  $\mu\text{M}$ ) and reductant  $\text{NH}_2\text{OH}$  (1.0 mM) which quantitatively reduces  $\text{Cu}^{\text{II}}$   
20 to  $\text{Cu}^{\text{I}}$  in the presence of a strong  $\text{Cu}^{\text{I}}$  ligand (eg Bca:  $\beta_{2,\text{Cu}(\text{I})} = 1.6 \times 10^{17}\text{ M}^{-2}$  (ref.<sup>60</sup>)). UV-  
21 visible absorbance was recorded at equilibrium to define  $\text{Cu}^{\text{I}}$  speciation ( $\text{Cu}^{\text{I}}\text{Bca}_2 \epsilon_{562} = 7,900$   
22  $\text{cm}^{-1}\text{ M}^{-1}$  (ref.<sup>60</sup>)) and data were fit using Dynafit<sup>57</sup> to a model describing 1:1 stoichiometry  
23  $\text{Cu}^{\text{I}}$ :protein and 1:2 stoichiometry  $\text{Cu}^{\text{I}}$ :Bca.

24  
25  $\text{Zn}^{\text{II}}$  was titrated into a solution containing quin-2 (10  $\mu\text{M}$ ), CobW (10  $\mu\text{M}$ ),  $\text{Mg}^{\text{II}}$  (2.7  
26 mM) and GTP (50  $\mu\text{M}$ ) and UV-visible absorbance recorded at equilibrium. Quin-2 was  
27 quantified using  $\epsilon_{261\text{ nm}} = 37,000\text{ cm}^{-1}\text{ M}^{-1}$  (ref.<sup>63</sup>).  $K_{\text{Zn}(\text{II})}$  for CobW- $\text{Mg}^{\text{II}}$ GTP was beyond the  
28 range of this experiment (significantly tighter than quin-2) and only a limiting affinity was  
29 determined ( $K_{\text{Zn}(\text{II})} < 10^{-12}\text{ M}$ ).

### 31 **Determination of $\text{Zn}^{\text{II}}$ affinity of $\text{Mg}^{\text{II}}$ GTP-CobW via inter-metal competition**

32  
33 Solutions containing CobW (17.9 – 20.4  $\mu\text{M}$ ),  $\text{Mg}^{\text{II}}$  (2.7 mM), GTP (200  $\mu\text{M}$ ) and ligand NTA  
34 (0.4 – 4.0 mM) were titrated with  $\text{Co}^{\text{II}}$  (0.3 – 3.0 mM) and  $\text{ZnSO}_4$  (15.3 – 25.5  $\mu\text{M}$ ) and UV-  
35 visible absorbance was recorded at equilibrium to determine  $\text{Co}^{\text{II}}$  occupancy of CobW ( $\epsilon_{339\text{ nm}}$   
36 = 2,800  $\text{cm}^{-1}\text{ M}^{-1}$  for  $\text{Co}^{\text{II}}\text{Mg}^{\text{II}}$ GTP-CobW). Details of individual experiments are in

1 Supplementary Table 4. The total concentration of Co<sup>II</sup> and Zn<sup>II</sup> in each solution was limiting,  
2 such that both metals were buffered by ligand NTA. Metal speciation was determined from  
3 the mass balance relationships given in equations (6-8) (cofactors Mg<sup>II</sup>GTP omitted for  
4 clarity). Thus,  $K_{Zn(II)}$  for CobW-Mg<sup>II</sup>GTP was calculated from the exchange equilibria ( $K_{ex}$ ) in  
5 Fig. 5a, relative to known  $K_{Co(II)}$  for the protein (Supplementary Table 3) and ligand  
6 dissociation constants (NTA  $K_{Zn(II)} = 1.18 \times 10^{-8}$  M,  $K_{Co(II)} = 2.24 \times 10^{-8}$  M (ref.<sup>60</sup>)). These  
7 calculations are valid given that  $[M]_{free} \ll [M]_{tot}$  ( $M = Co^{II}$  or  $Zn^{II}$ , buffered by excess NTA),  
8 the concentration of non-metalated protein is negligible (Supplementary Fig. 13) and  
9 potential ternary complexes involving metal, protein and NTA are transient species only with  
10 insignificant concentration at thermodynamic equilibrium (varying ratios of buffered metals,  
11  $[Co^{II}NTA]/[Zn^{II}NTA]$ , were used to confirm consistency of  $K_D$  values at multiple equilibria;  
12 see Fig. 5 and Supplementary Table 4).

13

$$14 \quad [Co^{II}NTA] = [Co^{II}]_{tot} - [Co^{II}CobW] \quad (6)$$

$$15 \quad [Zn^{II}CobW] = [CobW]_{tot} - [Co^{II}CobW] \quad (7)$$

$$16 \quad [Zn^{II}NTA] = [Zn^{II}]_{tot} - [Zn^{II}CobW] \quad (8)$$

17

## 18 **GTPase activity assays**

19

20 CobW (20 – 50  $\mu$ M) was incubated with Co<sup>II</sup> (0.9 equivalents Co<sup>II</sup>:protein) and GTP (200  $\mu$ M)  
21 in an anaerobic chamber in N<sub>2</sub>-purged, chelex-treated 10 mM HEPES pH 7.0, 100 mM NaCl,  
22 400 mM KCl. Aliquots of solution taken at various time intervals (0 – 390 mins) were loaded  
23 to a 5mL HiTrap Q HP column (GE Healthcare) equilibrated in buffer (20 mM HEPES pH  
24 7.0, 100 mM NaCl) and eluted with a linear NaCl gradient (100 – 500 mM NaCl). Nucleotides  
25 were detected by UV absorbance (254 nm or 280 nm) and the ratio of GTP:GDP in each  
26 sample was calculated by integration of the respective peak areas.

27

## 28 **Growth of *E. coli*\* strains**

29

30 *E. coli*\* strains used in this work are derived from *E. coli* MG1655 (DE3) engineered to  
31 contain the set of B<sub>12</sub> biosynthesis genes from *R. capsulatus* (described in refs.<sup>64,65</sup>), except  
32 *cobG* and *cobE* are *Brucella melitensis* homologs (described in ref.<sup>39</sup>). Chromosomally-  
33 integrated B<sub>12</sub> biosynthesis genes are IPTG-inducible under the control of the T7 promoter  
34 but in the current experiments IPTG was not added to cell cultures to avoid potential  
35 disruptions of cellular metal homeostasis caused by over-production of metalloproteins. All  
36 cultures and media were prepared in plasticware or acid-washed glassware to minimize

1 trace metal contamination. LB medium was inoculated with overnight culture of *E. coli*\*  
2 (OD<sub>600 nm</sub> = 0.025) and incubated at 37°C with shaking until OD<sub>600 nm</sub> reached ~ 0.2. Aliquots  
3 (5 mL or 50 mL) of this culture were treated with sterile Co<sup>II</sup>, H<sub>2</sub>O, EDTA or Zn<sup>II</sup> (100 ×  
4 concentrated stocks) to reach final concentrations as specified in figure legends (Figs. 7b, 8b  
5 and Supplementary Figs. 15 16a,b,d and 17c) and incubated under the same conditions for  
6 a further 1-4h. Samples used for RNA extraction were taken 1h after treatment. Samples for  
7 B<sub>12</sub> quantification and OD<sub>600 nm</sub> readings were taken 4h after treatment to ensure detectable  
8 corrinoid production.

9

#### 10 **Determination of transcript abundance in *E. coli*\***

11

12 Aliquots (1 mL) of *E. coli*\* culture from each growth condition were stabilised in RNAProtect  
13 Bacteria Reagent (2 mL; Qiagen) and cells pellets were frozen at -80°C prior to processing.  
14 RNA was extracted using an RNeasy Mini Kit (Qiagen) as described by the manufacturer.  
15 RNA was quantified by absorbance at 260 nm and treated with DNase I (2.5 U/μL;  
16 Fermentas). cDNA was generated using the ImProm-II Reverse Transcriptase System  
17 (Promega) with 300 ng RNA per reaction, and control reactions without reverse transcriptase  
18 were conducted in parallel. Transcript abundance was determined using primers 3 and 4 for  
19 *rcnA*, 5 and 6 for *zntA*, 7 and 8 for *znuA*, 9 and 10 for *rpoD*, each pair designed to amplify  
20 ~110 bp fragment (Supplementary Table 6). Quantitative PCR analysis was carried out in 20  
21 μL reactions using 5 ng cDNA, 0.8 μM of each appropriate primer and PowerUp SYBR  
22 Green Master Mix (Thermo Fisher Scientific). Three technical replicates of each sample (*ie*  
23 biological replicate) were analysed using a Rotor-Gene Q 2plex (Qiagen), plus control  
24 reactions without cDNA template for each primer pair. The fold change, relative to the mean  
25 of the control condition for each sensor, was calculated using the 2<sup>-ΔΔCT</sup> method<sup>66</sup>, with *rpoD*  
26 as the reference gene. C<sub>q</sub> values were calculated with LinRegPCR after correcting for  
27 amplicon efficiency<sup>67</sup>.

28

#### 29 **Determination of intracellular available ΔG<sub>Co(II)</sub> under bespoke conditions**

30

31 Fractional responses (θ<sub>D</sub>) of RcnR at bespoke growth conditions were calculated from  
32 transcript abundance of *rcnA* via equation (9):

33

$$34 \text{ Conditional } \theta_D = 0.99 - 0.98 \times \left( \frac{\text{fold-change}_{\text{obs}} - 1}{\text{fold-change}_{\text{max}} - 1} \right) \quad (9)$$

35

1 where fold-change<sub>obs</sub> is the observed fold-change in *rcnA* transcript abundance at the  
2 bespoke condition and fold-change<sub>max</sub> is the maximum fold-change in *rcnA* transcript  
3 abundance at the calibration limit (corresponding to maximum abundance); all fold-changes  
4 were determined relative to the defined control condition (untreated LB) corresponding to  
5 minimum *rcnA* transcript abundance (see Supplementary Fig. 15c). Equation (9) defines  
6 maximum and minimum transcript abundances as corresponding to  $\theta_D$  of 0.01 and 0.99,  
7 respectively (see Fig. 7a), and assumes a linear relationship between change in  $\theta_D$  and  
8 change in transcript abundance.

9

10 The intracellular available [Co<sup>II</sup>] concentration corresponding to each RcnR  $\theta_D$  was  
11 calculated as described in ref.<sup>27</sup> using properties determined for *Salmonella* RcnR to  
12 calculate the Co<sup>II</sup>-dependent response of *E. coli* RcnR (93% sequence identity). The  
13 intracellular available  $\Delta G_{Co(II)}$  for each condition was calculated using equation (10), where  
14 [Co<sup>II</sup>] is the intracellular available Co<sup>II</sup> concentration, R (gas constant) =  $8.314 \times 10^{-3}$  kJ K<sup>-1</sup>  
15 mol<sup>-1</sup> and T (temperature) = 298.15 K (see Supplementary Note 1).

16

$$17 \quad \text{Intracellular available } \Delta G_{Co(II)} = RT \ln[Co^{II}] \quad (10)$$

18

### 19 **Estimation of intracellular available $\Delta G_{Zn(II)}$ in LB media**

20

21 Fractional responses ( $\theta_D$ ) of Zur and ZntR in LB media were calculated from transcript  
22 abundance of *znuA* and *zntA*, via equations (9) and (11), respectively:

23

$$24 \quad \text{Conditional } \theta_D = 0.01 + 0.98 \times \left( \frac{\text{fold-change}_{\text{obs}} - 1}{\text{fold-change}_{\text{max}} - 1} \right) \quad (11)$$

25 where fold-change<sub>obs</sub> is the observed fold-change in transcript abundance in LB and fold-  
26 change<sub>max</sub> is the maximum fold-change in transcript abundance at the calibration limit  
27 (corresponding to maximum abundance); all fold-changes were determined relative to  
28 defined control conditions corresponding to minimum transcript abundance (see  
29 Supplementary Fig 16a,b). Equation (11) defines maximum and minimum transcript  
30 abundances as corresponding to  $\theta_D$  of 0.99 and 0.01, respectively, and assumes a linear  
31 relationship between change in  $\theta_D$  and change in transcript abundance.

32

33 The intracellular available [Zn<sup>II</sup>] concentration corresponding to each  $\theta_D$  was  
34 calculated as described in ref.<sup>27</sup> using properties determined for *Salmonella* homologs to  
35 calculate the Zn<sup>II</sup>-dependent responses of *E. coli* ZntR and Zur (both > 92% sequence  
36 identity). The intracellular available  $\Delta G_{Zn(II)}$  was calculated using equation (12), where [Zn<sup>II</sup>] is

1 the intracellular available Zn<sup>II</sup> concentration, R (gas constant) =  $8.314 \times 10^{-3} \text{ kJ K}^{-1} \text{ mol}^{-1}$  and  
2 T (temperature) = 298.15 K (see Supplementary Note 1).

3

$$4 \quad \text{Intracellular available } \Delta G_{\text{Zn(II)}} = RT \ln[\text{Zn}^{\text{II}}] \quad (12)$$

5

## 6 **Quantification of vitamin B<sub>12</sub> in *E. coli*\* cultures**

7

8 Aliquots (20 mL) of *E. coli*\* culture from each growth condition were taken, and cell pellets  
9 frozen at -20°C. To quantify corrin production (assumed to be predominantly B<sub>12</sub>, since *E.*  
10 *coli*\* contains genes for the complete pathway), *E. coli*\* pellets were thawed, resuspended in  
11 H<sub>2</sub>O (0.2 mL), boiled for 15 min (95°C) and centrifuged to remove cell debris. An aliquot (10  
12 µL) of each supernatant was applied to bioassay plates containing *Salmonella typhimurium*  
13 AR2680 ( $\Delta metE$ ,  $\Delta cbiB$ ) prepared as previously reported<sup>37</sup> and incubated at 37°C overnight.  
14 Plates were imaged together with a 1 cm<sup>2</sup> reference area on black background (see example  
15 in Supplementary Data 2) using a Gel-Doc XR + gel documentation system (BioRad).  
16 Images were analysed in MATLAB using the code in Supplementary Note 2 to determine the  
17 growth area (in cm<sup>2</sup>) of each sample. A calibration curve relating growth areas to B<sub>12</sub>  
18 concentration was generated using B<sub>12</sub> standards (cyanocobalamin; 1 – 100 nM; quantified  
19 by  $A_{360 \text{ nm}} = 27,500 \text{ cm}^{-1} \text{ M}^{-1}$  at pH 10 (ref.<sup>68</sup>)) in parallel with *E. coli*\* lysates, using the same  
20 batch of bioassay plates (Supplementary Fig. 17a-b). To determine the number of cells in  
21 each sample, solutions of *E. coli*\* at varying cell densities ( $OD_{600 \text{ nm}} = 0.2 - 0.9$ ) were  
22 prepared, serially diluted (2000-fold), and the number of cells per mL quantified using a  
23 CASY® cell counter. The resulting correlation factor ( $4.4 \pm 0.1 \times 10^8 \text{ cells mL}^{-1} OD_{600 \text{ nm}}^{-1}$ )  
24 was used to convert  $OD_{600 \text{ nm}}$  to cell number (Supplementary Fig. 17c,d).

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- 12

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9

## 10 **Author Contributions**

11 T.R.Y. conducted the *in vitro* metal-binding experiments, GTP-hydrolysis assays, *in vivo*  
12 gene expression experiments and B<sub>12</sub>-production experiments. T.R.Y. and M.A.M.  
13 developed experimental protocols for determining metal sensor responses by qPCR. M.A.M.  
14 derived equations for the metalation calculator and produced the spreadsheet. R.J.M. and  
15 D.O. generated the MATLAB code for analysis of B<sub>12</sub> bioassays. E.D. generated the CobW  
16 expression plasmid. E.D. and M.J.W. donated the B<sub>12</sub>-producing *E. Coli*\* strains and advised  
17 on B<sub>12</sub> biochemistry. E.D., M.J.W., and T.R.Y. co-designed the B<sub>12</sub>-production experiments.  
18 T.R.Y. and N.J.R. drafted the manuscript and, in conjunction with M.A.M and D.O.,  
19 interpreted the significance of the data. T.R.Y. and N.J.R. had overall responsibility for the  
20 design and management of the project. All authors reviewed the results and edited and  
21 approved the final version of the manuscript.

## 22 **Competing Financial Interests**

23 The authors declare no competing financial interests.

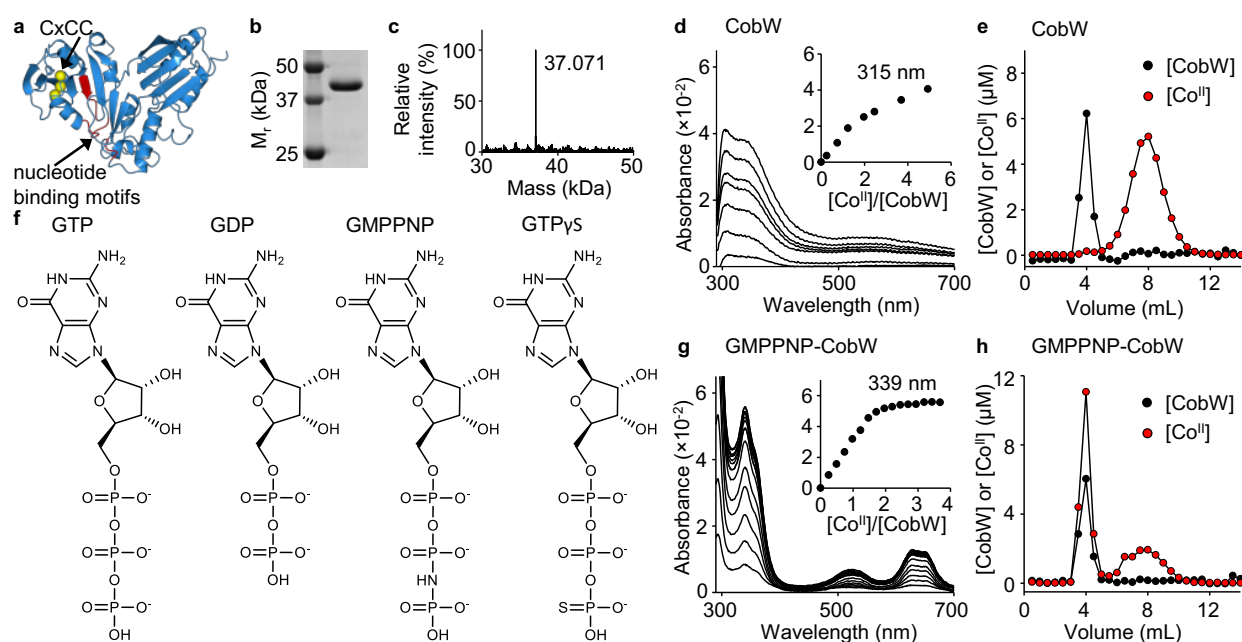
1 **Table 1** Calculated metal occupancies of CobW-Mg<sup>II</sup>GTP in an idealised cell <sup>a</sup>

Metal	Occupancy	
	Equation 2 <sup>b</sup>	Equation 4 <sup>c</sup>
Fe <sup>II</sup>	< 4.6 %	< 0.1 %
Co <sup>II</sup>	98.8 %	91.9 %
Zn <sup>II</sup>	86.2 %	6.9 %
Ni <sup>II</sup>	0.1 %	0.0 %
Cu <sup>I</sup>	0.5 %	0.0 %
TOTAL	190.3 %	98.9 %

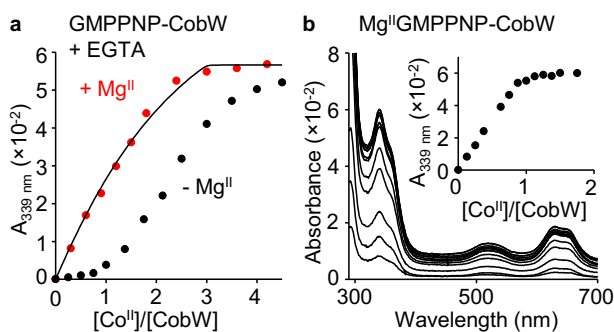
2 <sup>a</sup>Based on metal availabilities in *Salmonella* under idealised conditions (ref.<sup>27</sup>)

3 <sup>b</sup>Does not account for inter-metal competition between different metals for the same high-  
4 affinity site in Mg<sup>II</sup>GTP-CobW.

5 <sup>c</sup>Takes into account competition between multiple intracellular metals for the same site in  
6 Mg<sup>II</sup>GTP-CobW.

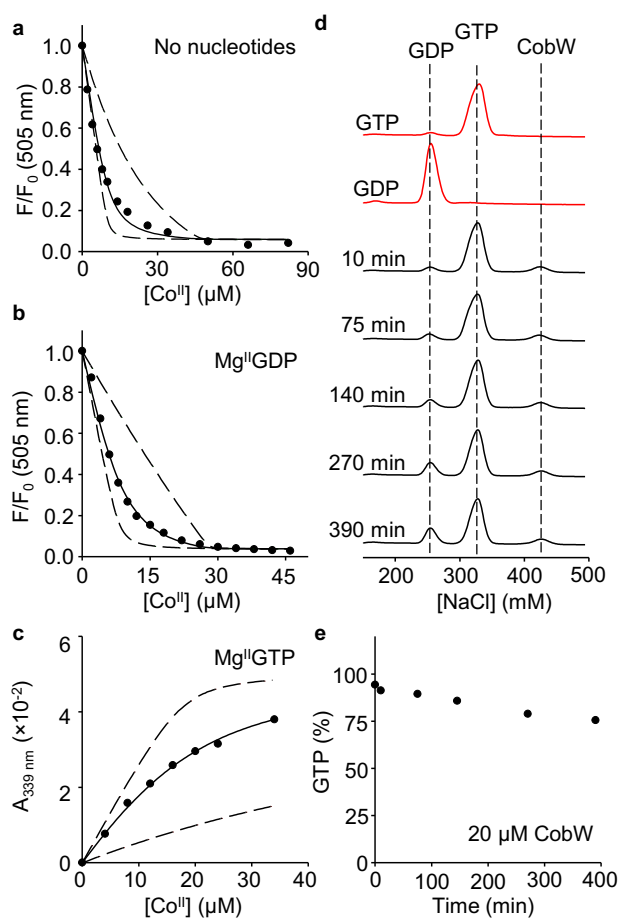


1  
2 **Fig 1. Co<sup>II</sup> binding to CobW is enhanced by guanine nucleotides.** **a** Homology model of  
3 CobW (generated with SWISS-MODEL<sup>69</sup> using *E. coli* YjiA PDB entry 1NIJ<sup>70</sup> as template)  
4 showing sulphur atoms from conserved CxCC motif (in yellow) and nucleotide-binding  
5 (GxxGxGK, hhhExxG, SKxD\*) motifs<sup>9,10</sup> (in red). \*Ordinarily NKxD but [ST]KxD observed in  
6 some COG0523 proteins<sup>9</sup>. **b** Purified CobW analysed by SDS-PAGE (full image in  
7 Supplementary Fig. 1). **c** ESI-MS analysis (de-convoluted spectra) of purified CobW. **d** Apo-  
8 subtracted spectra of Co<sup>II</sup>-titrated CobW (26.1 μM); feature at 315 nm (inset) shows a non-  
9 linear increase. **e** Representative (n=2) elution profile following gel-filtration of a mixture of  
10 CobW (10 μM) and Co<sup>II</sup> (30 μM) showing no co-migration of metal with protein. Fractions  
11 were analysed for protein by Bradford assay and for metal by ICP-MS. **f** Structures of  
12 nucleotides used in this work. **g** As in (**d**) for a mixture of CobW (24 μM) and GMPPNP (60  
13 μM); feature at 339 nm (inset) showing a linear increase saturating at 2:1 ratio Co<sup>II</sup>:CobW. **h**  
14 As in (**e**) for a mixture of CobW (10 μM), Co<sup>II</sup> (30 μM) and GMPPNP (30 μM) shows co-  
15 migration of 1.8 equivalents Co<sup>II</sup> per CobW (mean value from peak integration, n=2  
16 independent experiments).



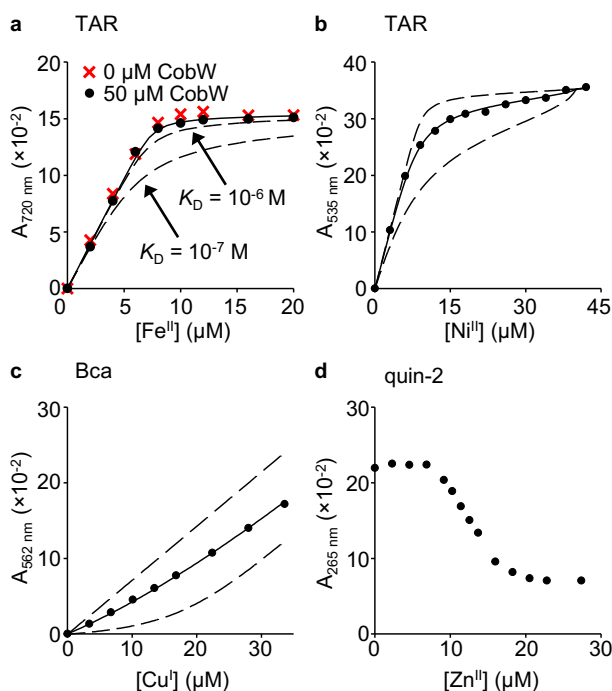
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2 **Fig. 2  $\text{Mg}^{\text{II}}$  is also required to assemble a high affinity  $\text{Co}^{\text{II}}$  site in CobW.** a Absorbance  
3 (relative to  $\text{Co}^{\text{II}}$ -free solution) of  $\text{Co}^{\text{II}}$ -titrated CobW (20  $\mu\text{M}$ ) with GMPPNP (60  $\mu\text{M}$ ) in  
4 competition with EGTA (40  $\mu\text{M}$ ); titrations performed in the absence (black) or presence  
5 (red) of  $\text{Mg}^{\text{II}}$  (2.7 mM, *ie* intracellular concentration in a bacterium<sup>27,30</sup>). Solid trace shows  
6 curve-fit of the experimental data to a model in which CobW binds one molar equivalent of  
7  $\text{Co}^{\text{II}}$  per protein monomer (in the presence of GMPPNP and  $\text{Mg}^{\text{II}}$ ). b Absorbance (relative to  
8  $\text{Co}^{\text{II}}$ -free solution) of  $\text{Co}^{\text{II}}$ -titrated CobW (20  $\mu\text{M}$ ) with GMPPNP (60  $\mu\text{M}$ ) and  $\text{Mg}^{\text{II}}$  (2.7 mM) in  
9 the absence of competing ligand; feature at 339 nm (inset) showing linear increase  
10 saturating at 1:1 ratio  $\text{Co}^{\text{II}}:\text{CobW}$ .



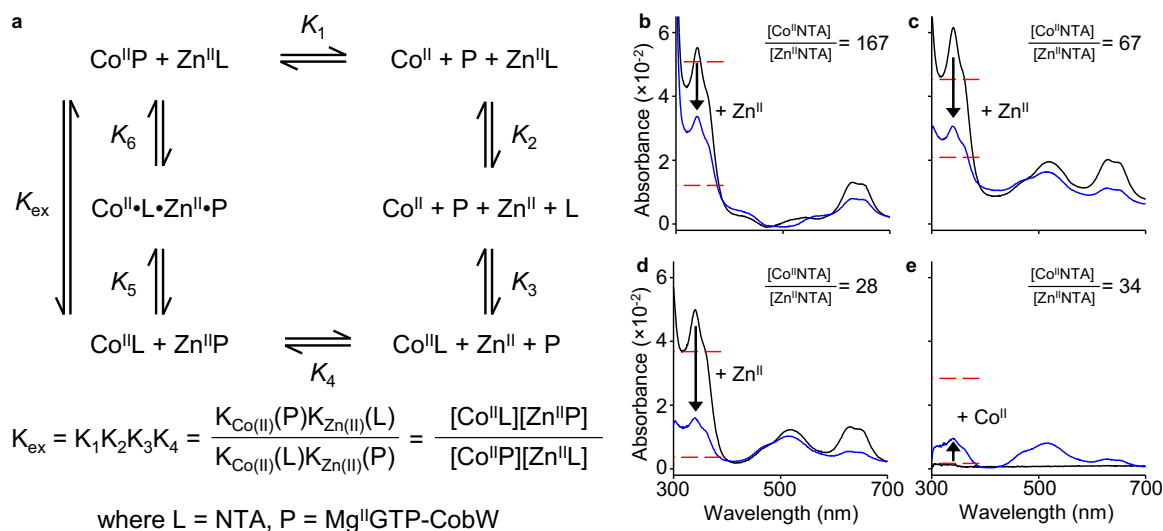
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2 **Fig. 3 The  $\gamma$ -phosphate group of GTP is necessary for high affinity  $Co^{II}$  binding.** a-c  
3 Representative  $K_{Co(II)}$  quantification for CobW in the absence or presence of nucleotides (n=3  
4 independent experiments, full details in Supplementary Fig. 4 and Supplementary Table 2).  
5 Solid traces show curve fits of experimental data to a model where CobW binds one molar  
6 equivalent  $Co^{II}$  per protein monomer. Dashed lines show simulated responses for  $K_{Co(II)}$   
7 tenfold tighter or weaker than the fitted value. **a** Fluorescence quenching of  $Co^{II}$ -titrated  
8 Fura-2 (10  $\mu M$ ) in competition with CobW alone (37  $\mu M$ ). **b** Fluorescence quenching of  $Co^{II}$ -  
9 titrated Fura-2 (8.1  $\mu M$ ) in competition with CobW (20  $\mu M$ ) in the presence of  $Mg^{II}$  (2.7 mM)  
10 and GDP (200  $\mu M$ ). **c** Absorbance (relative to  $Co^{II}$ -free solution) of  $Co^{II}$ -titrated CobW (18  
11  $\mu M$ ) in competition with EGTA (2.0 mM) in the presence of  $Mg^{II}$  (2.7 mM) and GTP (200  $\mu M$ ).  
12 **d** Analysis of GTP hydrolysis by anion-exchange chromatography. Control samples of GTP  
13 and GDP elute as distinct peaks (red traces) measured by absorbance at 254 nm. Black  
14 traces show the extent of nucleotide hydrolysis when a solution of GTP (200  $\mu M$ ) was  
15 incubated with CobW (20  $\mu M$ ),  $Mg^{II}$  (2.7 mM) and  $Co^{II}$  (18  $\mu M$ ) and analysed at time intervals  
16 indicated. **e** Analysis of data from (d) showing % GTP remaining over time. After 390 mins  
17 incubation nucleotides remain primarily (>75 %) unhydrolysed.



1

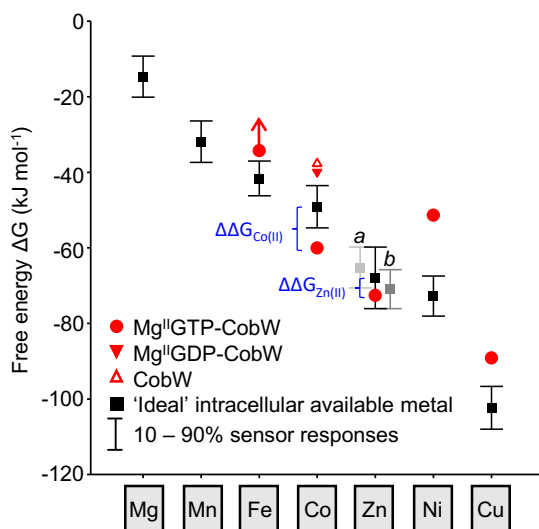
2 **Fig. 4 Binding of CobW-Mg<sup>II</sup>GTP to Fe<sup>II</sup>, Ni<sup>II</sup>, Cu<sup>I</sup> and Zn<sup>II</sup>.** **a** Absorbance upon Fe<sup>II</sup>-titration  
3 into a mixture of Tar (16 μM), Mg<sup>II</sup> (2.7 mM) and GTP (500 μM) in the absence or presence  
4 of CobW (50 μM). Dashed lines show simulated responses for specified  $K_{Fe(II)}$  of CobW-  
5 Mg<sup>II</sup>GTP, providing limiting  $K_{Fe(II)} \geq 10^{-6}$  M. Control Fe<sup>II</sup>-titration into a solution of Tar (16 μM)  
6 in buffer only (Supplementary Fig. 8a) confirmed that Mg<sup>II</sup> and GTP did not inhibit  
7 stoichiometric Fe<sup>II</sup>Tar<sub>2</sub> formation. **b** Absorbance change (relative to Ni<sup>II</sup>-free solution) of Ni<sup>II</sup>-  
8 titrated Tar (20 μM) in competition with CobW (30 μM) in the presence of Mg<sup>II</sup> (2.7 mM) and  
9 GTP (300 μM). **c** Absorbance of Cu<sup>I</sup>-titrated Bca (1.0 mM) in competition with CobW (20 μM)  
10 in the presence of Mg<sup>II</sup> (2.7 mM) and GTP (200 μM). In (**a-c**) solid traces show curve fits of  
11 experimental data to models where CobW binds one molar equivalent of metal per protein  
12 monomer. Supplementary Table 3 contains mean ± s.d.  $K_{metal}$  values from n=3 independent  
13 experiments (full details in Supplementary Figs. 8-12 and Supplementary Table 2). In (**b-c**)  
14 dashed lines show simulated responses for  $K_{metal}$  tenfold tighter or weaker than the fitted  
15 value. **d** Absorbance (relative to probe-free solution) upon titration of Zn<sup>II</sup> into a mixture of  
16 quin-2 (10 μM), Mg<sup>II</sup> (2.7 mM), GTP (100 μM) and CobW (10 μM).



1

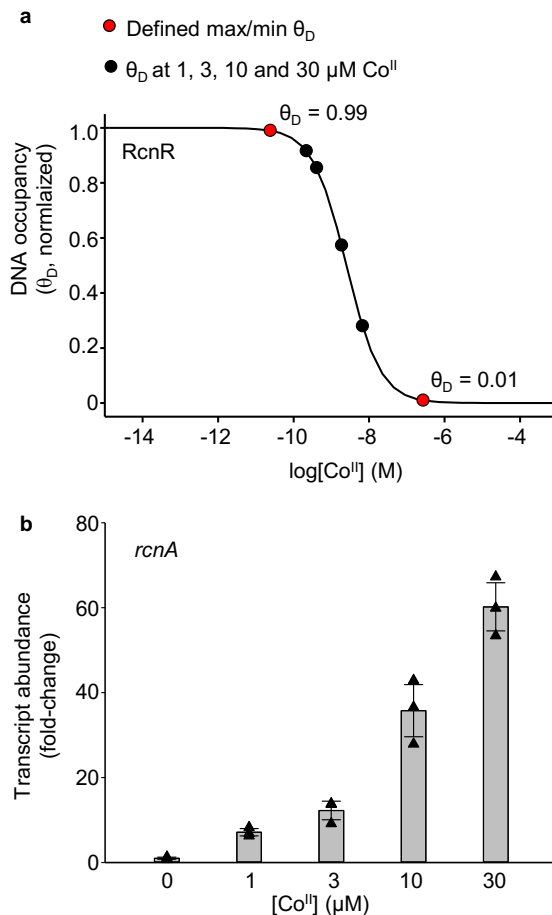
2 **Fig. 5 CobW-Mg<sup>II</sup>GTP binds Zn<sup>II</sup> with sub-picomolar affinity.** **a** Representation of the  
3 equilibrium for exchange of Co<sup>II</sup> and Zn<sup>II</sup> between ligand (L = NTA) and protein (P =  
4 Mg<sup>II</sup>GTP-CobW). **b-e** Absorbance (relative to metal-free solution) of solutions of CobW (17.9  
5 – 20.4 μM), Mg<sup>II</sup> (2.7 mM), GTP (200 μM) and NTA (0.4 – 4.0 mM) upon (**b – d**) first addition  
6 of Co<sup>II</sup> (black trace) then Zn<sup>II</sup> (blue trace) or (**e**) the reverse, at equilibrium. The absorbance  
7 peak at 339 nm corresponds to Co<sup>II</sup>-bound protein. An excess of ligand NTA was used to  
8 buffer both metals in each experiment: varying the ratios of ligand-bound metal ions  
9 ([Co<sup>II</sup>NTA]/[Zn<sup>II</sup>NTA] = 28 - 167) shifted the ratios of Co<sup>II</sup>- and Zn<sup>II</sup>-bound protein as predicted  
10 by the equilibrium exchange constant in (**a**). Consistent  $K_{\text{Zn}(\text{II})}$  values for Mg<sup>II</sup>GTP-CobW  
11 were generated at all tested conditions (Supplementary Table 4). Dashed red lines show  
12 expected  $A_{339 \text{ nm}}$  peak intensities for  $K_{\text{Zn}(\text{II})}$  of Mg<sup>II</sup>GTP-CobW 10-fold tighter or weaker than  
13 calculated values.





1

2 **Fig. 6 Mg<sup>II</sup>GTP-CobW is predicted to acquire Co<sup>II</sup> or Zn<sup>II</sup> in a bacterial cell.** Free-energy  
3 change ( $\Delta G$ ) for metal-binding to Mg<sup>II</sup>GTP-CobW plotted against the intracellular available  
4 free energies for metal-binding in a reference bacterial cytosol (values correspond to  
5 *Salmonella*) under idealised conditions (*ie* where each metal sensor undergoes half of its  
6 transcriptional response) (ref.<sup>27</sup>). Intracellular available  $\Delta G_{Zn(II)}$  is the mean of the values  
7 determined from the two Zn<sup>II</sup>-sensors ZntR (*a*) and Zur (*b*). Bars shows the change in  
8 intracellular available  $\Delta G$  as cognate sensors shifts from 10-90% of their responses. Free  
9 energy differences ( $\Delta\Delta G$ ) which favour acquisition of metals by Mg<sup>II</sup>GTP-CobW *in vivo* are  
10 indicated.  $\Delta G$  values for Co<sup>II</sup>-complexes of CobW alone and Mg<sup>II</sup>GDP-CobW are also  
11 shown. For Fe<sup>II</sup> binding to Mg<sup>II</sup>GTP-CobW, arrow indicates limiting  $\Delta G > -34.2$  kJ mol<sup>-1</sup>.



1

2 **Fig. 7 Calculations of conditional  $\text{Co}^{\text{II}}$  availabilities in  $\text{B}_{12}$ -producing *E. coli*\*. a**

3 Calculated relationship between intracellular  $\text{Co}^{\text{II}}$  availability and normalised DNA occupancy

4 ( $\theta_D$ ) by RcnR.  $\theta_D$  of 0 and 1 are the maximum and minimum calculated DNA occupancies.

5 The dynamic range (within which RcnR responds to changing intracellular  $\text{Co}^{\text{II}}$  availability)

6 has been defined as  $\theta_D$  of 0.01 to 0.99 (ie 1 – 99% of RcnR response). The calibrated

7 maximum and minimum fold changes in *rcnA* transcript abundance (ie boundary conditions,

8 see Supplementary Fig. 15) therefore correspond to  $\theta_D$  of 0.01 and 0.99 in these

9 calculations.  $\theta_D$  for each growth condition (black circles) was calculated from the qPCR

10 response in **b**, assuming a linear relationship between change in  $\theta_D$  and change in transcript

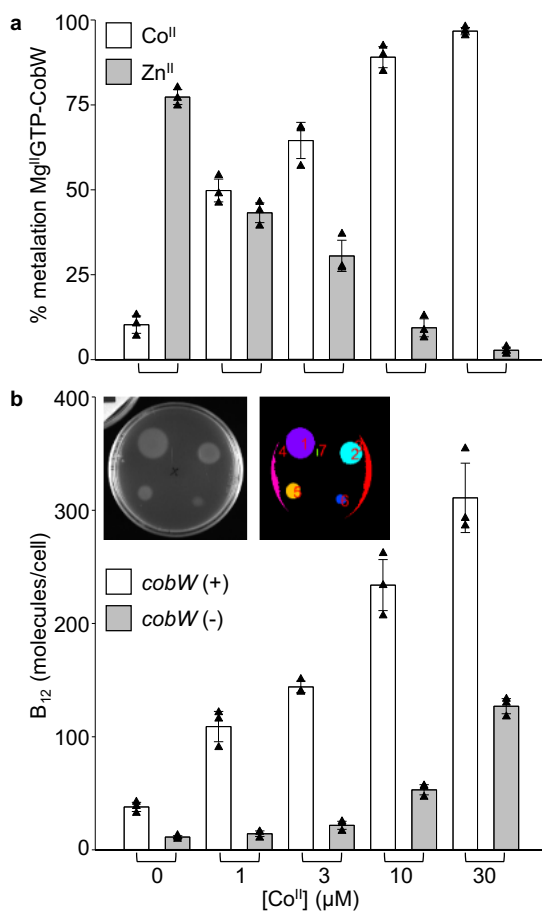
11 abundance (equation (9)). Corresponding  $\text{Co}^{\text{II}}$  availabilities were calculated as previously

12 described<sup>27</sup> and are listed in Supplementary Table 5. **b** Transcript abundance (relative to

13 untreated control) of the RcnR-regulated gene *rcnA* following 1h exposure of *E. coli*\* to

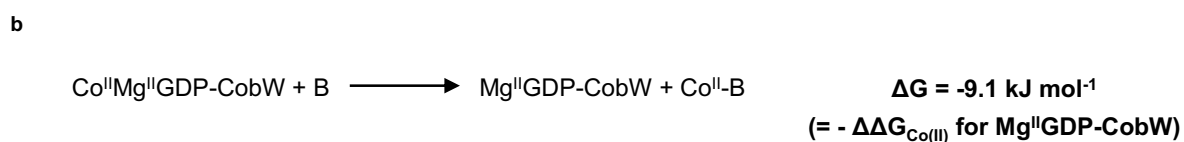
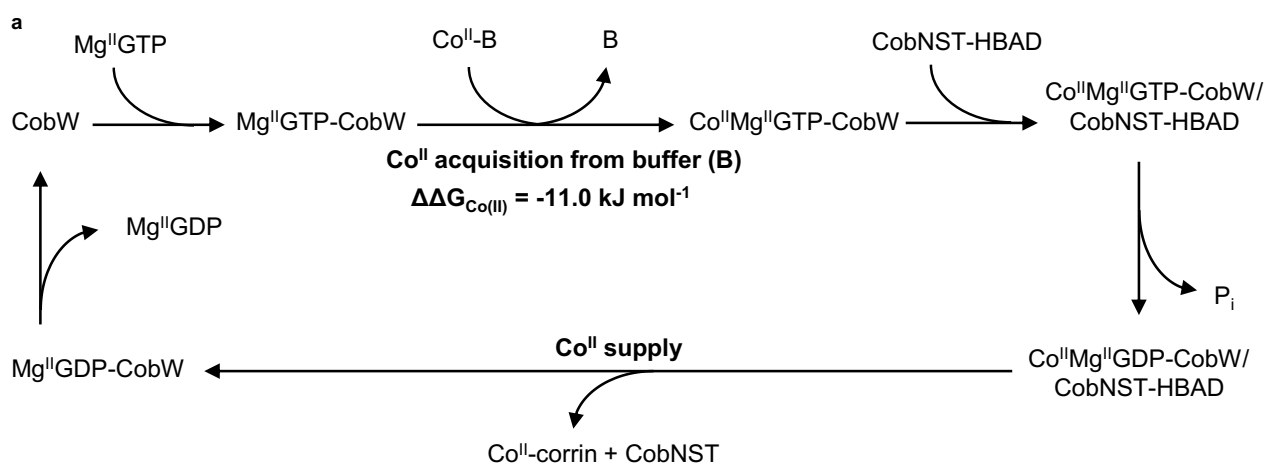
14 increasing  $[\text{Co}^{\text{II}}]$ , measured by qPCR. Data are the mean  $\pm$  s.d. of n=3 biologically

15 independent replicates. Triangle shapes represent individual experiments.



1

2 **Fig. 8 B<sub>12</sub> production follows predicted metalation of Mg<sup>II</sup>GTP-CobW.** **a** Predicted  
3 metalation of Mg<sup>II</sup>GTP-CobW with Co<sup>II</sup> and Zn<sup>II</sup> in samples treated with defined media [Co<sup>II</sup>].  
4 Intracellular  $\Delta G_{\text{Co(II)}}$  for each condition was calculated from *rcnA* expression (Fig. 7 and  
5 Supplementary Table 5). **b** B<sub>12</sub> produced by *E. coli*\* strains with and without *cobW* (open and  
6 grey bars, respectively) following 4h exposure to defined [Co<sup>II</sup>]. B<sub>12</sub> was detected using a  
7 *Salmonella* AR2680 bioassay<sup>37</sup> (detects corrins, expected to be predominantly B<sub>12</sub>; see  
8 Methods) and quantified by automated analysis of growth areas (Supplementary Fig. 17 and  
9 Supplementary Note 2). Inset shows original image and detected areas for representative  
10 (n=3) bioassay plate of B<sub>12</sub> calibration standards. All data are the mean  $\pm$  s.d. of n=3  
11 biologically independent replicates (with errors in **a** propagated from qPCR data in Fig 7b).  
12 Triangles represent individual experiments.



1

2 **Fig. 9 Proposed mechanism of CobW.** a Binding of  $\text{Mg}^{\text{II}}\text{GTP}$  enables CobW to acquire  $\text{Co}^{\text{II}}$   
3 from intracellular buffer ligands (B) followed by interaction with the CobNST  
4 cobaltocheletase bound to its substrate hydrogenobyric acid *a,c*-diamide (HBAD). GTP  
5 hydrolysis will trigger  $\text{Co}^{\text{II}}$  release to CobNST-HBAD, since the reaction in **b** is  
6 thermodynamically favourable, for incorporation into the corrin ring of vitamin B<sub>12</sub>.