Ribosome-Dependent ATPase Interacts with Conserved Membrane Protein in *Escherichia coli* to Modulate Protein Synthesis and Oxidative Phosphorylation

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Abstract

Elongation factor RbbA is required for ATP-dependent deacyl-tRNA release presumably after each peptide bond formation; however, there is no information about the cellular role. Proteomic analysis in *Escherichia coli* revealed that RbbA co-purifies with a conserved inner membrane protein of unknown function, YhjD. Both proteins are also physically associated with the 30S ribosome and members of the lipopolysaccharide transport machinery. Genome-wide genetic screens of *rbbA* and *yhjD* deletion mutants revealed aggravating genetic interactions with mutants deficient in the electron transport chain. Cells lacking both *rbbA* and *yhjD* exhibited reduced cell division, respiration and global protein synthesis as well as increased sensitivity to antibiotics targeting the ETC and the accuracy of protein synthesis. Our results suggest that RbbA appears to function together with YhjD as part of a regulatory network that impacts bacterial oxidative phosphorylation and translation efficiency.

Introduction

Protein synthesis on ribosomes requires GTPase and ATPase factors. During protein chain elongation, EF-Tu, a GTPase recruits the activated aminoacyl-tRNA to the ribosomal A-site. RbbA or Ribosome Bound ATPase, acts in conjunction with EF-Tu to poise the aminoacyl-tRNA on the ribosomal A-site [1]. After peptide bond formation by the peptidyl transferase, the growing peptide is bound to the tRNA in the A-site and the deacyl-tRNA to the P-site. Subsequently, EF-G, a ribosome-associated GTPase, promotes the translocation of the ribosome and the tRNAs one codon at a time relative to the mRNA in the 5’ to 3’ direction, transferring the newly formed peptidyl-tRNA to the P-site, the deacyl-tRNA to the E-site and exposing the ribosomal A-site to the next aminoacyl-tRNA [2].

The ribosome-dependent ATPase, RbbA in *Escherichia coli*, markedly stimulates synthesis in reconstituted extracts [3]. RbbA is encoded within its own operon by a gene non-essential for growth [4]. The amino acid sequence of RbbA reveals two ATP binding motifs, two Walker motifs that define most ABC transporters [1], one RNA-binding motif and six predicted ATP binding motifs, two Walker motifs that define most ABC transporters at neutral pH [4]. The amino acid sequence of RbbA reveals two encoded within its own operon by a gene non-essential for growth [5]. RbbA markedly stimulates synthesis in reconstituted extracts [3] as well as stimulates ejection of deacyl-tRNA by accelerating binding of EF-TuGTPaminoacyl-tRNA to ribosomes [1]. Indeed, chemical protection footprints indicate that RbbA binds directly to the ribosomal E-site [1]. The E-site is involved in deacyl-tRNA ejection, translocation and reading frame maintenance [5]. The ribosome and RbbA-dependent hydrolysis of ATP as well as the transfer of acylated aminoacyl-tRNAs is inhibited by the ATP analogue, AMPPpC, suggesting that the hydrolysis of ATP is indeed required during synthesis [6]. If, in fact, RbbA stimulates the ribosome-dependent hydrolysis of ATP after each peptide bond is formed upon release of each deacyl-tRNA, then the energy requirements for synthesis correspondingly increase. In this context, and considering the total metabolic, energy demand imposed by the translation process [7], it seems reasonable to assume that this process is associated with an energy source to rapidly replenish energy and maintain cellular viability.

To gain insight into the cellular role of RbbA, we performed genome-wide physical and genetic interactions involving RbbA from *E. coli*. We found that RbbA bound to the membrane where it reciprocally co-purifies with an inner membrane (IM) protein of unknown function, YhjD. Both these tagged proteins physically interact with members of the lipopolysaccharide (LPS) transport pathway and localize to the 30S ribosomal subunits. Genome-wide genetic screens of *rbbA* and *yhjD* displayed aggravating interactions with members of the electron transport chain (ETC). Strains...
lacking rbbA and yhjD showed defects in growth, cell division, respiration and protein synthesis and increased sensitivity to chemical agents that block the ETC and to antibiotics affecting translational fidelity and translocation. Collectively, these and supporting data suggest that RbbA has a dual role in the cell, which, in conjunction with YhjD, links protein synthesis to several membrane processes including LPS transport and energy regeneration.

Results

RbbA Physically Associates with Protein Synthesis and Membrane Processes

To probe the cellular aspects of RbbA function, we performed an unbiased protein interaction screen to identify its physical interaction partners in vivo. Full-length RbbA was affinity-tagged at its C-terminal end by recombining a SPA (Sequential Peptide Affinity) cassette (consisting of a triple FLAG tag and a calmodulin binding peptide) into the chromosome [8]. SPA-tagged RbbA was extracted from the membranes of log-phase cultures in the absence of detergents as well as using three different mild non-ionic detergents [Triton X 100, DDM (n-dodecyl-β-D-maltopyranoside), and C12E10 (Octaethylene glycol monododecylether)] at 1% concentrations, following which the stably associated proteins were affinity-purified in two steps on anti-FLAG and calmodulin resins and then identified using tandem mass spectrometry (LCMS) and peptide mass fingerprinting (MALDI-TOF MS). Three purifications with different detergents were used [9] to optimize the solubilization and partly validate interacting proteins.

Purification of RbbA in the detergent extracts detected a 91 kDa full-length RbbA, while the protein was not detected in purifications without detergents, suggesting that it is tightly bound to the membrane fraction (Table S1). The 91 kDa RbbA also occurs tightly bound to the ribosomes [10] because the conditions used to extract RbbA from the membranes were expected to disrupt ribosome structure and may thus not be seen in the detergent free-extract. To minimize potential non-specific interactions, we considered only proteins that co-purified with RbbA in at least two different detergents as bona fide interactors (Table S1). Using this stringent filtering criteria, we found RbbA bound to protein synthesis elongation factor EF-Tu (e.g. TuA and TuB), 30S (e.g. RpsE, RpsJ, RpsM, RpsN, RpsP, RpsS and RpsT) subunit ribosomal (r) proteins, to other cytoplasmic proteins involved in tRNA (e.g., CCA) and rRNA (e.g. Rng, RlmL) processing (Text S1) and with the release factor PrfA (Table S1). The endogenous affinity-tagged RbbA also co-purified efficiently with several energy regeneration (e.g. AceE, FdoH and FruR) and membrane (e.g. LolB, Trg, MrcB, SecD, WcaM, LptD) components (Text S1) including an outer membrane (OM; LptD) and with two IM (LptB and MsbA) LPS transporters in the detergent extracts (Table S1). The later observation is consistent with the recent implication on the function of YhjD protein in LPS transport [11,12]. Conversely, the affinity-tagged RbbA bound only to the LptD protein in detergent extracts. Although the mass spectrometry failed to detect the two other LPS components with RbbA, we were able to confirm these associations, including LptD by immunoprecipitation from cell-lysates derived from strains containing C-terminal affinity-tagged MsbA, LptB and LptD proteins (Figure 2A). Thus, the stable association of these two affinity-tagged proteins with LPS transport components suggests the possibility that they might cooperate with LPS proteins to mobilize phospholipids and alter the fluidity of the membrane by providing a mechanism to move LPS molecules into and/or across the OM barrier.

Intracellular Localization of RbbA and YhjD

The protein interaction data suggested a potential role for RbbA and YhjD in membrane processes. To find whether RbbA and YhjD localized to the membrane, we constructed C-terminal fusions of RbbA and YhjD to yellow fluorescent protein (YFP). A strain producing the YFP fusion to MinD was used as a control. MinD is an ATPase that, when bound to ATP, localizes peripherally to the cytoplasmic membrane at one end of the two cell poles [14,15]. Similar fluorescence patterns were observed in the RbbA-YFP and YhjD-YFP strains with about 43% of the >200 cells analyzed showing a fluorescent zone at one end of the cell (Figure 2B). In addition, ~13% of the cells had fluorescent zones at both poles, suggesting that RbbA and YhjD most likely
associate with the cell membrane or possibly anchor to the membrane surface. The similarity in fluorescence patterns of RbbA and YhjD with a cell division protein suggests that RbbA and YhjD might also cross talk to cell division.

Since translation affects many aspects of cell division [16], the morphology of the cells was examined at different temperatures in the \( rbbA^{D} \) and \( yhjD^{D} \) mutants. As shown in Figure 2C, the \( rbbA^{D} \) and \( yhjD^{D} \) single mutants appear to be slightly elongated; with average cell length of the mutant cells (\( 5.0 \pm 0.7 \) mm) being almost 0.8 fold than that of the wild type cells (from 4.1 \( \pm 0.4 \) mm to 3.4 \( \pm 0.4 \) mm) at both 15 and 32°C. Remarkably, the double mutant appears to be even more elongated at both 15 and 32°C, with average cell lengths ranging from 9.0 \( \pm 0.32 \) to 7.2 \( \pm 0.35 \) mm, and resembled the \( minD^{D} \) (\( \sim 11.3 \pm 1.49 \) to 9.9 \( \pm 0.98 \) mm) control mutant [17]. The phenotype of the double mutant is more evident at 15°C compared to other temperatures. At 44°C, the cells from these deletion strains appeared shortened, restricted in cell size and unviable. These data reconfirm the attachment of RbbA and YhjD to the membrane and imply a connection of these two genes in cell division and protein synthesis.

Genetic Interactions Suggest Involvement of RbbA and YhjD in Energy Generation, Protein Synthesis and Cell Division

Since genetic interactions are an informative means to discover functional relationships among genes [18], we used a recently developed eSGA (E. coli Synthetic Genetic Array) approach [19] to identify E. coli genes that genetically interact with \( rbbA \) and \( yhjD \). In this conjugation-based assay, we generated double mutants by conjugating \( rbbA \) or \( yhjD \) donor strain (marked with \( D::CmR \)) with single gene deletions of almost all other non-essential E. coli genes as well as hypomorphic alleles of selected essential E. coli genes (recipients marked with \( D::KanR \)) (Table S2). The colony growth and relative fitness of the resulting double mutants surviving dual drug selection was then examined by digital imaging. Using a statistical interaction score (S) to quantify both the strength and confidence of the interactions between each E. coli mutant gene pair, we detected cases of both aggravating (synthetic sick or lethal (SSL)) as well as alleviating (i.e. positive or suppressing) interactions. Two genes are said to have aggravating or alleviating genetic interactions when the double mutant grows significantly weaker than the single mutants.
Figure 2. Localization of RbbA and YhjD on membranes. (A) Immunoblot analysis of the indicated His6-RbbA fusion protein in whole cell lysates (WCL, panel II) and anti-FLAG immunoprecipitates (IP, panel II) from an untagged DY330 strain and E. coli expressing a SPA-tagged MsbA, LptB and LptD proteins. Expression of affinity-tagged proteins from MsbA, LptB and LptD in cell lysates (panel I) is monitored using M2 anti-FLAG antibody.

(Differential interference contrast images of temperature induced changes of cell morphology and the average cell length of the poles (bottom panel) for the indicated YFP-strain is an average of at least four experiments. Error bars indicate mean and LptD proteins. Expression of affinity-tagged proteins from MsbA, LptB and LptD in cell lysates (panel I) is monitored using M2 anti-FLAG antibody. The unrelated SPA-tagged YchM strain served as control. Molecular masses are shown in kDa. (B) Intracellular localization of RbbA-YFP and YhjD-YFP using MinD-YFP fusion as control. Arrows show the YFP localization at the cell poles (top panel). Percentage YFP cells in one or two ends of the cell poles (bottom panel) for the indicated YFP-strain is an average of at least four experiments. Error bars indicate mean ± SD. Scale bar equals 2 μm. (C) Differential interference contrast images of temperature induced changes of cell morphology and the average cell length of the rbbA-yhjD double mutant and their respective single mutants at indicated temperatures are shown on the right side of panel C. The minDΔ served as control. Scale bar equals 2 μm.

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The genetic interactions were re-examined in more detail to confirm some of the whole genome deletion screen results of RbbA and YhjD with the ETC pathway and to confirm the interaction of rbbA with yhjD or vice-versa. Thus, we individually deleted 8 of the 13 nuo-subunits of NDH1 (e.g., nuoA, nuoB, nuoF, nuoJ, nuoK, nuoL, nuoM and nuoN) from complex 1, 4 of the sdh-subunit genes of succinate dehydrogenases (sdhCDAB) from complex II, and 4 of the 4 cyo-subunit genes of bo terminal oxidase from complex IV (cyoA,B,C,D) and conjugated the resulting F- recipients with rbbA and yhjD donor mutants (Figure 3B). Consistent with our rbbA and yhjD whole genome deletion screens, the recipient NDH1 and sdh complexes bearing individual gene deletions exhibited SSL phenotypes when combined with RbbA and YhjD donor mutants. Accordingly, the RbbA mutant exhibited a SSL phenotype with members of complex IV of the ETC. Conversely, no apparent effect on growth was observed with the YhjD donor and the recipient electron transport complex IV mutants. However, there was a slight growth defect consistently apparent between the YhjD donor and the AcyoA and AgyoA recipient mutants (Figure 3B). In addition, the YhjD recipient and the RbbA donor mutant or vice-versa, exhibited a significant synthetic sick growth defect (Figure 3B). We also confirmed that these observed genetic interactions were not a consequence of recombinant suppression resulting from gene proximity because no noticeable effects on growth were observed when RbbA or YhjD donor mutants were combined with deletions of functionally unrelated genes flanking worse or better, respectively than expected based on the phenotypes of the corresponding single mutants [19]. Aggravating or alleviating genetic interactions suggest that the protein products of the two genes are functionally related and are usually interpreted in terms of redundancy (parallel pathways) or similarity (same pathway) of function, respectively [20].

As for the protein interaction experiments, we also observed genetic interactions of rbbAΔ and yhjDΔ with deletions of several energy regeneration genes. These included SSL interactions with members of the ETC: NADH dehydrogenases (namely NDH-I and NDH-II from complex I), succinate dehydrogenases (sdhCDAB from complex II), cytochrome bo terminal oxidase complex (cyoA,B,C,D from complex IV), ATP synthase (atpAC from complex V), iron-sulfur (Fe-S) cluster proteins and with a few other catabolic genes encoding enzymes which reduce the NDH pool and reduce the electron flux through the ETC (Figure 3A, Table S2). In addition, a synthetic sick interaction was observed between the RbbA mutant as donor and YhjD as recipient or vice-versa (Table S2). Over-representation analysis using GO annotation terms showed that the interacting genes identified in the rbbAΔ and yhjDΔ deletion screens were enriched significantly (q-value <0.05) for genes involved in the ETC, tricarboxylic acid cycle, ATP-synthesis coupled electron transport, lipid biosynthesis, fatty acid synthesis, rRNA and tRNA processing, as well as other processes linked to membrane, protein synthesis and energy metabolism (Tables S3 and S4, see Text S3).
Figure 3. Functional redundancy of RbbA and YhjD with oxidative phosphorylation genes. (A) Diagrammatic representation of the ETC system of *E. coli* modeled based on [55]. The diagram shows the SSL interactions from rbbA and yhjD donor deletion strains with members of the pathway: Fe-S cluster, complex I, II, IV, V and some key genes encoding catabolic enzymes. The cytochrome bc1 or cytochrome c oxidases of complex III does not occur in *E. coli* [56]. Q: ubiquinone; FMN: flavin mononucleotide; FAD: flavin adenine dinucleotide. Blue represents aggravating (negative S score) interaction. (B) Chromosomal positions of rbbA and yhjD (green arrows), individual gene deletions in nuo (complex I), sdh (complex II), and cyo (complex IV) operons (purple arrows), and other gene (red arrows) deletions used as donors (marked with CmR) or recipients (marked with KanR) in RbbA and YhjD Affect Protein Synthesis and ETC.
RbbA and YhjD Mutants Impair Translational Fidelity In Vivo

Since RbbA and YhjD physically interact with t-proteins, we further assessed their possible function in protein synthesis by comparing the [35S]-methionine incorporation into total proteins for the rbbA-yhjD double mutant and the corresponding single mutants in wild type cells at 32°C for 30 min. The total protein synthesized in the rbbA-yhjD double mutant was significantly lower than that observed with each single mutant, and was also significantly lower than the wild type (Figure 4A). Thus, the coordinate behavior of rbbA and yhjD suggests that both genes affect translation in a manner reminiscent of genes that suppress common functions.

Because the rbbA and yhjD mutants appeared defective in the synthesis of proteins (Figure 4A), we assessed the translational fidelity of the mutants in vivo by examining the expression of full-length β-galactosidase (β-gal) from a template harboring out-of-frame nonsense codons in its coding sequence. As shown in Figure 4B, compared to the wild type, the rbbA and yhjD mutants did not exhibit significant read-through of either the amber (UAG) or the opal (UGA) mutants of β-gal. However, the rbbA-yhjD double mutant exhibited higher read-through of both UAG and UGA β-gal mutants. Western-blot analysis of the cell lysates from the single and double gene deletions transformed with the two different reporter plasmids verified the expected presence of the truncated β-gal specified by the UAG or +1 frame-shift mutations. However, the full-length β-gal is synthesized significantly only by the double mutant, which also encoded correspondingly less of the truncated protein (Figure 4C). Thus, it appears that nonsense codons are indeed mistranslated in the double mutant. Further study of β-gal +1 and -1 frame-shift mutations revealed that, relative to the wild type, the single mutants were only slightly affected while the double mutant exhibited significantly higher miscoding of both +1 and -1 frame-shift mutations in β-gal. These results suggest that the double mutant has significant lesions in translational miscoding.

Inhibition of Electron Transport Impairs RbbA and YhjD Functions as well as Protein Synthesis and Translational Fidelity

The genetic interactions of RbbA and YhjD suggested a functional relation to oxidative phosphorylation. This was also suggested by the finding that the single and double mutants grew poorly in glycerol media compared to glucose implying that they
Figure 4. Protein synthesis, miscoding and ETC are impaired by deletions of rbbA and yhjD. (A) Incorporation of [35S]-methionine into total proteins of the wild type and the indicated deletion strains in the absence and presence of sodium azide (500 μM) or CCCP (10 μM). Error bars indicate mean ± SD. (B) Elevated rates of frame-shifting and stop-codon read-through in wild type and in the indicated deletion strains in the absence and presence of sodium azide (500 μM) or CCCP (10 μM). Error bars indicate mean ± SD. The β-gal activity is normalized to the activity of β-lactamase that has been used as an internal control. (C) Immunoblot of the full-length and truncated β-gal is assayed after transforming the reporter plasmid pSG400 carrying a premature UAG stop codon expressed in rbbA-yhjD double mutant (Lane 4) and in the rbbA (Lane 2) and yhjD (Lane 3) single mutants. The assay was also performed with another reporter plasmid 4pCCCU containing +1 frame-shift expressed in rbbA-yhjD double mutant (Lane 8) and in the rbbA (Lane 6) and yhjD (Lane 7) single mutants. The wild type and plasmids pSG400 (Lane 1) and 4pCCCU (Lane 5) served as controls. (D) Growth sensitivity assay of the indicated deletion strains in the presence or absence of antibiotics affecting miscoding, translocation...
were compromised in aerobic respiration (Figure 4E). To verify the functional linkage of these proteins with the ETC we blocked the electron flow of the oxidative phosphorylation chain using piericidin A, a selective inhibitor of NDH (Complex I) [30] where 90% of the membrane potential due to the redox reaction of complex I activity has previously been shown to be inhibited in E. coli [31,32]. Remarkably, piericidin A inhibited the growth of rbbA-yhjD double mutant in the whole cell extract to the same extent as their single mutants (Figure 4F). The observed phenotype is indeed caused by deleting rbbA or yhjD because a pBAD-plasmid expressing RbbA or YhjD under the control of an arabinose-inducible promoter, but not a control plasmid without the respective genes, restored the antibiotic resistance of rbbA or yhjD null strains to wild type levels (Figure 4F; only rbbA complementation is shown). Thus, it appears that the growth phenotype observed with the rbbA-yhjD double mutant and their respective single mutants may be linked to the electron transport pathway.

To verify the functional linkage between protein synthesis and the ETC, we challenged the rbbA-yhjD double mutant and their respective single mutants with CCCP (carbonylcyanide-m-chlorophenylhydrazone), a proton-ionophore which not only diffuses across the IM and the periplasmic space in the protonated form (domains II-III in EF-3, underlying their structure and strengthening the connection between RbbA and EF-3 (Figure S3A and S3B). There are, however, features which differ in the sequences of these proteins. The N-terminus of EF-3 has a HEAT domain which is absent in RbbA (Figure S3B), and was shown by Cryo-EM analysis to bind to the head of the 80S and reach the L1 stalk of the yeast 60S ribosomal subunit E-site, suggesting that it may participate in release of deacyl-rRNA from ribosomes during each step of elongation [39]. Although RbbA lacks the HEAT domain, it interacts directly with the E-site and has been shown to accelerate release of deacyl-rRNA from ribosomes during protein chain elongation [1]. The C-terminus of EF-3 is a stretch of dispensable basic amino acids. In contrast, RbbA has six predicted transmembrane helices (residues 563-906 of the 911 amino acids) at its C-terminus, pointing to its potential to interact with membranes. Although these features suggest a dual localization of RbbA on ribosomes and on membranes, the cellular role of RbbA is yet not fully understood. Here, we use biochemical and genetic approaches to examine the possible connection of RbbA to protein synthesis, membrane processes and energy sources.

The physical interaction study we report shows that RbbA binds to the membrane and reciprocally co-purifies with an IM protein of unknown function, YhjD, implicated in LPS export across the IM [12]. Mutations based on single amino acid substitutions in either MsbA, an IM, essential ABC transporter in the LPS export pathway or in YhjD suppress the lethal consequence of an incomplete lipid A precursor IVA [11]. However, the physical evidence we report shows that RbbA, as well as YhjD, co-purifies with other members of the LPS export pathway (i.e. MsbA, LptB and LptD), suggesting that RbbA and YhjD, together with an as-yet-unidentified transmembrane partner, may form a membrane-associated complex with LPS proteins and thus both proteins are likely to have a role in LPS transport across the IM. Remarkably, both proteins interact with the same 30S ribosomal subunit proteins, with a related number of ABC transporters, and with proteins that are integral to the E. coli membrane (Table S1).

Alterations in membrane composition due to mutations have effects upon membrane protein folding, electron transport, initiation of DNA replication, protein synthesis and cell division [40]. Consistent with this notion, the genome wide genetic screen of the rbbA mutant displayed a SSL interaction with yhjD and with members of complexes I, II and IV of the ETC. Phospholipids that reside in the membrane are vital to the function of the proton gradients responsible for the synthesis of ATP by the ETC via ATP synthase. In fact, disruption of phospholipids of the ETC is expected to impair the proton gradient, thus inactivating the synthesis of ATP [41]. Thus, the SSL phenotype of the ETC imparted by deletion of the rbbA and yhjD genes can be partly explained by the effect of these proteins on phospholipids of the ETC. Our observations from the genetic interactions complement the biochemical results demonstrating that RbbA is functionally an important component of the membrane bound YhjD. The regeneration of the active or ATP form of rbbA may occur by virtue of its proximity to the yhjD membrane protein and in turn to the ETC. Interestingly, phospholipids are known to accelerate the rate of ATP:ADP exchange [42] supporting the suggestion that the attachment of RbbA to the membranes may indeed enhance its
ability to exchange bound ADP to the active bound ATP form which is needed for protein synthesis. Moreover, RbbA and YhjD also appear to affect cell division. The similarity in the YFP localization patterns of RbbA and YhjD with a MinD cell division protein reconfirmed the attachment of these proteins to the membrane as well their role in cell division. The minD mutants apparently allow polar divisions because of their affinity for the phospholipid tubes which occur at the cell poles, and restrict the potential for division to mid cell [43]. The observation that RbbA and YhjD physically bind to LPS proteins and exhibit a similar cell division phenotype as MinD may also be explained by alterations in phospholipids. In fact, the conversion of membrane bound MinD-ADP to the active MinD-ATP occurs in a phospholipid dependent fashion [44]. The appearance of more elongated cells in the RbbA and YhjD double mutant under permissive growth conditions (i.e., at 15°C and 30°C), also suggest a role for these proteins in cell division. It is important to restate that the appearance of an elongated phenotype in the double mutant is not just an artifact of cell death taking place at lower or at normal growth temperatures. Thus, it is likely that RbbA and YhjD play redundant roles in membrane biology necessary for the completion of cell division. A role of RbbA and YhjD in cell division is also suggested by its aggravating genetic interactions with various known cell division factors (e.g., minC, divis). Another explanation for the possible involvement of these two proteins in cell division is that the ATP supply might be lower prior to division events, leading to curtailment of replication and protein synthesis. Such a phenomenon takes place in eukaryotic cells where the amount of ATP is greatly reduced in the G1 phase of the growth which precedes cell division [45]. In some cases, new protein synthesis might also be required prior to the onset of DNA replication which precedes cell division [46]. Since total protein synthesis is greatly reduced in the rbbA yhjD double mutant at 32°C (Figure 4A), it is possible that the impaired cell division observed in the double mutant is due to limitation of certain proteins needed for DNA replication prior to the cell division. Curiously, the ability of RbbA to promote cell division at 15 and 32°C, appears to function in cooperation with the ETC and requires a functional YhjD.

Apart from their role in membrane biogenesis, the presence of an RNA binding motif inscribed in both RbbA and YhjD sequences as well as the reciprocal physical interaction of these proteins suggested that like RbbA, YhjD may also function in protein synthesis. Our physical evidence indicates that these two proteins localize to the 30S ribosomal subunits. Further, the rbbA and yhjD double mutant and their respective single mutant cells showed comparable level of sensitivity to antibiotics targeting translational fidelity. This suggests that YhjD indeed functions in protein synthesis in cooperation with RbbA. In vitro studies on RbbA suggest a role in synthesis accuracy as it restrains miscoding of β-gal synthesis which was not evident in the single mutants. These results suggest that there may be functions compensating for miscoding when there is loss-of -function in one of the alleles but such compensatory mechanisms may not keep pace with the loss of both functions and thus result in an increased error rate observed with the double mutant. For example, it is entirely possible that EF-Tu, which is essential for the accuracy of protein synthesis and functions with RbbA, may suppress the loss of the rbbA gene. In turn, the yhjD gene might suppress some of the functions affected by loss of the yhjD gene. Another possibility is that the reduced ATP concentration may suffice to reduce the translational errors observed in the single mutants but may not adequately compensate for the miscoding events observed when these errors are compounded in the double mutant. In any case, the decrease in the cellular supply of ATP derived from the ETC is expected to exacerbate at least some of the translation and fidelity defects observed with the mutants reported here.

We consider the possible repercussions of the effects of these proteins on membrane and protein synthesis processes. First, these proteins might alter the biosynthesis of several integral membrane proteins by membrane bound ribosomes, which requires their insertion into a phospholipid bilayer, a process governed by membrane-bound transporters which facilitate threading of appropriate polypeptides through the membrane [47,48]. Second, it is possible that RbbA and YhjD act as chaperones during membrane synthesis and/or assembly. Thus, it is conceivable that the localization of these proteins on ribosomes and on membranes may be used to transport LPS through membranes, thus creating conformational changes in the membrane that are transmitted to ribosomes. The attachment of ribosomes to membranes in the process of protein secretion and during membrane biogenesis is well known [48] but possible connectivity between OM signals and the translation process have not been described. Nonetheless, it may be that the heterogeneity of ribosomes implicated as a regulatory feature of the particles [49] is used to bind ribosomes to specific IM sites that are involved in the formation of respiratory chain complexes, ribosome biogenesis and/or sensors that transmit extracellular stimuli from ribosomes to the translation machinery. Such an attachment of ribosomes to the IM may occur at discrete sites on the membrane to insure the entry of proteins in a defined orientation for proper assembly of membrane structures.

The evidence we present implicates a direct association of RbbA and YhjD through physical and genetic interactions as well as through the common membrane and ribosome targets. We suggest that the association of these proteins, however disparate be their detailed mechanism, is part of a cellular link between the energy requirement in protein synthesis and the energy supplied by oxidative phosphorylation. It seems entirely possible that these proteins are part of a network of interacting pathways that connect external stimuli from the membrane to protein synthesis and possibly mediate regulation of growth and cell division. It is necessary for the cell to commit to diverse functions in compliance with its energy supply. Thus, the linkage of protein synthesis to the ATP supplied by the ETC may be essential to cellular survival. The finding that RbbA and YhjD are genetically and physically linked to the ETC in the cell points such a functional relationship.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

The wild type and deletion strains used in this study are listed in Table S5. Deletion strains were from the Keio knock-out library [50]. Hfr donor strains were constructed using λ-Red recombina-
Tagging and Purification
The endogenous RbbA and YhjD were C-terminally tagged using a sequential peptide affinity (SPA) dual tagging system and purified essentially as previously described [51], except in all purification steps 1% Triton or DDM or C12E8 mild non-ionic detergents were added to disrupt the association of RbbA with membrane fraction. The analysis of mass spectrometry data was essentially as previously described [51].

Immunoprecipitation
In the case of overproduced His6-tagging and purification, the E. coli open reading frames cloned into a high copy pCA24N plasmid [52] with a chaperomembrane selectable marker was used to express C-terminal His6 - tagged fusion protein under the control of an IPTG inducible promoter. After 5 hrs induction at 30°C to an OD of ~0.6, cells were harvested and lysed by sonication and the bait proteins purified by affinity chromatography under non-denaturing conditions as essentially as previously described [51]. Immunoblots were performed essentially as previously described [51] with antiserum against FLAG and His epitopes.

Yellow Fluorescent Protein and Cell Morphology Studies
C-terminal fusions of chromosomal RbbA, YhjD and MinD YFP strains were constructed in DY330 background by efficiently converting the respective E. coli SPA-tagged strains via λ-RED recombination system [51]. Briefly, PCR amplification was performed on an YFP-Cm resistance cassette (gift from Paul Choi and Sunney Xie, Harvard University) as a template. The PCR was performed using ~40 bp primers homology to the insertion site (SPA) and ~20 bp annealing to Cm resistance cassette. The YFP-fusion strains were grown exponentially in LB medium at 32°C. For cell morphological studies, the overnight deletion strain cultures on LB medium were sub-cultured and grown until the A600 value of the cultures reached 0.3, 10 μg/ml, ampicillin (100 μg/ml), as required.

Sucrose Gradient Centrifugation
The crude S30 extracts were loaded on linear sucrose density gradients as described [53], and 47% sucrose was used as cushion and ribosomes or subunits were sedimented at 4°C for 3 hrs.

Genome-wide eSGA Screens
A full-genome eSGA screen using rbbADΔ::CmR and yhjDΔ::CmR in Hfr Cavalli as donor was carried out and analyzed as previously described [19], as were with various mini-array crosses. Interac- tion scores (S) were calculated as previously described [19] to quantify the strength and confidence of the genetic interaction for each mutant gene pair. Gene set enrichment analysis was performed on the interaction S scores to determine the significant functionally related gene sets for GO terms spanning various biological processes (see Text S3).

In Vivo Assays on Respiratory Competence and on Fidelity and Translocation
Growth of the E. coli wild type and deletion strains under starvation conditions were assessed by growing the cultures exponentially in LB media. The exponential phased cultures were serially diluted and plated onto M9 minimal media plates supplemented with 2% glucose or glycerol. For the drug sensitivity assays, exponentially growing wild type and deletion strains in LB medium were serially diluted and pinned onto LB plates in the absence or presence of the indicated concentrations of the antibiotics targeting the ETC or fidelity and translocation. Plates were incubated for 48 hrs at 32°C before scoring for any growth defects.

Incorporation of Amino acids into Protein and Frame- shift Mutations of rbbA and yhjD Deleted Strains
The E. coli wild type and deletion strains were grown logarithmically in M9 minimal media without methionine. When the A600 value of the cultures reached 0.3, 10 μg/ml of [35S]methionine was incorporated into the growing cells. The translation reactions were incubated at 32°C and samples were drawn at 30 min. The rate of protein synthesis was determined as described previously [13]. Translation fidelity assay on the deletion strains were performed essentially as described [13] using previously reported expression plasmids [54], with the following modifications. Briefly, the E. coli cells were grown at 30°C to OD600 0.4–0.6. Cell pellets were resuspended in 0.1 M phosphate buffer pH 7.5 and lysed by sonication. β-galactosidase assays were performed using the O-nitrophenyl-α-D-galactopyranoside (ONPG) method and the β-lactamase activities were measured using a CENTA kit (Calbiochem). The activity of β-galactosidase was normalized to that of β-lactamase.

Each experiment was repeated independently at least six times. Incorporation of [35S]-methionine into total proteins and β-gal activity in the wild type and in the deletion strains treated with specific ETC inhibitors (sodium azide or CCCP) of ETC was assessed in the same manner as described above.

Supporting Information

Figure S1 Comparative Sequence Alignments of RbbA and YhjD. Amino acid sequence alignments of E. coli RbbA and YhjD proteins were determined by the CLC sequence viewer program. The RNA binding and two ATP binding (ATP-1 and ATP-2) motifs shared by the amino acid sequence of YhjD and RbbA are boxed in blue. The letters in red indicate the conserved amino acids.

Figure S2 RbbA and YhjD show Reduced Protein Synthesis and their Growth is Perturbed with Antibiotics
Targeting Fidelity and Translocation. (A, B) Serial-dilution assay showing the perturbed growth of rbbA-yhjD double mutant and their corresponding single mutants in the presence of antibiotics targeting ribosomal A-site, translocation and ribosome recycling (Panel A) and in the presence of aminoglycosides targeting fidelity and decoding process of protein synthesis (Panel B) (see main text for details). The MIC of the antibiotics is shown in parenthesis. The controls were same as in Figure 4D.

Figure S3 Comparative sequence alignments and folding patterns of RbbA and EF-3. (A) Amino acid sequence alignments of E. coli RbbA and S. cerevisae EF-3 proteins were

Supporting Information
determined by the CLC sequence viewer ver 6.0 program. The two common Walker ATP binding motifs (ATP-1 and ATP-2) are underlined in blue and the blue rectangular box shows the common t-RNA binding motif. The letters in red indicate the conserved amino acids. B) The crystal structures of RbbA and EF-3 determined at 2.7 Å resolutions was modeled using the SWISS-PROT folding program. The model used to draft the folding pattern of RbbA is based on sequence homology and strict folding pattern similarity to the ABC1 transporter of Pseudomonas abyssi [57]. The positions of the RNA and ATP binding motifs and the location of N- and C-terminus of the RbbA and the folding pattern of the N-terminal end of RbbA lacking the HEAT domain (residues 1 to 321) are shown on the left panel. The crystal structure of EF-3 shown in right panel represents the common ATP and RNA binding motifs as well as the HEAT domain (residues 1-321) at the N-terminus of the structure.

Table S1: Protein-protein interactions from endogenous affinity tagged RbbA and YhjD in the presence and absence of various detergents.

Table S2: Genetic Interaction Scores of Hfr rbbDA and yhjDA Donor Mutants Screened against the Recipient F- Keio Non-essential Single Gene Knock-Out Library and 149 Potential Hypomorph Essential Genes.

Table S3: Over-representation Analysis using Gene Ontology Terms on the Genetic Interaction Score from rbbDA Genome-Wide Genetic Screen.

References

1. Xu J, Kiel MC, Golshani A, Chosay JG, Aoki H, et al. (2006) Molecular localization of a ribosome-dependent ATPase on Escherichia coli ribosomes. Nucleic Acids Res 34: 1150–1165.
2. Ramakrishnan V (2002) Ribosome structure and the mechanism of translation. Cell 108: 557–572.
3. Ganoza MC, Cunningham C, Green R M (1995) A new factor from Escherichia coli affects translation of mRNA. J Biol Chem 270: 26377–26381.
4. Baba T, Ara T, Haegawa M, Takai Y, Okumura Y, et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2: 2006 0008.
5. Decraer A, Sjoen M, Holbrook ED, Fredrick K (2009) A role for the 30S subunit E site in maintenance of the translational reading frame. RNA 15: 255–265.
6. Ganoza MC, Kiel MC (2001) A ribosomal ATPase is a target for hygromycin B inhibition on Escherichia coli ribosomes. Antimicrob Agents Chemother 45: 2813–2819.
7. Jwett MC, Miller ML, Chen Y, Swartz JR (2009) Continued protein synthesis at low [ATP] and [GTP] enables cell adaptation during energy limitation. J Bacteriol 191: 1083–1091.
8. Butler G, Peregrin-Alvarez JM, Li J, Yang W, Yang X, et al. (2005) Interaction network containing conserved and essential protein complexes in Escherichia coli. Nature 433: 531–537.
9. Diaz-Mejia JJ, Babu M, Emili A (2009) Computational and experimental approaches to chart the Escherichia coli envelope-associated proteome and interaction. FEMS Microbiology Reviews 33: 66–97.
10. Kiel MC, Ganoza MC (2001) Functional interactions of an Escherichia coli ribosomal ATPase. Eur J Biochem 268: 278–286.
11. Maniat C, Meredith TG, Aggarwal P, Kuhl A, Kirchhoff P, et al. (2008) Single amino acid substitutions in either YhjD or MbaA confer viability to 3-deoxymanno-oct-2-ulose acid-depleted Escherichia coli. Mol Microbiol 67: 633–648.
12. Tran AX, Trent MS, Whitfield C (2000) The LptA protein of Escherichia coli is a periplasmic lipid A-binding protein involved in the lipopolysaccharide export pathway. J Biol Chem 275: 20342–20349.
13. Hu P, Janga SC, Babu M, Diaz-Mejia JJ, Butland G, et al. (2009) Global functional atlas of Escherichia coli encompassing previously uncharacterized genes. PLoS Biol 7: e123.
14. de Beer PA, Crossley RE, Hand AR, Rothfield LJ (1991) The MinD protein is required as a membrane ATPase for the correct placement of the Escherichia coli division site. EMBO J 10: 4571–4580.

Table S4: Over-representation Analysis using Gene Ontology Terms on the Genetic Interaction Score from yhjDA Genome-Wide Genetic Screen.

Table S5: Bacterial Strains used in this Study.

Text S1: RbbA Associates with Proteins Inolved in Protein Synthesis and Membrane Processes.

Text S2: ABC Transporters and Energy-Linked Pathway Proteins Co-purifies with YhjD.

Text S3: Over-representation Analysis using Gene Ontology Terms on the Genetic Interaction Score from rbbDA and yhjDA Genome-Wide Genetic Screens.

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Author Contributions

Conceived and designed the experiments: MB MCG. Performed the experiments: MB HA WQC A. Gasarova GP BL NS MJ MCG. Analyzed the data: MB MCG. Contributed reagents/materials/analysis tools: A. Golshani AE JFG MCG. Wrote the paper: MB MCG. Provided critical suggestions: A. Golshani AE JFG.
30. Cox GR, Newton NA, Gibson F, Snowsell AM, Hamilton JA (1970) The function of ubiquinone in Escherichia coli. Biochem J 117: 551–562.
31. Friedrich T, van Heek P, Leif H, Ohnishi T, Forche E, et al. (1994) Two-binding sites of inhibitors in NADH:ubiquinone oxidoreductase (complex I). Relationship of one site with the ubiquinone-binding site of bacterial glucose:ubiquinone oxidoreductase. Eur J Biochem 219: 691–698.
32. Stolpe S, Friedrich T (2004) The Escherichia coli NADH:ubiquinone oxidoreductase (complex I) is a primary proton pump but may be capable of secondary sodium antiport. J Biol Chem 279: 18377–18383.
33. Krulwich TA, Quirk PG, Guffanti AA (1990) Uncoupler-resistant mutants of bacteria. Microbiol Rev 54: 52–65.
34. Strahil H, Hameen LW (2010) Membrane potential is important for bacterial cell division. Proc Natl Acad Sci U S A 107: 12291–12296.
35. Resnick M, Schuldiner S, Bercovier H (1985) Bacterial membrane potential analyzed by spectrofluorometry. Curr Microbiol 12: 183–186.
36. van Dalen A, Schreppl H, Killian JA, de Kruijff B (2000) Efficient membrane assembly of the KcsA potassium channel in Escherichia coli requires the protonotive force. EMBO Rep 1: 340–346.
37. Ganoza MC, Kiel MC, Aoki H (2002) Evolutionary conservation of reactions in translation. Microbiol Mol Biol Rev 66: 460–495.
38. Thomsen ND, Berger JM (2008) Structural frameworks for considering microbial protein- and nucleic acid-dependent motor ATPases. Mol Microbiol 69: 1071–1090.
39. Anderson CE, Becker T, Blau M, Anand M, Halic M, et al. (2006) Structure of eEF3 and the mechanism of transfer RNA release from the E-site. Nature 443: 663–668.
40. Zhang YM, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6: 222–233.
41. Mitchell P (1961) Coupling of phosphorylation to electron transport and hydrogen transfer by a chemiosmotic mechanism. Nature 191: 144–148.
42. Crooke E, Castuma CE, Kornberg A (1992) The chromosome origin of Escherichia coli stabilizes DnaA protein during rejuvenation by phospholipid. J Biol Chem 267: 16779–16782.
43. Norris V, Woldringh C, Mileykovskaya E (2004) A hypothesis to explain division site selection in Escherichia coli by combining nuclear occlusion and Min. FEBS Lett 561: 3–10.
44. Hu Z, Latkemahns J (2001) Topological regulation of cell division in E. coli. spatiotemporal oscillation of MinD requires stimulation of its ATPase by MinE and phospholipid. Mol Cell 7: 1337–1543.
45. Marcussen M, Larsen PJ (1996) Cell cycle-dependent regulation of cellular ATP concentration, and depolymerization of the interphase microtubular network induced by elevated cellular ATP concentration in whole fibroblasts. Cell Mol Cytokeleten 33: 94–99.
46. Jones NC, Donachic WD (1973) Chromosome replication, transcription and control of cell division in Escherichia coli. Nat New Biol 243: 100–103.
47. Lam VQ, Akopian D, Rome M, Henningsen D, Shsn SO (2010) Lipid activation of the signal recognition particle receptor provides spatial coordina- tion of protein targeting. J Cell Biol 190: 629–635.
48. Muller M, Koch HG, Beck K, Schafer U (2003) Protein traffic in bacteria: multiple routes from the ribosome to and across the membrane. Prog Nucleic Acid Res Mol Biol 66: 107–157.
49. Mauro VP, Edelman GM (2002) The ribosome filter hypothesis. Proc Natl Acad Sci U S A 99: 12031–12036.
50. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2: 2006.0008.
51. Babu M, Butland G, Pogoussi O, Li J, Greenblatt JF, et al. (2009) Sequential peptide affinity purification system for the systematic isolation and identification of protein complexes from Escherichia coli. Methods Mol Biol 564: 373–400.
52. Kitagawa M, Ara T, Ariluzaman M, Ioka-Nakamichi T, Inamoto E, et al. (2005) Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. DNA Res 12: 291–299.
53. Jiang M, Sullivan SM, Walker AK, Strahler JR, Andrews PC, et al. (2007) Identification of novel Escherichia coli ribosome-associated proteins using isobaric tags and multidimensional protein identification techniques. J Bacteriol 189: 3434–3444.
54. Thompson J, O’Connor M, Mills JA, Dahlberg AE (2002) The protein synthesis inhibitors, oxazolidinones and chloramphenicol, cause extensive translational inaccuracy in vivo. J Mol Biol 322: 273–279.
55. Greiss HS, Hottes AK, Tavaoie S (2009) Genetic architecture of intrinsic antibiotic susceptibility. PLoS One 4: e629.
56. Musser SM, Chan SI (1998) Evolution of the cytochrome c oxidase proton pump. J Mol Evol 46: 508–520.
57. Kaeser A, Schle A, Hopfner KP (2008) X-ray structure of the complete ABC enzyme ABCE1 from Pyrococcus abyssi. J Biol Chem 283: 7962–7971.