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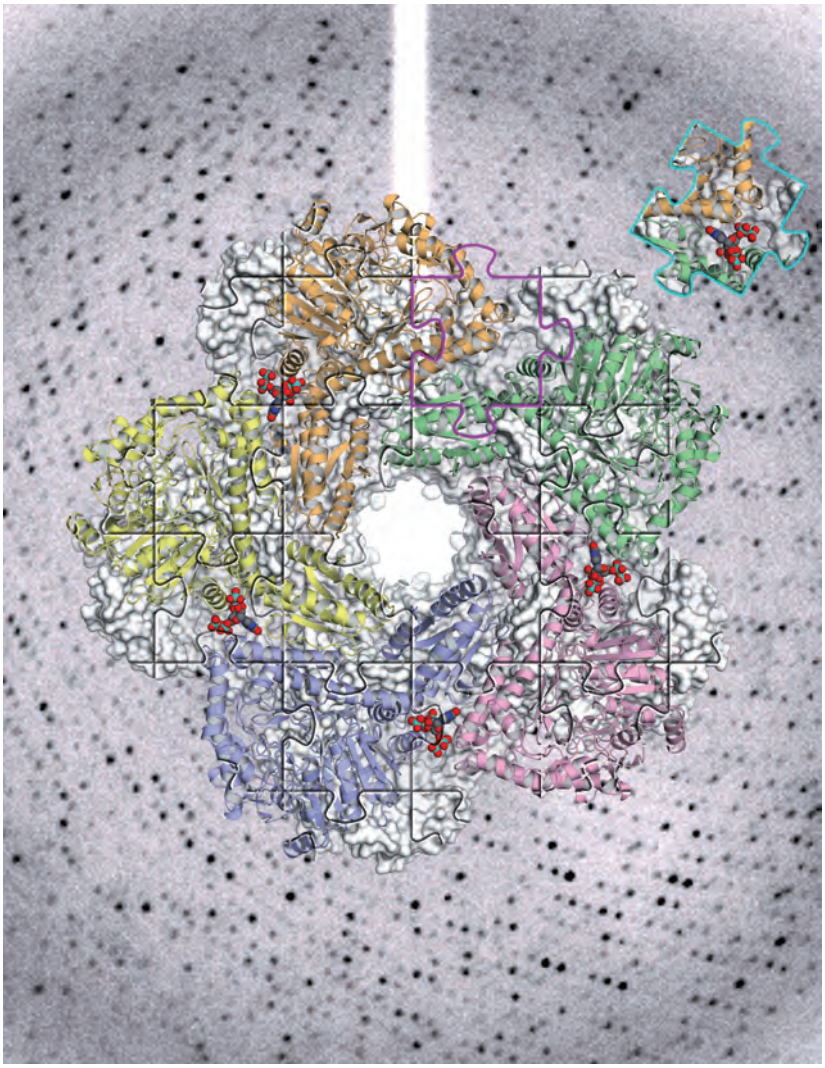

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MicroReview

Direct binding targets of the stringent response alarmone (p)ppGpp

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Summary

The *Escherichia coli* stringent response, mediated by the alarmone ppGpp, is responsible for the reorganization of cellular transcription upon nutritional starvation and other stresses. These transcriptional changes occur mainly as a result of the direct effects of ppGpp and its partner transcription factor DksA on RNA polymerase. An often overlooked feature of the stringent response is the direct targeting of other proteins by ppGpp. Here we review the literature on proteins that are known to bind ppGpp and, based on sequence homology, X-ray crystal structures and *in silico* docking, we propose new potential protein binding targets for ppGpp. These proteins were found to fall into five main categories: (i) cellular GTPases, (ii) proteins involved in nucleotide metabolism, (iii) proteins involved in lipid metabolism, (iv) general metabolic proteins and (v) PLP-dependent basic aliphatic amino acid decarboxylases. Bioinformatic rationale is provided for expanding the role of ppGpp in regulating the activities of the cellular GTPases. Proteins involved in nucleotide and lipid metabolism and general metabolic proteins provide an interesting set of structurally varied stringent response targets. While the inhibition of some PLP-dependent decarboxylases by ppGpp suggests the existence of cross-talk between the acid stress and stringent response systems.

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Introduction

The *Escherichia coli* stringent response is a sophisticated and rapidly activated system which is induced in response to a number of nutritional or environmental stresses, and that mediates the transition between exponential and stationary phase growth (Cashel *et al*., 1996; Nystrom, 2004; Potrykus and Cashel, 2008). The stringent response effects are potentiated primarily through the unusual guanosine nucleotides: guanosine tetraphosphate ppGpp [guanosine 3′, 5′-bis(diphosphate)] and guanosine pentaphosphate pppGpp (guanosine 3′-diphosphate, 5′-triphosphate), collectively known as (p)ppGpp. In the cell, pppGpp is synthesized from GTP and ATP via the action of two paralogous enzymes RelA and SpoT (Cashel *et al*., 1996), which belong to a widely distributed family of RelA/SpoT homologue proteins (Atkinson *et al*., 2011). Subsequently, pppGpp is converted to ppGpp through the action of the pppGpp 5′-phosphohydrolase GppA enzyme (Fig. 1) (Hara and Sy, 1983). The protein domain boundaries and X-ray crystal structures of the N-terminal (p)ppGpp binding domains of both the RelA/SpoT homologue protein Rel_{Seq} from *Streptococcus equisimilis* (Hogg *et al*., 2004) and the GppA paralogue PPX_{Aae} from *Aquifex aeolicus* (Kristensen *et al*., 2008) are shown in Fig. 1A–D. RelA is associated with ribosomes through its C-terminus and is responsible for (p)ppGpp synthesis in response to amino acid limitation (Fig. 1E) (Wendrich *et al*., 2002). Recent single molecule studies have shown that alarmone synthesis occurs upon release of RelA from the ribosome during the stringent response (English *et al*., 2011). Cytoplasmic SpoT is responsible for the basal synthesis of (p)ppGpp during growth and for (p)ppGpp degradation (Gentry and Cashel, 1995). SpoT is also responsible for (p)ppGpp synthesis in response to a number of stress conditions (Cashel *et al*., 1996). Under fatty acid starvation conditions, and potentially under carbon-source starvation conditions, the acyl carrier protein (ACP) binds to and has been proposed to activate SpoT (Fig. 1E) (Battesti and Bouveret, 2006).

The stringent response was historically identified by the rapid downregulation of stable RNA (rRNA and tRNA)

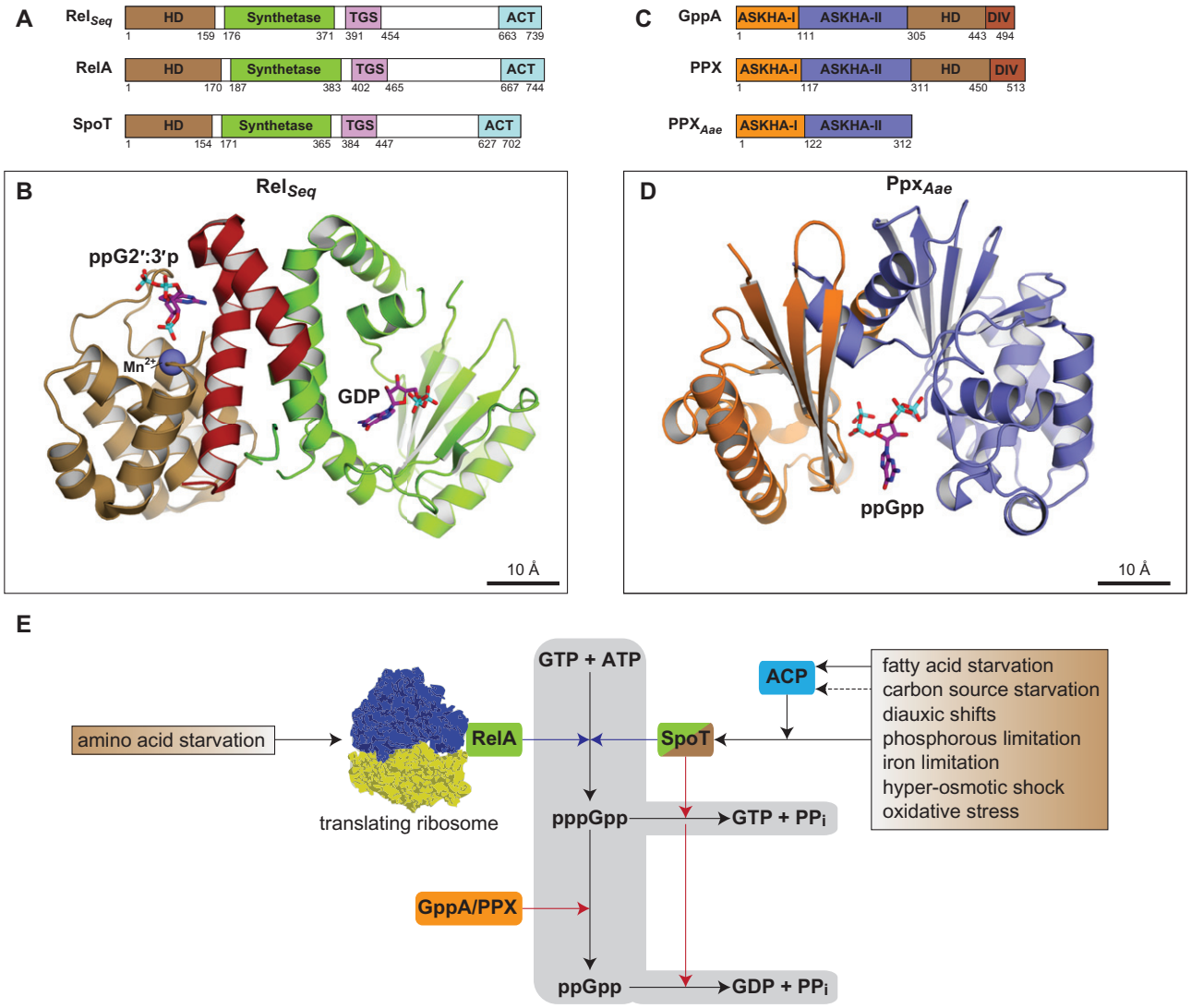

Fig. 1. Proteins involved in (p)ppGpp metabolism.

A. Schematic of the domain boundaries of the *Streptococcus equisimilis* Rel_{Seq} (Hogg *et al.*, 2004) and *E. coli* RelA and SpoT proteins (Metzger *et al.*, 1989). The following domains are indicated: HD-type (p)ppGpp hydrolase domain (brown) (Aravind and Koonin, 1998); nucleotidyltransferase-type (p)ppGpp synthetase domain (green) (Hogg *et al.*, 2004); TGS domain (purple) (a small domain found in threonine aminoacyl-tRNA synthetase ThrRS, GTPases and SpoT) (Wolf *et al.*, 1999); and ACT domain (cyan) (aspartate kinase, chorismate mutase and TyrA domain) (Aravind and Koonin, 1999).

B. Cartoon representation of the X-ray crystal structure (PDB ID: 1VJ7) of Rel_{Seq} HD and synthetase domains coloured as in (A). The helices coloured red form part of the conserved three-helix bundle that mediates communication between the HD and synthetase domains (Hogg *et al.*, 2004). The bound GDP and ppG2′:3′p (a derivative of ppGpp) are illustrated as sticks with carbon atoms coloured purple, oxygen atoms coloured red, nitrogen atoms coloured blue, and phosphorous atoms coloured cyan. The bound Mn^{2+} ion is shown as a blue sphere. All X-ray structure images were generated using PyMOL (DeLano, 2002).

C. The domain boundaries for the paralogous GppA and the polyphosphatases PPX from *E. coli* (Kuroda *et al.*, 1997) and PPX from *Aquifex aeolicus* (PPX_{Aqa}) are shown. The PPX and GppA enzymes have a pppGpp 5′-phosphohydrolase activity (Hara and Sy, 1983; Kristensen *et al.*, 2008). The following domains are indicated: ASKHA-I (orange) and ASKHA-II (blue) (acetate and sugar kinase/Hsp70/actin) superfamily domains (Reizer *et al.*, 1993); the HD domain III (brown); and the C-terminal domain IV (DIV) (maroon) (Alvarado *et al.*, 2006; Rangarajan *et al.*, 2006).

D. Cartoon representation of the X-ray crystal structure (PDB ID: 2J4R) of the PPX_{Aae} is shown with the domains coloured as indicated in (C) and the bound ppGpp shown as a stick figure and coloured as in (B).

E. A schematic diagram showing the pathway for (p)ppGpp synthesis. Blue arrows indicate synthetic reactions and red arrows indicate degradative reactions. Activation of the ribosome-bound RelA is via amino acid starvation (Wendrich *et al.*, 2002). SpoT is activated in response to a number of stresses including fatty acid starvation (Battesti and Bouveret, 2006), carbon source starvation (Xiao *et al.*, 1991), diauxic shifts (Harshman and Yamazaki, 1971), phosphorous limitation (Spira *et al.*, 1995; Spira and Yagil, 1998; Bougdour and Gottesman, 2007), iron limitation (Vinella *et al.*, 2005), hyper-osmotic shock (Harshman and Yamazaki, 1972; Cashel *et al.*, 1996), and oxidative stress (Chang *et al.*, 2002). ACP senses fatty acid and potentially carbon source starvation and activates ppGpp production by SpoT.

Table 1. Summary of processes affected by the stringent response.

Process	References
Downregulated proliferative processes	
Cell division	Schreiber *et al.* (1991); Ferullo and Lovett (2008); Traxler *et al.* (2008)
Cell motility (fimbriae and flagellar)	Aberg *et al.* (2006); Magnusson *et al.* (2007)
DNA replication	Hernandez and Bremer (1993); Schreiber *et al.* (1995); Wang *et al.* (2007); Ferullo and Lovett (2008); Traxler *et al.* (2008)
rRNA and tRNA synthesis	Hernandez and Bremer (1993); Cashel *et al.* (1996); Traxler *et al.* (2008)
Ribosome synthesis	Cashel *et al.* (1996); Zhang *et al.* (2006); Lemke *et al.* (2011)
Protein synthesis	Svitil *et al.* (1993)
Translation initiation and elongation	Rojas *et al.* (1984); Cashel *et al.* (1996); Milon *et al.* (2006); Bremer and Dennis (2008)
Nucleotide biosynthesis	Hochstadt-Ozer and Cashel (1972); Fast and Skold (1977); Morton and Parsons (1977); Pao and Dyess (1981); Cashel *et al.* (1996); Traxler *et al.* (2008)
Metabolite transport	Hochstadt-Ozer and Cashel (1972); Hochstadt (1978)
Phospholipid synthesis	Merlie and Pizer (1973); Polakis *et al.* (1973); Lueking and Goldfine (1975); Heath *et al.* (1994)
Oxidative metabolism	Chang *et al.* (2002)
Upregulated stress response processes	
Amino acid biosynthesis	Cashel *et al.* (1996); Tedin and Norel (2001); Barker *et al.* (2001b); Magnusson *et al.* (2005); Paul *et al.* (2005)
σ^S synthesis	Gentry *et al.* (1993); Chang *et al.* (2002)
Universal stress protein synthesis	Kvint *et al.* (2000); Gustavsson *et al.* (2002); Trautinger *et al.* (2005)
Carbohydrate metabolism	Dietzler and Leckie (1977); Traxler *et al.* (2006); 2008)
Virulence gene expression	Magnusson *et al.* (2005); Nakanishi *et al.* (2006)
Toxin/antitoxin systems	Chang *et al.* (2002)
Antibiotic resistance	Rodionov and Ishiguro (1995); Greenway and England (1999); Korch *et al.* (2003)
Cyclopropane fatty acid synthesis	Eichel *et al.* (1999)
Chaperones and proteolysis systems	Cashel *et al.* (1996); Chang *et al.* (2002); Yang and Ishiguro (2003)

Cellular processes that are downregulated or upregulated during the stringent response are shown.

genes when cells grown in rich media encountered amino acid starvation (Stent and Brenner, 1961; Dennis *et al.*, 2004). Subsequently, it was shown that the stringent response results in global genetic and physiological changes to cellular metabolism (Cashel *et al.*, 1996; Nystrom, 2004; Magnusson *et al.*, 2005; Potrykus *et al.*, 2011; Traxler *et al.*, 2011), listed in Table 1. As the master regulator of the stringent response, (p)ppGpp has two major categories of effects (i) modification of gene transcription and (ii) direct interaction with target proteins. The effects of (p)ppGpp on gene transcription has been extensively studied and reviewed (Cashel *et al.*, 1996; Dennis *et al.*, 2004; Nystrom, 2004; Magnusson *et al.*, 2005; Potrykus and Cashel, 2008). In *E. coli*, alterations in gene expression profiles during the stringent response are the result of interactions between the RNA polymerase (RNAP), ppGpp, and a specific transcription factor DksA. During the stringent response, ppGpp and DksA are able to facilitate opposing effects on transcription: downregulation of highly expressed stable RNA (rRNA and tRNA) and cell proliferation genes and simultaneous upregulation of stress and starvation genes (Magnusson *et al.*, 2005). A binding site for ppGpp was observed in a *Thermus thermophilus* RNAP-ppGpp co-crystal structure (Artsimovitch *et al.*, 2004), but subsequent analysis of the *E. coli* RNAP has shown that this site is probably not responsible for mediating RNAP regulation by ppGpp (Kasai *et al.*, 2006; Vrentas *et al.*, 2008).

While the global effects of the stringent response are mediated via changes in the transcription profile of the cell, there are a number of specific proteins that are directly targeted by ppGpp. Apart from the proteins that are involved in (p)ppGpp synthesis and degradation (RelA, SpoT, GppA) (Fig. 1) and the main target of (p)ppGpp regulation (RNAP), we identified five major categories of *E. coli* (p)ppGpp targets based on literature reports, bioinformatics, and *in silico* docking analysis: (i) cellular GTPases, (ii) proteins involved in nucleotide metabolism, (iii) proteins involved in lipid metabolism, (iv) general metabolic proteins and (v) the basic aliphatic amino acid decarboxylases.

GTPases

The GTPase superfamily of proteins are found in all kingdoms of life, and in prokaryotes they function in translation, cell cycle regulation, protein translocation, and other essential but poorly characterized cellular functions (Caldon and March, 2003; Brown, 2005; Margus *et al.*, 2007). The GTPases share a number of common GTP binding motifs that include: (G_1) P-loop [$GX_4GK(S/T)$] involved in binding 5′α- and 5′β-phosphates of GTP; (G_2)

conserved T involved in Mg^{2+} binding; (G_3) Walker B [DX_2G] involved in binding Mg^{2+} and the 5′γ-phosphate of GTP; (G_4) [(N/T)(K/Q)XD] involved in binding the guanosine ring; and (G_5) [poor consensus] involved in stabilizing G_4 residues (Bourne *et al*., 1991; Brown, 2005). In many (but not all) cases, the GTPase activity cycle is regulated by two types of proteins: a GTP-bound protein is stimulated to hydrolyse GTP to GDP upon the binding of a GTPase activating protein (GAP); subsequently, the GDP is released upon interaction with a guanine nucleotide release protein (GNRP)/guanine nucleotide exchange factor (GEF) (Bourne *et al*., 1991; Caldon and March, 2003).

The *E. coli* GTPases can be divided into three major categories: the translation elongation-factor group (CysN, EFG, TypA/BipA, LepA, EF-Tu, RF3, SelB, IF2) (Margus *et al*., 2007), the cell-signalling and cell division Era/Obg group (Der/EngA, EngB, EngD, Era, HflX, MnmE/TrmE, Obg, YfjP, YkfA, RsgA) (Caldon and March, 2003), and the protein translocation FtsY/Ffh group (FtsY, Ffh) (Caldon and March, 2003). A number of other GTPases (FeoB, PurA) are also present but have not been categorized. Figure 2A shows the domain organization of the *E. coli* GTPases and Fig. 2B shows a sequence alignment of the GTP binding motifs and highlights the residue conservation. The strong amino acid conservation at the GTP binding site and the similarities in structure between GTP and ppGpp suggest that these proteins could bind ppGpp; there are reports of such interactions for the five GTPases discussed below.

The translation elongation and initiation GTPases EFG, EF-Tu, and IF2 are large, multi-domain proteins that have homologous GTPase domains followed by a short β-barrel domain (DII) (Fig. 2A) (Margus *et al*., 2007). EFG GTPase activity powers the translocation of the ribosome during protein synthesis (al-Karadaghi *et al*., 1996). A complex between EFG and ppGpp has been postulated and ppGpp has been suggested to inhibit EFG activity (Table 2) (Rojas *et al*., 1984). The translation elongation factor EF-Tu is one of the most common proteins in the cell. GTP-bound EF-Tu ferries aminoacylated-tRNA to the A-site of the translating ribosome and, upon recognition of the correct codon/anticodon pair, EF-Tu is released from the tRNA by GTP hydrolysis (Song *et al*., 1999). The tightly bound EF-Tu-GDP complex is recycled via the action of the EF-Ts GNRP. The X-ray crystal structure of GDP-bound EF-Tu (Abel *et al*., 1996) is shown in Fig. 2C. EF-Tu can bind ppGpp alone (Legault *et al*., 1972; Miller *et al*., 1973; Hamel and Cashel, 1974; Rojas *et al*., 1984), in complex with aa-tRNA (Pingoud *et al*., 1983) or in complex with the EF-Ts (Rojas *et al*., 1984). It has been proposed that EF-Tu bound to ppGpp increases the translation fidelity during stress and starvation conditions (Rojas *et al*., 1984; Dix and Thompson, 1986).

Initiation factor 2 (IF2) is a GTPase that binds to the initiator fMet-$tRNA^{fMet}$ during assembly of the translating ribosome. IF2 is GTP-bound during active cell growth but its activity is inhibited by ppGpp binding under stress conditions (Legault *et al*., 1972). A nuclear magnetic resonance (NMR) solution structure of the GTPase domain of *Bacillus stearothermophilus* IF2 in complex with ppGpp has been reported (Milon *et al*., 2006) and ppGpp was found to bind at the same site as GTP/GDP. The binding of the 5′α- and 5′β-phosphates and the guanosine base are essentially the same for both GTP and ppGpp, while in the latter case the 3′α- and β-phosphates project away from the binding site and are thought to interfere with IF2-interacting partners (fMet-$tRNA^{fMet}$ or ribosomal proteins).

Obg also known as CgtA/YhbZ is an essential GTPase that may function in DNA replication, in ribosome assembly through interaction with the 50S ribosomal subunit, and in the stringent response by interacting with SpoT (Wout *et al*., 2004; Persky *et al*., 2009). Obg has a moderate affinity for GTP/GDP, a high exchange rate and a weak GTPase activity (Wout *et al*., 2004). In addition, the protein has been shown to bind (p)ppGpp and to influence the balance of pppGpp/ppGpp in the cell, suggesting that Obg functions as a pppGpp 5′-phosphohydrolase (Persky *et al*., 2009). A crystal structure of residues 1–342 of the Obg homologue from *Bacillus subtilis* (Obg_{Bs}) in complex with ppGpp has been determined (Buglino *et al*., 2002) and is shown in Fig. 2D. The binding of ppGpp was found to be dependent on the 5′α- and 5′β-phosphates and the guanosine base while the 3′α- and 3′β-phosphates are not directly coordinated and face away from the binding site.

The adenylosuccinate synthetase PurA is involved in *de novo* ATP biosynthesis and catalyses the following GTP-dependent reaction: inosine monophosphate + L-aspartate + GTP ↔ adenylosuccinate + GDP + phosphate (Honzatko and Fromm, 1999). PurA activity can be inhibited by ppGpp (Table 2) (Gallant *et al*., 1971; Stayton and Fromm, 1979; Pao and Dyess, 1981) and co-crystal structures of GDP- and ppGpp-bound PurA have been determined (Honzatko and Fromm, 1999; Hou *et al*., 1999) (Fig. 2E and F). The alarmone was bound as, and potentially converted to, a ppG2′:3′p derivative of ppGpp in the GTP binding site, suggestive of a more complex inhibition mechanism (Hou *et al*., 1999).

To examine if the ppGpp binding interactions that have been observed for Obg_{Bs} and PurA (Fig. 2D and F) are structurally conserved, we performed *in silico* docking experiments with ppGpp and the X-ray crystal structures of the *E. coli* or homologous GTPases listed in Fig. 2A. For each model, ppGpp was docked onto the position of the natural substrate GTP/GDP or a substrate analogue (depending on their availability in the PDB file) and molecular dynamics simulations were performed to relax

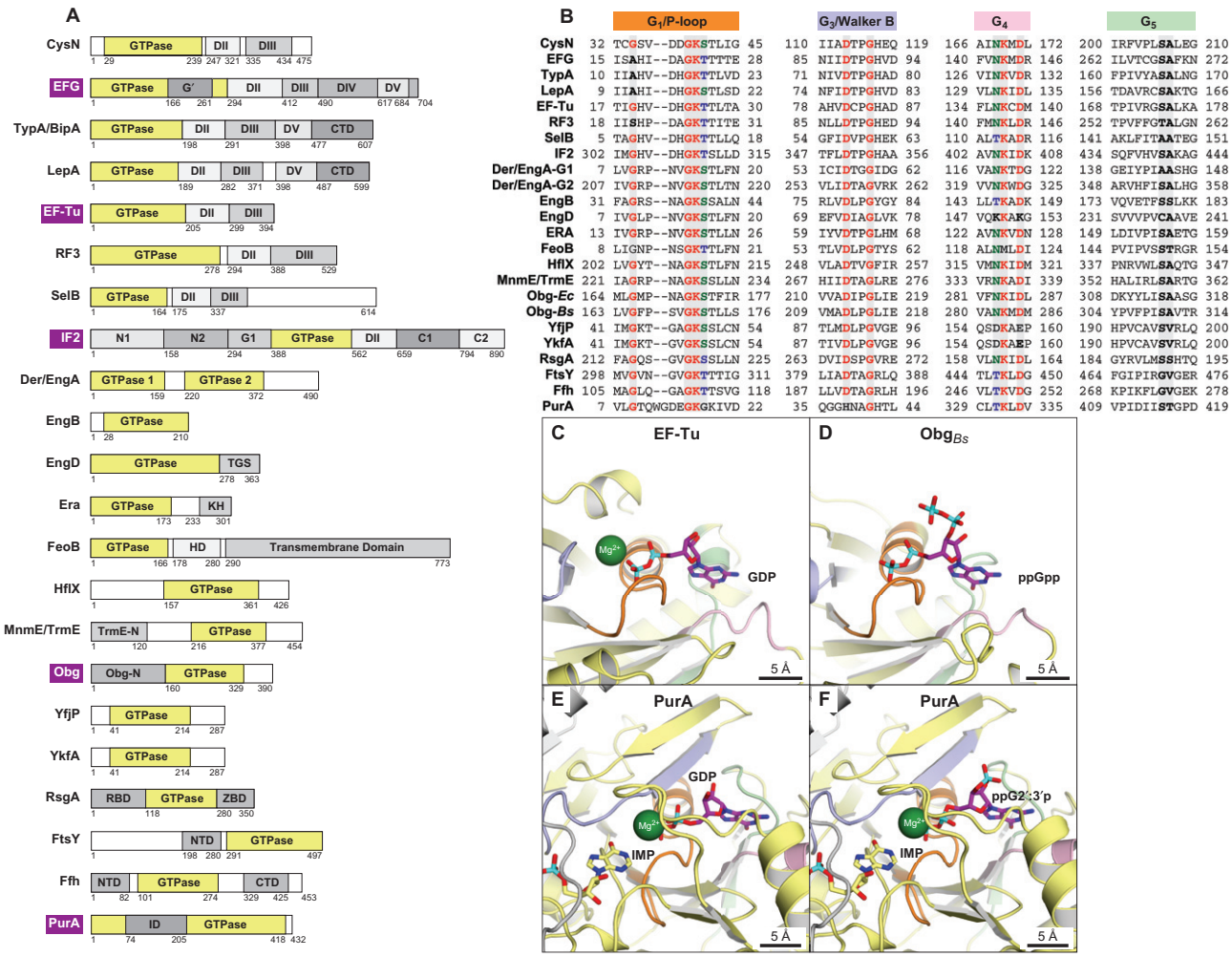

Fig. 2. *E. coli* GTPases as direct targets of ppGpp action.

A. Domain organization of the various *E. coli* GTPases is shown. Each protein contains a GTPase domain (coloured yellow) that has the conserved GTP binding motifs. Proteins that have been shown to interact with ppGpp are highlighted in purple. The following is a list of the UniProt (UniProt Consortium, 2011) accession numbers in parentheses for each protein followed where applicable by the full domain name(s). For the translation/elongation GTPases (CysN, EFG, TypA/BipA, LepA, EF-Tu, RF3, SelB), the GTPase domain is followed by two conserved domains: DII – domain II and DIII – domain III. CysN (P23845); EFG (P0A6M8) G′ – GTPase prime insertion domain, DIV – domain IV, DV – domain V; TypA/BipA (P32132) DV – domain V, CTD – C-terminal domain; LepA (P60785) DV – domain V, CTD – C-terminal domain; EF-Tu (P0CE47); RF3 (P0A7I4), SelB (P14081); IF2 (P0A705) N1 – N-terminal domain 1, N2 – N-terminal domain 2, G1 – pre-GTPase domain, DII – domain II, C1 – penultimate C-terminal domain, C2 – C-terminal domain; Der/EngA (P0A6P5); EngB/YihA (P0A6P7); EngD (P0ABU2) TGS – ThrRS, GTPases, and SpoT domain; Era (P06616) KH – K-homology RNA binding domain; FeoB (P33650) HD – His-Asp metal binding domain; HflX (P25519); MnmE/TrmE (P25522) TrmE-N–N-terminal TrmE domain; Obg/CgtA (P42641) Obg-N – Obg-fold N-terminal domain; YfjP (P52131); YkfA (P75678); RsgA (P39286) RBD – OB-fold RNA binding domain, CTD – C-terminal domain; FtsY (P10121) NTD – N-terminal domain; Ffh (P0AGD7) NTD – N-terminal domain, CTD – C-terminal domain, and PurA (P0A7D4) ID – insertion domain.

B. A multiple sequence alignment of the GTPase domains was determined using *MUSCLE* (Edgar, 2004) and the results were manually verified using *JALVIEW* (Clamp *et al.*, 2004). The conserved GTPase-features including G_1/P-loop, G_3, G_4 and G_5 signature sequences and residues that make up these signature sequences are shown in bold. Highly conserved residues are coloured red and residues that vary between one of two predominant residues in a position are shown as green and blue. The protein names are abbreviated as in (A) except for Obg-*Ec* (*E. coli* Obg – P42641) and Obg-*Bs* (*Bacillus subtilis* Obg – P20964).

C–F. Cartoon representation of the X-ray crystal structures of (C) EF-Tu bound to GDP (PDB ID: 1DG1) (Abel *et al.*, 1996), (D) Obg from *B. subtilis* (Obg_{Bs}) bound to ppGpp (PDB ID: 1LNZ) (Buglino *et al.*, 2002), (E) PurA bound to GDP (PDB ID: 1CIB) or (F) ppG2′:3′p (PDB ID: 1CH8) (Hou *et al.*, 1999). In all cases, the GTPase domain is coloured yellow and the specific GTP binding motifs are indicated as follows: G_1/P-loop – orange, G_3 – light blue, G_4 – pink, and G_5 – light green. The guanosine nucleotide is shown as a stick figure with oxygen atoms coloured red, nitrogen atoms coloured blue, phosphorous atoms coloured cyan, and carbon atoms coloured purple. Where applicable, the Mg^{2+} ion is shown as a green sphere.

Table 2. Measured binding affinities of (p)ppGpp and other substrates to *E. coli* proteins.

Protein	Affinity (μM)	Notes	Reference
(1) GTPases			
EFG	60	K_i – ppGpp	Rojas *et al.* (1984)
EF-Tu	0.7[a]	K_i – ppGpp	Rojas *et al.* (1984)
	40[b]	K_i – ppGpp	Rojas *et al.* (1984)
	0.008	K_d – ppGpp	Miller *et al.* (1973)
	0.002	K_d – GDP	Bourne *et al.* (1991)
RF3	0.006	K_d – GDP	Gao *et al.* (2007)
SelB	0.74	K_d – GTP	Thanbichler *et al.* (2000)
	13.4	K_d – GDP	Thanbichler *et al.* (2000)
Der/EngA	143	K_M – GTP	Bharat *et al.* (2006)
EngB/YihA	27	K_d – GTP	Lehoux *et al.* (2003)
	3	K_d – GDP	Lehoux *et al.* (2003)
MnmE/TrmE	0.57	K_d – GDP	Scrima and Wittinghofer (2006)
Obg	1.6	K_i – ppGpp	Persky *et al.* (2009)
	1.6	K_i – GDP	Persky *et al.* (2009)
	8	K_d – GDP	Wout *et al.* (2004)
RsgA	120	K_M – GTP	Daigle *et al.* (2002)
Ffh	7	K_M – GTP	Powers and Walter (1995)
PurA	140	K_i – ppGpp	Pao and Dyess (1981)
	50	K_i – ppGpp	Stayton and Fromm (1979)
	12	K_i – GDP	Stayton and Fromm (1979)
(2) Nucleotide metabolism			
DnaG	250	K_i – ppGpp	Wang *et al.* (2007)
	120	K_i – pppGpp	Wang *et al.* (2007)
	200	> 50% inhibition – ppGpp	Maciag *et al.* (2010)
	400	> 50% inhibition – pppGpp	Maciag *et al.* (2010)
	500	> 50% inhibition – GDP	Maciag *et al.* (2010)
GuaB	50	K_i – ppGpp	Pao and Dyess (1981)
	30	K_i – ppGpp	Gallant *et al.* (1971)
	80	K_i – GMP	Gallant *et al.* (1971)
	11	K_M – IMP	Gilbert *et al.* (1979)
GuaC	6.9	K_M – GMP	Martinelli *et al.* (2011)
Gpt	4.3	K_M – guanine	Vos *et al.* (1998)
	39	K_M – xanthine	Liu and Milman (1983)
	140	K_M – PRPP	Guddat *et al.* (2002)
Apt	1500	> 50% inhibition – ppGpp	Hochstadt-Ozer and Cashel (1972)
	11	K_M – adenine	Hochstadt-Ozer and Stadtman (1971)
	180	K_M – PRPP	Hochstadt-Ozer and Stadtman (1971)
Upp	2	K_M – uracil	Fast and Skold (1977)
	300	K_M – PRPP	Rasmussen *et al.* (1986)
Hpt	85	> 50% inhibition – ppGpp	Hochstadt-Ozer and Cashel (1972)
	12.5	K_M – hypoxanthine	Guddat *et al.* (2002)
	192	K_M – PRPP	Guddat *et al.* (2002)
PyrE	30	K_M – orotate	Shimosaka *et al.* (1985)
	40	K_M – PRPP	Shimosaka *et al.* (1985)
HisG	76	K_i – ppGpp[c]	Morton and Parsons (1977)
(3) Lipid metabolism and (4) general metabolic proteins			
PgsA	4000	> 50% inhibition – ppGpp	Merlie and Pizer (1973)
GdhA	30	K_d – ppGpp	Maurizi and Rasulova (2002)
(5) Amino acid decarboxylases			
LdcI	0.013	K_{d1} – ppGpp	Kanjee *et al.* (2011b)
	0.685	K_{d2}– ppGpp	Kanjee *et al.* (2011b)
LdcC	0.1–0.5	K_d – ppGpp	Kanjee *et al.* (2011a)
SpeF	12.2	K_d – ppGpp	Kanjee *et al.* (2011a)
SpeC	0.599	K_d – ppGpp	Kanjee *et al.* (2011a)
	0.025	K_{d1} – GTP	Kanjee *et al.* (2011a)
	0.403	K_{d2} – GTP	Kanjee *et al.* (2011a)

a. K_i in the absence of EF-Ts (Rojas *et al.*, 1984).
b. K_i in the presence of EF-Ts (Rojas *et al.*, 1984).
c. K_i in the presence of 100 μM histidine (Morton and Parsons, 1977).
The interaction affinity for ppGpp and/or enzyme substrates for the five different classes of target proteins is shown. Where possible, the reported interaction affinity measurement is the dissociation constant K_d, or where this value was not available, the measured inhibition constant K_i, the Michaelis constant K_M, or the concentration giving greater than 50% inhibition are reported. Proteins that are inhibited by (p)ppGpp are highlighted in purple.

the system (Fig. S1). Additionally a measure of the thermodynamic favourability of the interaction between ppGpp and the target protein was calculated and in all cases a negative ΔG was obtained, indicating a favourable interaction (Tables S1 and S2). While the results of the docking experiments should be treated with caution as they are based on an *in silico* simulation, we can infer several useful trends related to the interaction between ppGpp and the target proteins. When ppGpp was docked into the GTPase active site, the guanosine base, ribose ring and 5′-phosphates occupy very similar positions to the natural substrate/product (GTP/GDP), while the 3′-phosphates tend to point away from the active site and, hence, away from the surface of the protein (Fig. S1). The 3′-phosphates generally do not make significant contacts with the protein, but where they do, they tend to be coordinated most often by residues from the X_3 position of the G_1 motif (mostly acidic or polar residues) (see Fig. 2B) and/or from G_3 loop residues, as well as, from residues outside of the conserved GTPase motifs (see Table S3). The position of the 3′-phosphates directed away from the protein surface may sterically interfere with the binding of GEF and GNRF proteins that help to regulate GTPase activity.

ppGpp binding may also result in competitive inhibition with GTP/GDP. A comparison of the reported K_i values for ppGpp and K_d or K_M values for GTP/GDP for the various GTPases (Table 2) indicates that all of the guanosine nucleotides bind with a similar range of affinities, mostly in the low micromolar to nanomolar range. It is significant that, where data are available for the same enzyme, ppGpp does not bind comparatively better than GTP/GDP. This suggests that any inhibitory effect on GTPase activity would be easily reversible and would likely only be significant when the intracellular concentration of ppGpp is very high, such as during the peak of the stringent response where ppGpp concentrations may reach millimolar amounts (Cashel *et al*., 1996). This is important as many of the GTPases are essential for cell growth (Caldon and March, 2003; Brown, 2005; Margus *et al*., 2007) and a reversible inhibition by ppGpp, resulting in a transient reduction in protein translation and translocation, increased translation fidelity and reduced cell division rates, would serve to complement the transcriptional effects of the stringent response that contribute to the shift from exponential to stationary phase growth.

Nucleotide and lipid metabolism

During the stringent response one of the major effects is the downregulation of genes involved in nucleotide and lipid biosynthesis (Table 1) (Cashel *et al*., 1996; Traxler *et al*., 2008). In addition, there are direct effects of ppGpp on a number of enzymes involved in nucleotide and lipid metabolism, which are listed schematically in Fig. 3. These enzymes belong to different functional classes and are, therefore, likely to interact with ppGpp via different mechanisms. At present there are no co-crystal structures available for any of these enzymes bound to ppGpp, so a docking approach was used in order to gain some insights into these interactions. A common theme revealed by the docking experiments is the potential for ppGpp to bind at a nucleotide/nucleotide analogue binding site and to potentially act as a competitive inhibitor.

DNA primase (DnaG) is a multi-domain enzyme that is a component of the replisome and interacts with the DnaB helicase, single stranded binding protein, and DNA polymerase III. Direct inhibition of DnaG by pppGpp was first observed in *B. subtilis* where pppGpp was found to be more inhibitory than ppGpp (Table 2) (Wang *et al*., 2007). A subsequent study of *E. coli* DnaG indicated that, in the presence of DnaB, ppGpp was a more potent inhibitor than pppGpp (Table 2) (Maciag *et al*., 2010). DnaG was inhibited by concentrations of (p)ppGpp in the 0.2–1.0 mM range, which are readily reached during the stringent response (Cashel *et al*., 1996; Buckstein *et al*., 2007; Traxler *et al*., 2008). ppGpp was successfully docked into the proposed nucleotide binding site (Keck *et al*., 2000) (Fig. S2A) at the interface between the α/β and topoisomerase/primase domains (Fig. 3) and, thus, may function by competitive inhibition. Inhibition of DNA primase would serve to strongly reduce the rate of *de novo* DNA synthesis and, hence, of cell division.

The nucleotide pyrophosphohydrolase MazG functions in regulating programmed cell death in *E. coli* and is negatively regulated by the MazEF toxin-antitoxin system (Lee *et al*., 2008). MazG has low levels of ppGpp pyrophosphohydrolase activity *in vitro*, but this activity is insufficient to complement a deletion of *spoT*, which causes a toxic accumulation of ppGpp (Xiao *et al*., 1991). ppGpp was docked at the ATP binding pocket of MazG (Fig. S2B).

The first step in the guanosine nucleotide *de novo* biosynthesis pathway is catalysed by inosine monophosphate dehydrogenase (GuaB). GuaB activity is inhibited by ppGpp with a K_i of ~ 50 μM, indicating that GuaB will be efficiently inhibited during the stringent response (Table 2) (Gallant *et al*., 1971; Gilbert *et al*., 1979; Pao and Dyess, 1981). Inhibition of GuaB results in decreased pools of GTP and this has been implicated in mediating effects of the stringent response including induction of sporulation in *B. subtilis* (Ochi *et al*., 1982) and modulating RNAP activity in *T. thermophilus* (Kasai *et al*., 2006). A homologous enzyme guanosine-5′-monophosphate dehydrogenase (GuaC), which is involved in the purine salvage pathway, may also be a target of (p)ppGpp as the enzymes share a common TIM-barrel fold (Andrews and Guest, 1988). (p)ppGpp may act as a competitive inhibitor by binding to the guanosine binding site present in these

Domain Structure	Function	Effect of (p)ppGpp
Nucleotide Metabolism		
DnaG (ZBD, α/β, TOPRIM, 3HB, DBD; 1, 111, 240, 368, 428, 582)	DNA Primase	**Inhibited**
MazG (NTD, CTD; 1, 123, 141, 263)	NTP pyrophosphohydrolase	**low ppGpp degradation activity**
GuaB (CBS, TIM; 1, 89, 215, 488)	Inosine-5′-monophosphate dehydrogenase	**Inhibited**
GuaC (TIM; 1, 347)	Guanosine-5′-monophosphate dehydrogenase	(Inhibited)
Gpt (PRT-I; 1, 152)	Xanthine-guanine phosphoribosyltransferase	**Inhibited**
Apt (PRT-I; 1, 183)	Adenine phosphoribosyltransferase	**Inhibited**
Upp (PRT-I; 1, 208)	Uracil phosphoribosyltransferase	**Inhibited**
Hpt (PRT-I; 1, 178)	Hypoxantine phosphoribosyltransferase	**Inhibited**
PyrE (PRT-I; 1, 213)	Orotate phosphoribosyltransferase	(Inhibited)
PurF (GPATase, PRT-I; 1, 268, 278, 394, 505)	Glutamine phosphoribosylpyrophosphate amidotransferase	(Inhibited)
HisG (PBP-a, PBP-b, FDX; 1, 104, 191, 225, 299)	ATP phosphoribosyltransferase	**Inhibited**
Lipid Metabolism		
PlsB (LPLAT; 1, 279, 480, 807)	Glycerol-3-phosphate acyltransferase	**Inhibited**
PgsA (CDP-OH; 1, 182)	Phosphatidylglycerophosphate synthase	**Inhibited**
YnjF (CDP-OH; 1, 206)	Inner membrane protein	(Inhibited)
AccA (α/β; 1, 109, 228, 319)	Acetyl-CoA carboxylase carboxytransferase α-subunit	**Inhibited**
AccD (ZBD, α/β; 1, 55, 116, 228, 304)	Acetyl-CoA carboxylase carboxytransferase β-subunit	**Inhibited**
FabA (α+β; 1, 28, 172)	β-hydroxyacyl-ACP dehydratase	**Inhibited**
FabZ (α+β; 1, 17, 151)	β-hydroxyacyl-ACP dehydratase	**Inhibited**
Other Enzymes		
GdhA (DI, DII; 1, 211, 423, 447)	Glutamate dehydrogenase	**Inhibited**
GlgC (ADP-G-PP, AT; 1, 328, 431)	Glucose-1-phosphate adenylyltransferase	**Inhibited**
Ppc (PEPC; 1, 883)	Phosphoenolpyruvate carboxylase	**Activated**

Fig. 3. Known and potential targets of direct (p)ppGpp action are involved in nucleotide and lipid metabolism. Domain organization of proteins involved in nucleotide and lipid metabolism that are either known to interact with ppGpp (labelled purple) or based on sequence homology are potential targets of ppGpp. For each protein, two columns of additional information are provided: (i) the general cellular function and (ii) the effect of (p)ppGpp on its activity. Proteins with demonstrated inhibition and activation in the presence of (p)ppGpp are indicated in bold and those with inferred inhibition are shown in brackets. The following is a list of the UniProt (UniProt Consortium, 2011) accession numbers in parentheses for each protein followed where applicable by the full domain name(s). DnaG (P0ABS5): ZBD – zinc binding domain, α/β subdomain, TOPRIM – topoisomerase/primase, 3HB – 3-helix bundle, DBD – DnaB-interacting domain; MazG (P0AEY3) NTD – N-terminal domain, CTD – C-terminal domain; GuaB (P0ADG7) TIM-barrel catalytic domain, CBS – tandem cystathione β-synthase domain, GuaC (P60560) TIM-barrel catalytic domain. The following proteins contain a type I phosphoribosyltransferase domain (PRT-I): Gpt (P0A9M5), Apt (P69503), Upp (P0A8F0), Hpt (P0A9M2), PyrE (P0A7E3), and PurF (P0AG16). PurF also has glutamine phosphoribosylpyrophosphate amidotransferase (GPATase) domain. HisG (P60757) contains a type IV phosphoribosyltransferase domain that consists of PBP-a, PBP-b – periplasmic binding protein domains and FDX – ferredoxin-like domain; PlsB (P0A7A7) LPLAT – lysophospholipid acyltransferase of glycerophospholipid biosynthesis; PgsA (P0ABF8) CDP-OH – cytosine diphosphate-alcohol phosphatidyltransferase; YnjF (P76226) CDP-OH – cytosine diphosphate-alcohol phosphatidyltransferase; AccA (P0ABD5) α/β spiral domain; AccD (P0A9Q5) ZBD – zinc binding domain, α/β spiral domain; FabA (P0A6Q3) α + β hot-dog domain; FabZ (P0A6Q6) α + β hot-dog domain; GdhA (P00370) DI – domain I, DII – domain II; GlgC (P0A6V1) ADP-G-PP – ADP-glucose-pyrophosphorylase domain, AT – glucose-1-phosphate adenylyltransferase; Ppc (P00864) PEPC – phosphoenolpyruvate carboxylase domain.

enzymes, which is observed in the docked models of ppGpp with GuaB (Fig. S2C) and GuaC (Fig. S2D).

A number of phosphoribosyltransferases (PRTases) have been implicated in the stringent response. There are four major categories of PRTases: class I to IV (Lohkamp *et al.*, 2004). Class I PRTases (PRT-I) contain a unique conserved motif [VL(IVL)VDDX$_4$G] that is involved in binding to phosphoribosylpyrophosphate (PRPP). Four PRT-I enzymes involved in purine and pyrimidine salvage pathways: xanthine-guanine PRTase (Gpt), adenine

PRTase (Apt), uracil PRTase (Upp) and hypoxanthine PRTase (Hpt) are inhibited by (p)ppGpp (Hochstadt-Ozer and Cashel, 1972; Fast and Skold, 1977; Morton and Parsons, 1977). There are two other *E. coli* PRT-I enzymes that may also be inhibited by (p)ppGpp: orotate PRTase (PyrE), and glutamine phosphoribosylpyrophosphate amidotransferase (PurF). We have docked ppGpp at the active sites of the various PRT-I enzymes (Fig. S2E–J) and the alarmone seems to adopt a conformation similar to the expected transition state intermediate formed between PRPP and the purine/pyrimidine base (see Fig. S3). The K_M values for the various substrates and PRPP for these enzymes are generally within the low to moderate micromolar range and where available, measures of ppGpp binding are either of similar or lower affinity to the *bona fide* substrates (Table 2). The class IV PRTase HisG (ATP PRTase), which catalyses the first step in histidine biosynthesis, is also inhibited by the activity of (p)ppGpp (Morton and Parsons, 1977). This protein has a different domain architecture from the PRT-I enzymes (Fig. 3) but does contain PRPP and ATP binding sites either of which may be possible targets for (p)ppGpp binding and inhibition (Fig. S2K). There is both *in vivo* (Barker *et al.*, 2001a) and *in vitro* (Paul *et al.*, 2005) evidence that the P*hisG* promoter is upregulated by (p)ppGpp and DksA during the stringent response, so it is important to determine the extent of (p)ppGpp-based inhibition of this enzyme.

The enzyme responsible for the first step of lipid biosynthesis is the membrane-bound glycerol-3-phosphate acyltransfersae (PlsB) (Fig. 3). PlsB activity is directly inhibited by low millimolar quantities of (p)ppGpp (Merlie and Pizer, 1973; Heath *et al.*, 1994). As there is no current structure for this integral membrane protein, predicting the site of action of (p)ppGpp is not currently feasible. A down-stream enzyme that catalyses the first step of phospholipid biosynthesis is phosphatidylglycerophosphate synthase (PgsA) (Fig. 3) and this membrane protein is similarly inhibited by (p)ppGpp (Merlie and Pizer, 1973). A homologous predicted membrane protein YnjF (Fig. 3) is also present in *E. coli* and while the function of this protein is currently not known with certainty, it is expected to function as a phosphatidyl transferase and may also be inhibited by (p)ppGpp.

A key enzyme in the bacterial type-II fatty acid biosynthesis (FAS-II) pathway is the acetyl-CoA carboxylase complex that consists of three separate protein complexes: the dimeric biotinoyl carboxyl carrier protein (BCCP) $(AccB)_2$; dimeric biotin carboxylase (BC) $(AccC)_2$ and the heterotetrameric acetyl-CoA carboxytransferase (CT) $(AccA)_2(AccD)_2$ (Bilder *et al.*, 2006). BC catalyses the ATP-dependent addition of HCO_3^- to BCCP-biotin, generating BCCP-biotin-CO_2. The carbonyl group is subsequently transferred to acetyl-CoA to generate malonyl-CoA via CT. The AccA and AccD proteins that make up the CT complex are homologous and the CT complex is inhibited by low millimolar concentrations of (p)ppGpp (Polakis *et al.*, 1973). Two other FAS-II enzymes involved in the formation of unsaturated fatty acids are also inhibited by low millimolar concentrations (p)ppGpp (Stein and Bloch, 1976): the homologous FabA and FabZ proteins that contain a unique $\alpha + \beta$ hot-dog topology and catalyse β-hydroxyacyl-ACP dehydratase reactions (Leesong *et al.*, 1996).

A number of other metabolic enzymes are also regulated by (p)ppGpp and include the $NADP^+$-dependent glutamate dehydrogenase (GdhA) that is inhibited by (p)ppGpp (Maurizi and Rasulova, 2002), the first enzyme in the glycogen biosynthesis pathway glucose-1-phosphate adenylyltransferase (GlgC) that is inhibited by low millimolar concentrations of (p)ppGpp (Dietzler and Leckie, 1977), and the metabolic enzyme phosphoenolpyruvate carboxylase (Ppc) that, uniquely among the enzymes considered, is activated by (p)ppGpp (Pao and Dyess, 1981). Interestingly, GlgC transcription is activated by ppGpp (Romeo and Preiss, 1989) and translation is blocked through the action of the carbon storage regulator CsrA (Romeo *et al.*, 1993), suggesting a complex regulatory network and potentially opposing effects of ppGpp for this enzyme.

Docking of ppGpp to AccA, FabA, GdhA, GlgC and Ppc was performed (Fig. S4A–E) but the docking procedure was more challenging in several of the cases (AccA, FabA, Ppc) where a suitable substrate on which to model ppGpp was unavailable. In order to deal with this, the *DOCK6* (Lang *et al.*, 2009) software package was used to obtain suitable docking conformations. In addition, all the enzymes are from different structural classes, thus further complicating a search for a common mechanism of action of ppGpp. More accurate measurements of the actual affinity of ppGpp for these enzymes would be necessary to determine the extent of direct inhibition during the stringent response.

Basic aliphatic amino acid decarboxylases

Escherichia coli possesses five enzymes belonging to the prokaryotic ornithine decarboxylase (pODC) subclass of Fold Type I pyridoxal-5′-phosphate (PLP)-dependent decarboxylases: inducible lysine decarboxylase (LdcI), constitutive lysine decarboxylase (LdcC), inducible arginine decarboxylase (AdiA), inducible ornithine decarboxylase (SpeF) and constitutive ornithine decarboxylase (SpeC) (Fig. 4A) (Kanjee *et al.*, 2011a). These multidomain enzymes form large oligomeric complexes consisting of dimers (SpeF, SpeC) or decamers (LdcI, LdcC, AdiA). The inducible enzymes are involved in the acid stress response while the constitutive enzymes, particularly SpeC, are important in polyamine production.

In the recently determined X-ray crystal structure of LdcI by our group, the enzyme was found to bind ppGpp

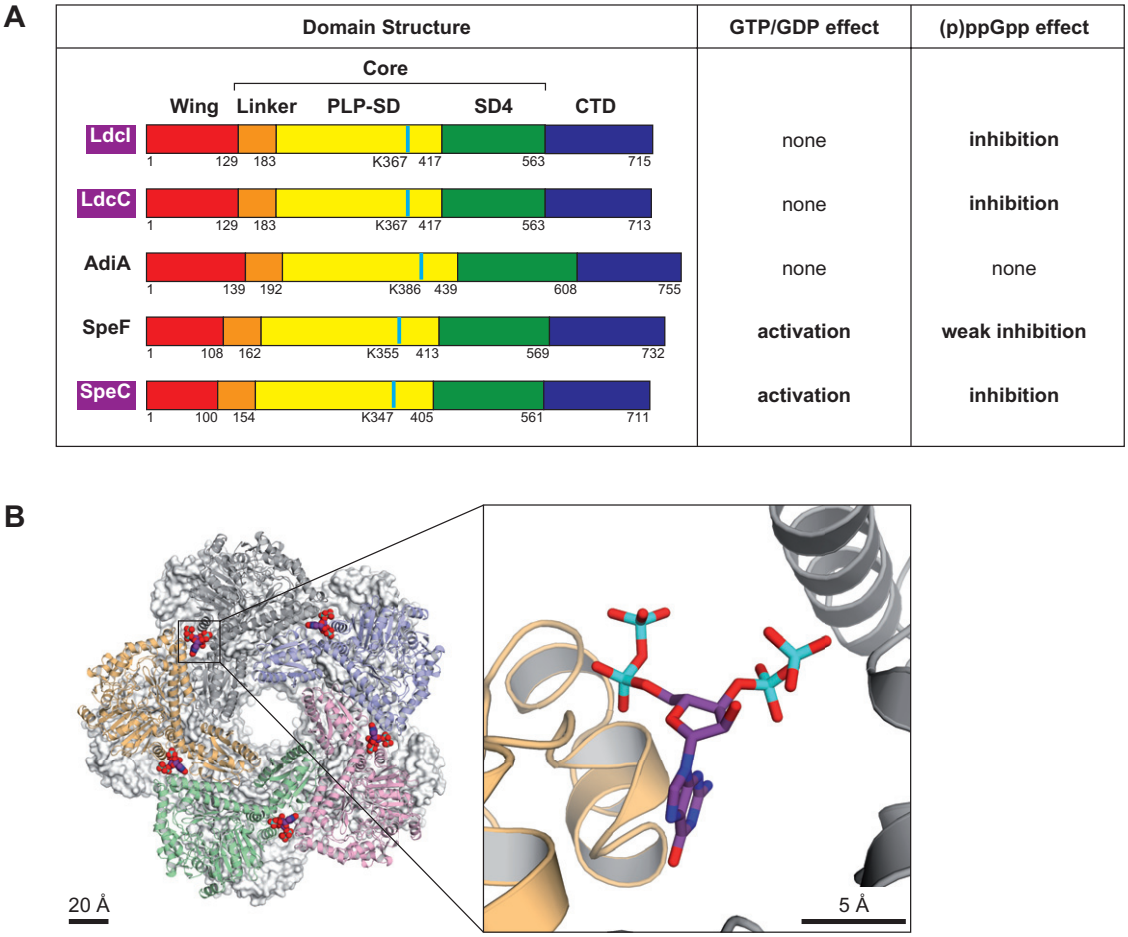

Fig. 4. Regulation of lysine and ornithine decarboxylases by (p)ppGpp.
A. The domain organization of the five related PLP-dependent basic aliphatic amino acid decarboxylases is shown along with the effects of GTP/GPD and (p)ppGpp on enzyme activities (Kanjee *et al.*, 2011a). Each of the decarboxylases shares a common domain architecture consisting of: an N-terminal Wing domain; a Core domain made up of a short α-helical linker, a PLP binding subdomain (PLP-SD) and a subdomain four/aspartate aminotransferase small domain (SD4); and a C-terminal domain (CTD). The following is a list of the UniProt (UniProt Consortium, 2011) accession numbers in parentheses for each protein: LdcI (P0A9H3), LdcC (P52095), AdiA (P28629), SpeF (P24169) and SpeC (P21169).
B. X-ray crystal structure of the *E. coli* LdcI decamer (PDB ID: 3N75) (Kanjee *et al.*, 2011b) with each monomer in the top ring highlighted in a different colour and shown as a cartoon. The bottom ring monomers are shown in surface representation. The five ppGpp molecules that interact with the top ring are indicated. The insert shows a close-up of one of the ppGpp binding sites. The guanosine nucleotide is shown as a stick figure with oxygen atoms coloured red, nitrogen atoms coloured blue, phosphorous atoms coloured cyan, and carbon atoms coloured purple.

with high affinity (Table 2) at specific sites between neighbouring monomers in the LdcI decamer (Fig. 4B) (Kanjee *et al.*, 2011b). Furthermore, it was found that LdcI activity was specifically inhibited by ppGpp and pppGpp over a range of pH values *in vitro* and *in vivo*. Of the related *E. coli* decarboxylases, it was found that LdcC was similarly inhibited by (p)ppGpp (Kanjee *et al.*, 2011a). SpeF and SpeC were both activated by GTP and GDP, while SpeC was inhibited by (p)ppGpp. The arginine decarboxylase AdiA was unaffected by any of the guanosine nucleotides (Fig. 4A). Inhibition of these decarboxylases by ppGpp likely results in the conservation of amino acids when the stringent response is activated under acid stress conditions and, thus, serves as an additional means of regulating decarboxylation activity (Kanjee *et al.*, 2011a). Further experimentation is required to elucidate the exact mechanism of inhibition by (p)ppGpp and to elucidate the wider effects this inhibition has on cellular adaptation to acid stress.

Conclusion

DNA microarray experiments comparing wild-type and either Δ*relA* or ppGpp^0 strains have extended our understanding of the global transcriptional changes that take place upon the induction of the stringent response (Chang

et al., 2002; Traxler *et al.*, 2006; 2008; 2011; Durfee *et al.*, 2008). Direct interaction of proteins with ppGpp provides a central regulatory framework for many different types of processes, and this is exemplified by the transcriptional effects of ppGpp (and DksA) on RNAP. These transcriptional effects serve to manage a core set of genes that are involved in the shift between exponential phase growth conditions and stationary phase stress response conditions (Nystrom, 2004).

Based on the reported inhibition constants for ppGpp (Table 2) it is likely that, in the majority of cases, inhibition by ppGpp is transient, reversible and dependent on the high concentrations of ppGpp reached during the peak of the stringent response. The potential consequences on cell physiology of inhibition of these proteins by ppGpp are likely to be complex but may serve to complement the transcriptional effects of ppGpp on RNAP. Inhibition of the cellular GTPases may result in an overall decrease in protein translation and cell growth rates. Inhibition of enzymes involved in nucleotide and lipid metabolism is also consistent with reduction in the cell division rates as there is a reduced demand for producing nucleotides for DNA replication and stable RNA transcription and lower need for lipids to form new membranes. Similarly inhibition by ppGpp of certain metabolic enzymes and the amino acid decarboxylases would serve to conserve nutrients and amino acids during conditions of nutrient deprivation. Direct targeting of enzymes by (p)ppGpp may have evolved as a mechanism to specifically extend stringent control to and enable a rapid and reversible control of metabolic and stress response processes and help to fine-tune the effects of the stringent response.

In order to identify the direct targets of ppGpp, we have used reports from the existing literature as well as bioinformatic approaches (sequence alignments and *in silico* docking) in order to compile a list of proteins that are known or speculated to be regulated by the alarmone. While we have made every attempt to be rigorous in our analysis and selection of protein targets, we cannot be certain that all of these proteins are *bona fide* targets. As such, further structural and biochemical investigations into the known and proposed enzymes targeted by (p)ppGpp are essential to more completely define the role of this unusual nucleotide in regulating the stringent response.

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Supporting information

Additional supporting information may be found in the online version of this article.

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Supplementary Table 1. Protein Data Bank (PDB) files used for *in silico* docking.

Protein	PDB ID	Source organism	Reference
GTPases			
CysN	1ZUN	*Pseudomonas aeruginosa*	(Mougous *et al.*, 2006)
EFG	1DAR	*Thermus thermophilus*	(al-Karadaghi *et al.*, 1996)
LepA	3CB4	*E. coli*	(Evans *et al.*, 2008)
EF-Tu	1EFC	*E. coli*	(Song *et al.*, 1999)
RF3	2H5E	*E. coli*	(Gao *et al.*, 2007)
Der	1MKY	*Thermotoga maritime*	(Robinson *et al.*, 2002)
EngB	1PUI	*E. coli*	(Kniewel *et al.*)
EngD	1JAL	*Haemophilus influenza*	(Teplyakov *et al.*, 2003)
Era	3IEU	*E. coli*	(Tu *et al.*, 2009)
FeoB	3HYT	*E. coli*	(Guilfoyle *et al.*, 2009)
MnmE/TrmE	2GJ8	*E. coli*	(Scrima & Wittinghofer, 2006)
Obg	1LNZ	*B. subtilis*	(Buglino *et al.*, 2002)
RsgA	2RCN	*S. enterica*	(Nichols *et al.*, 2007)
FtsY	2XXA	*E. coli*	(Ataide *et al.*, 2011)
Ffh	2XXA	*E. coli*	(Ataide et al., 2011)
PurA	1CIB	*E. coli*	(Hou *et al.*, 1999)
Nucleotide Metabolism Proteins			
DnaG	1DD9	*E. coli*	(Keck *et al.*, 2000)
MazG	3CRA	*E. coli*	(Lee *et al.*, 2008)
GuaB	3TSD	*Bacillus anthracis*	(Kim *et al.*)
GuaC	2A7R	*Homo sapiens*	(Li *et al.*, 2006)
Gpt	1A95	*E. coli*	(Vos *et al.*, 1998)
Apt	2DY0	*E. coli*	(Shimizu)
Upp	2EHJ	*E. coli*	(Lokanath *et al.*)
Hpt	1G9T	*E. coli*	(Guddat *et al.*, 2002)
PyrE	1ORO	*E. coli*	(Henriksen *et al.*, 1996)
PurF	1ECB	*E. coli*	(Krahn *et al.*, 1997)
HisG	1Q1K	*E. coli*	(Lohkamp *et al.*, 2004)
Lipid Metabolism Proteins			
AccA	2F9Y	*E. coli*	(Bilder *et al.*, 2006)
FabA	1MKA	*E. coli*	(Leesong *et al.*, 1996)
GdhA	1BGV	*Clostridium symbiosum*	(Stillman *et al.*, 1993)
GlgC	3BRK	*Agrobacter tumefasciens*	(Cupp-Vickery *et al.*, 2008)
Ppc	1FIY	*E. coli*	(Kai *et al.*, 1999)

Supplementary Table 2. Calculation of ppGpp binding energy in docking experiments

The binding free energy (ΔG_{bind}) between a ligand and a receptor to form a complex is calculated using molecular mechanics Poisson−Boltzmann surface area (MMPBSA) and molecular mechanics Generalized Born surface area (MMGBSA) methods (Srinivasan *et al.*, 1998), as follows:

$$\Delta G_{bind} = \Delta E_{MM} + \Delta G_{solv} - T\Delta S$$

where ΔE_{MM}, ΔG_{solv} and $-T\Delta S$ are MM energy, the solvation free energy and the conformational entropy, respectively. To calculate ΔE_{MM} and ΔG_{solv}, 2 ns MD simulation was performed with explicit water molecules and 200 snapshots were extracted from stored structures every 10 ps during the simulation. Normal-mode analysis was applied to calculate the conformational entropy from 20 snapshots extracted from structures at every 100 ps during the simulation. ΔG_{bind} value indicates the binding capability to target proteins. Therefore, if ΔG_{bind} value is negative, binding of ppGpp to target proteins is favorable. All the complexes show negative ΔG_{bind} values, and the target proteins are, hence, likely to form a complex with ppGpp.

	ΔG (kcal/mol)	
Protein	**ΔG_{mmgbsa}**	**ΔG_{mmpbsa}**
GTPases		
CysN	-79.2	-104.2
EFG	-85.9	-108.9
LepA	-19.2	-34.3
EF-Tu	-95.2	-101.4
RF3	-76.1	-92.5
Der	-191.6	-224.2
EngB	-41.7	-67.3
EngD	-49.9	-61.1
Era	-82.6	-100.0

FeoB	-86.0	-85.4
MnmE/TrmE	-89.5	-89.6
Obg	-76.7	-83.5
RsgA	-69.9	-85.0
FtsY	-133.7	-140.5
Ffh	-11.0	-10.4
PurA	-63.9	-51.7
Nucleotide Metabolism Proteins		
DnaG	-16.7	-36.8
MazG	-75.4	-85.3
GuaB	-69.8	-91.8
GuaC	-8.6	-4.3
Gpt	-106.8	-115.1
Apt	-69.3	-81.2
Upp	-95.4	-122.9
Hpt	-100.5	-119.8
PyrE	-38.4	-48.1
PurF	-32.1	-60.9
HisG	-3.1	-11.4
Lipid Metabolism Proteins		
AccAD	-21.5	-36.6
FabA	-19.3	-46.4
GdhA	-23.5	-36.1
GlgC	1.4	-17.3
Ppc	-123.3	-143.4

Supplementary Table 3. Amino acids involved in binding ppGpp from docked models.

This table lists the amino acid residues found within a 5 Å distance of the docked ppGpp molecule. Residues have been color coded as follows: green indicates binding to the guanosine base, pink indicates binding to the ribose ring, cyan indicates binding to the 5′-phosphates, and yellow indicates binding to the 3′-phosphates.

Supplementary Figure 1. *In silico* Docking of ppGpp to GTPases

To perform the docking, the target proteins were identified either from the wild type *E. coli* X-ray crystal structures or from homologous proteins in the PDB (Berman *et al.*, 2000) (see Supplementary Table 1). The *SWISSMODEL* web service (Kiefer *et al.*, 2009) was used to insert or delete loop structures where appropriate and in the case of homologous models, the amino acid sequence was modified to match the *E. coli* protein sequence. Where GTP/GDP or substrate analogues were present in the crystal structures, these molecules were used as templates for positioning ppGpp. To do this, the guanosine base, ribose ring and phosphate groups of ppGpp were superimposed on the equivalent purine/pyrimidine base, ribose sugar moiety and phosphoric acid positions of the templates. To eliminate steric clashes and to relax the docked model, a molecular dynamics (MD) simulation was performed with explicit water molecules using the *AMBER* package (Weiner *et al.*, 1986). Prior to the MD simulation, the ligand/protein complexes were optimized for 10,000 steps with a conjugate gradient method and then the system was heated to 300 K for 50 ps with protein and ligand constraints. After the optimization and heating procedures, a 600 ps MD simulation in the canonical ensemble (NPT) at 300 K controlled by the Langevin thermostat was performed. A snapshot at 600 ps of MD simulation was optimized for a 2,000 step conjugate gradient method *in vacuo*. A further binding free-energy calculation was performed between the ppGpp and the target protein and the negative ΔG values indicated thermodynamically favourable binding conditions (Supplementary Table 2). A snap shot at the end of the simulation was used to analyze the contact residues with ppGpp in the target proteins (see Supplementary Table 3). In all cases, the cartoon models are colored as in Figure 2C-F. Structure cartoons were generated in *PYMOL* (DeLano, 2002). Scale bar represents 5 Å.

Supplementary Figure 2. *In silico* Docking of ppGpp to Nucleotide Metabolism Proteins

Docking of ppGpp to the nucleotide metabolism proteins was performed as described in Supplementary Figure 1 with the following modification. Where nucleotide ligand groups were not available as templates for docking, the *DOCK6* (Lang *et al.*, 2009) software package was used to obtain suitable docking conformations. Structure cartoons were generated in *PYMOL* (DeLano, 2002) and coloured as for Figure 3. Scale bar represents 5 Å. A list of the ΔG binding energies is available in Supplementary Table 2 and a list of ppGpp contact residues is available in Supplementary Table 3.

Supplementary Figure 3. ppGpp, substrate and product binding to Gpt

(A) The docked model of ppGpp bound at the active site of *E. coli* Gpt is shown. For comparison also shown are the X-ray crystal structures of *E. coli* Gpt bound to: (B) CPRPP (PRPP analogue carboxylic PRPP) and guanine from PDB ID: 1A95 (Vos et al., 1998); (C) CPRPP and xanthine from PDB ID: 1A96 (Vos et al., 1998); and (D) guanosine 5′-monophosphate (GMP) (Vos et al., 1998).

Supplementary Figure 4. *In silico* Docking of ppGpp to Lipid and General Metabolism Proteins

Docking of ppGpp to the lipid and general metabolism proteins was performed as described in

Supplementary Figure 2. Structure cartoons were generated in *PYMOL* (DeLano, 2002) and coloured as for Figure 3. Scale bar represents 5 Å. A list of the ΔG binding energies is available in Supplementary Table 2 and a list of ppGpp contact residues is available in Supplementary Table 3.

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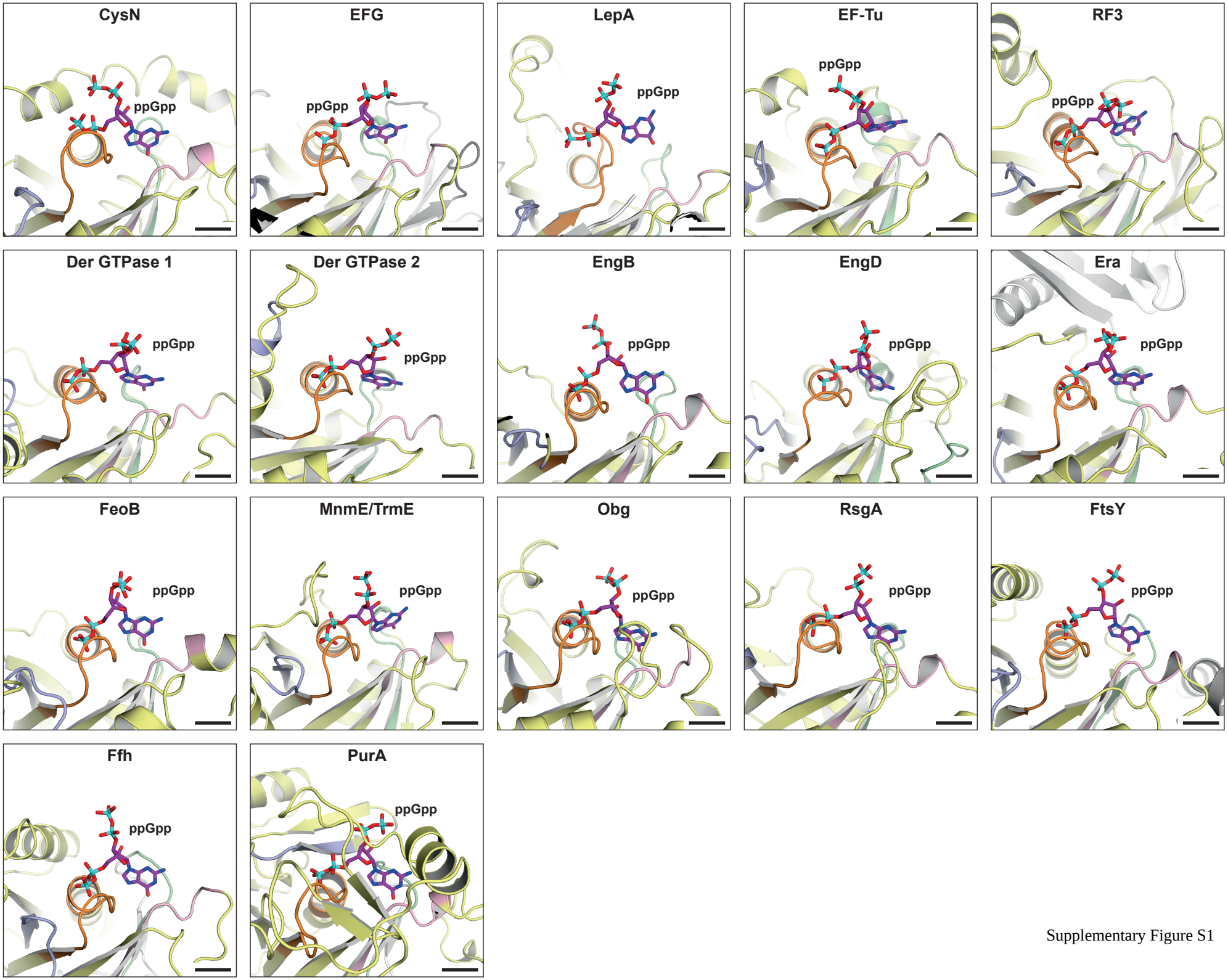
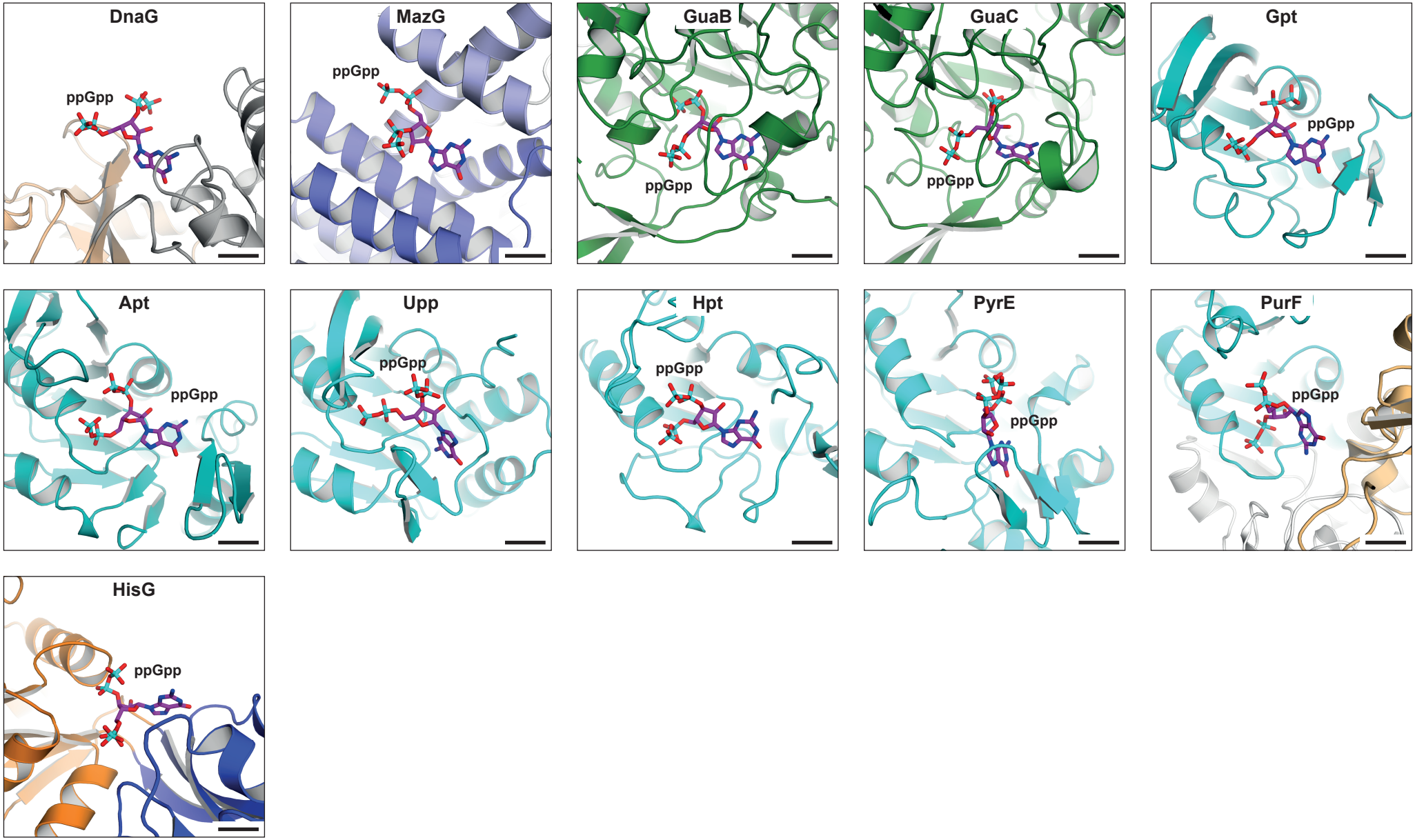


Supplementary Figure S1



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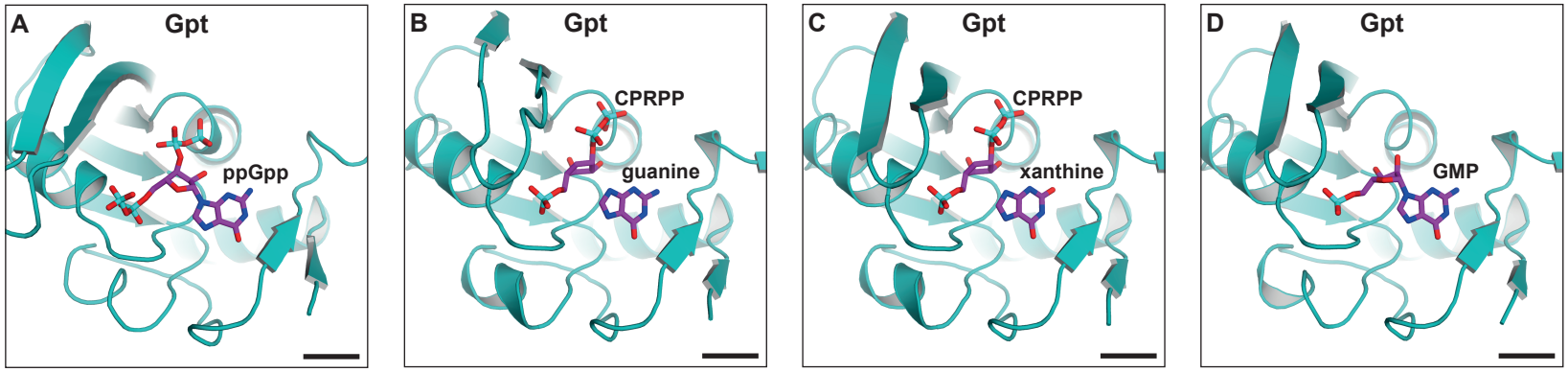

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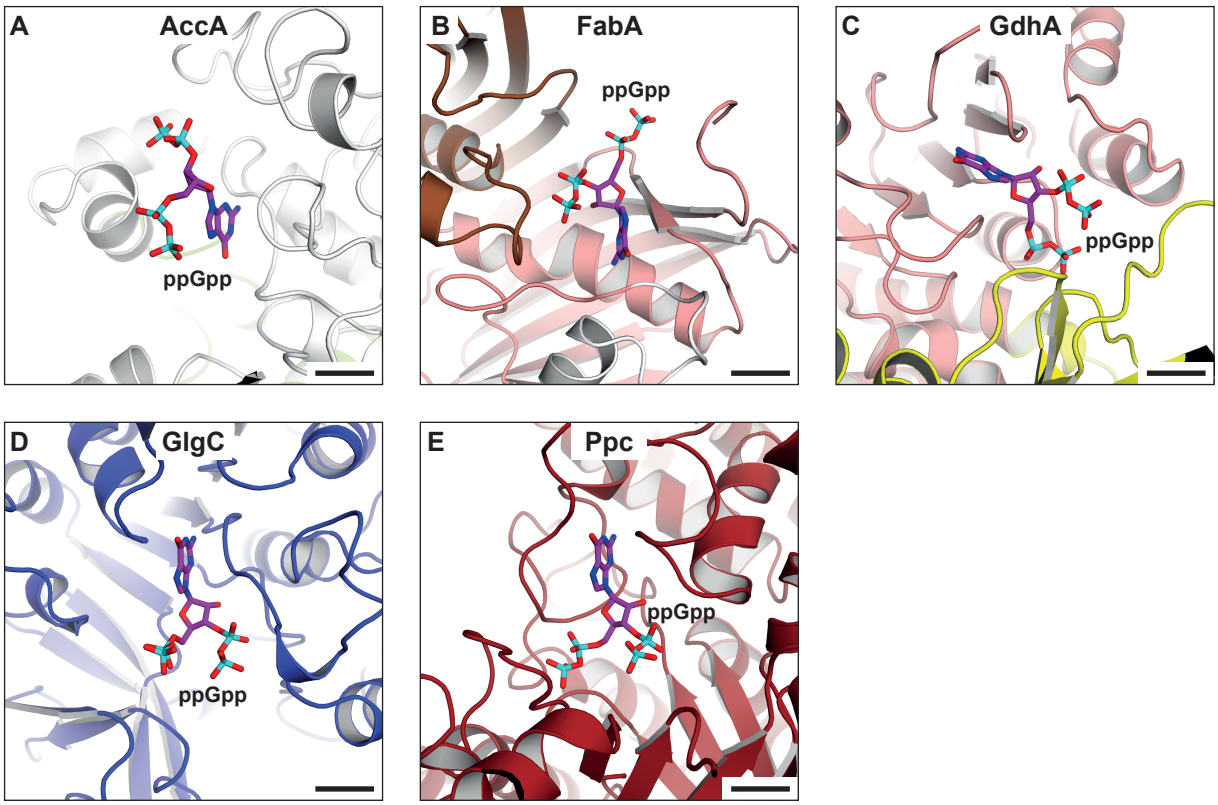

Supplementary Figure S4