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The RelA hydrolase domain acts as a molecular switch for (p)ppGpp synthesis

Anurag Kumar Sinha & Kristoffer Skovbo Winther

Bacteria synthesize guanosine tetra- and penta phosphate (commonly referred to as (p)ppGpp) in response to environmental stresses. (p)ppGpp reprograms cell physiology and is essential for stress survival, virulence and antibiotic tolerance. Proteins of the RSH superfamily (RelA/SpoT Homologues) are ubiquitously distributed and hydrolyze or synthesize (p)ppGpp. Structural studies have suggested that the shift between hydrolysis and synthesis is governed by conformational antagonism between the two active sites in RSHs. RelA proteins of γ-proteobacteria exclusively synthesize (p)ppGpp and encode an inactive pseudo-hydrolase domain. Escherichia coli RelA synthesizes (p)ppGpp in response to amino acid starvation with cognate uncharged tRNA at the ribosomal A-site, however, mechanistic details to the regulation of the enzymatic activity remain elusive. Here, we show a role of the enzymatically inactive hydrolase domain in modulating the activity of the synthetase domain of RelA. Using mutagenesis screening and functional studies, we identify a loop region (residues 114–130) in the hydrolase domain, which controls the synthetase activity. We show that a synthetase-inactive loop mutant of RelA is not affected for tRNA binding, but binds the ribosome less efficiently than wild type RelA. Our data support the model that the hydrolase domain acts as a molecular switch to regulate the synthetase activity.
Bacteria have evolved intricate mechanisms and responses to adapt quickly to changing and stressful environments. One of such bacterial responses is the universal stringent response. The stringent response is induced in response to amino acid starvation\(^1\), fatty acid limitation\(^1\), iron limitation\(^3\), heat shock\(^6\), glucose starvation\(^7\), nitrogen starvation\(^8\), phosphate starvation\(^9\) and other stress conditions\(^10\). The stringent response reprograms cell physiology, which facilitates stress adaptation and survival under harsh environmental conditions\(^11\). Furthermore, the stringent response is essential for virulence and has been shown to mediate antibiotic tolerance\(^12,13\). Derivatives of GDP/GTP, guanosine tetra- and pentaphosphate (collectively referred to as (p)ppGpp or alarmones), are the effector molecules of the stringent response and are synthesized/hydrolyzed by the RSH superfamily (RelA/SpoT homologues) proteins. The most commonly distributed protein of this family is the bifunctional Rel protein, which has both (p)ppGpp synthetase and hydrolase activities.

In γ-proteobacteria such as in Escherichia coli, the rel gene has been duplicated to form relA and spoT\(^14\). The RelA protein has only (p)ppGpp synthetase activity but carries an inactive pseudo-hydrolase domain, whereas, SpoT is a weak (p)ppGpp synthetase and exhibits strong hydrolase activity\(^11\). Hence SpoT is essential for cell growth unless the RelA synthetase function is compromised, as SpoT is necessary for (p)ppGpp hydrolysis\(^15\). Weak SpoT-dependent (p)ppGpp synthesis has been reported under multiple starvation conditions; however, RelA exclusively synthesizes (p)ppGpp in response to amino acid starvation\(^10,11\). These metabolic cues are not mutually exclusive and accumulating evidence suggest that diverse starvation signals including glucose and fatty acid starvation can indirectly lead to conditions that trigger the RelA-dependent stringent response\(^47,16,17\).

RelA activation occurs when RelA binds with an uncharged tRNA at an empty A-site of a stalled ribosome, which leads to induction of (p)ppGpp synthesis\(^18\). Cryo-EM structures of RelA in complex with uncharged tRNA and the ribosome have revealed that the C-terminal Zinc-finger domain (ZFD) and RNA recognition motif (RRM) of RelA are responsible for ribosome binding at helix 38, the A-site finger of 23 S ribosomal RNA in the 50 S ribosomal subunit (Fig. 1a). The C-terminal TGS domain (ThrRS, GTPase, SpoT/RelA) is primarily involved in the recognition and binding to uncharged tRNA\(^19-21\). All three domains enclose the A-site tRNA, and expose the N-terminal synthetase (SYN) and inactive hydrolase (pseudo-HD) domains on the surface of the ribosome. Recently, it was demonstrated using an in vivo UV crosslinking and analysis of cDNAs (CRAC) approach that RelA interacts with the ribosome as a RelA•tRNA complex\(^6\). RelA is thought to bind with tRNA at ribosomal A-sites during amino acid starvation, when EF-Tu•GTP•tRNA ternary complexes are scarce\(^18\).

In bifunctional RSHs, including Rel, (p)ppGpp hydrolysis or synthesis is governed by conformational antagonism between the active sites of the hydrolase and synthase domains\(^22-24\). Specifically, switching between hydrolase domain ON or OFF determines if the synthetase domain will be OFF or ON. More recently, it has also been shown that the TGS domain of Bacillus subtilis Rel is directly involved in the repression of the synthetase domain, which keeps the enzyme in a hydrolase ON-state in the absence of decacylated tRNA and the vacant ribosomal A-site\(^25\).

The switch-ON signal for SpoT enzymes is still not clear, but for bifunctional Rel proteins, (p)ppGpp synthesis results from an accumulation of uncharged tRNA during amino acid starvation\(^26-28\). SpoT does not respond to amino acid starvation like Rel or RelA, its hydrolysis or synthesis activity is instead governed by the interaction with auxiliary protein regulators. These factors have been reported to either stimulate SpoT hydrolase or synthetase activity under diverse conditions either with high intracellular GTP levels or under fatty acid, carbon or phosphate starvation\(^3,29-31\). In all cases, direct interaction of regulators with specific domains of SpoT control switching of hydrolase/synthetase from ON/OFF to OFF/ON and vice versa. E. coli RelA contains a pseudo-HD, which is conserved but lacks the essential residues needed for (p)ppGpp hydrolysis\(^14,32\).
ppGpp and pppGpp are indicated. For TLCs of biological replicates see Supplementary Fig. 1i.

**α** of helices (WP_003096603.1) Ccr Caulobacter crescentus

Klebsiella pneumoniae

equisimilis (Q54089)

SpoT: WP_010895463.1) (NP_461877.1)

RelA: NP_417264.1, SpoT: NP_418107.1)

alignment of selected RelA, Rel and SpoT sequences.

resistance) and plates were incubated at 30 °C (See Supplementary Fig. 1f

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Mutants were isolated and screened on SMG plates. H-loop mutants showing growth defects were subsequently isolated and sequenced (Fig. 2b). Interestingly, a majority of substitutions (21 different, independently isolated mutants) were present in the start of the loop between residues 110–123 and had either one or two amino acid changes. Remarkably, substitutions primarily generated proline or charged amino acids such as glutamate or lysine. Four different mutants were selected from the primary screen (L119M, A121E, M113K and Q118L) and re-introduced at the chromosomal relA locus (Fig. 2c). As a control 1116L was used, which has previously been isolated in the study by Montero et al. We observed that all the tested mutants affected RelA complementation on SMG plates, however, A121E drastically hampered RelA activity (Fig. 2c). Similar results were obtained when these mutations were introduced into untagged RelA (Supplementary Fig. 2a) or when assaying AT resistance (Supplementary Fig. 1g). Furthermore, comparable protein levels of RelA::HTF, RelAA121E::HTF and RelAI116L::HTF in response to amino acid starvation were confirmed by western analysis (Supplementary Fig. 1h, compare lanes 3–4 with 5–6 and 9–10). Consistent to their growth phenotype on SMG plates, MG1655 relA1116L::HTF and relAA121E::HTF showed reduced (p)ppGpp synthesis in response to isoleucine starvation as measured by thin layer chromatography (Fig. 2d, for TLCs see Supplementary Fig. 1i–l). Particularly, MG1655 relAA121E::HTF showed a little (p)ppGpp accumulation after 30 min (30% of wild-type after 30 min). In conclusion, we have isolated a hydrolase domain H-loop substitution mutant, RelAA121E which affects (p)ppGpp synthesis.

RelAA121E substitution mutant interacts with uncharged tRNA and the ribosome. We have implemented a crosslinking methodology, crosslinking and analysis of cDNA (CRAC) (Supplementary Fig. 3a), which allows mapping of protein-RNA interactions with single nucleotide resolution, in living cells. We applied this approach to investigate the interaction of the H-loop RelA mutant with the tRNA and the ribosome. In response to isoleucine starvation, wild-type RelA crosslinking increases with uncharged isoleucine tRNA, tRNAileTUV, and the ribosome. Using false discovery rate analysis (FDR) we previously identified three sites on the ribosome, which showed statistically increased crosslinking with RelA after isoleucine starvation. These sites are: the A-site finger (ASF) of 23S rRNA, Helix 15 (H15) of 16S rRNA and the Sarcin-Ricin Loop (SRL) of 23S rRNA (Fig. 3a). Consistent with the cryo-EM structure of RelA bound to the ribosome along with the uncharged tRNA, we observed crosslinking between the ZFD and RRM domains of RelA with the ASF of 23S rRNA, and the TGS domain of RelA with H15 of 16S rRNA. These interactions are consistent with the RelA accommodation in the A-site during isoleucine starvation. Here we performed CRAC to compare interactions of the RelA::HTF and RelAA121E::HTF with the tRNA and the ribosome.
The interaction site for RelA C-terminal ZFD and RRM domains is helix 38 of 23S rRNA A-site finger (ASF), which bridges the A-site between the ribosomal subunits. RelA and RelA\textsuperscript{A121E} showed similar fold increase in interaction with ASF after isoleucine starvation, 4.7- and 5.1-fold respectively (Fig. 3b and Supplementary Fig. 3b and 3e). After confirmation that both RelA::HTF and RelA\textsuperscript{A121E::HTF} bind to similar sites on the ribosome, we analysed the crosslinking pattern at the specific residue level. In CRAC method, crosslinking pattern can easily be scored as reverse transcription mutations (RT-mutations) that occur at high frequency at the crosslinking sites. The most common mutations observed are deletions or substitutions in the cDNA at the crosslinking sites\textsuperscript{2,39}. Crosslink mediated increase in both deletions and substitutions were clearly observed in the ASF region of 23S rRNA in response to isoleucine starvation (Supplementary Fig. 4a–d for all RT-mutation heatmaps). We primarily observed substitutions at A887 and deletions at U884-C888 showing crosslinking pattern of the ZFD domain (Fig. 4a–b). These mutations were similar for both RelA and RelA\textsuperscript{A121E}, suggesting similar binding of the ZFD domain to the ASF. The signature of RRM domain interactions with ASF is deletions at position U894-A896 (Fig. 4a–b)\textsuperscript{2}. Though the number of cDNA reads from RelA\textsuperscript{A121E} aligning to ASF crosslinking is lower than the RelA, the crosslinking patterns were the same. Furthermore, crosslinking of the TGS domain to Helix 15 of 16S rRNA resulted in substitutions at U368 and deletions at position G359, A364, U365 and A367 (Fig. 4c–d and Supplementary Fig. 4e–h). At Helix 15, we observed slightly lower crosslinking with RelA\textsuperscript{A121E} as compared to RelA, but with similar crosslinking pattern. Another crosslinking site in the ribosome is the SRL of 23S rRNA, which showed increased amounts of substitutions at position G359, A364, U365 and A367 (Fig. 4c–d and Supplementary Fig. 4e–h). Again, the crosslinking pattern is the same between the two strains, but the number of reads was lower in RelA\textsuperscript{A121E}. Additionally, as expected from the enrichment data, crosslinking to tRNA\textsuperscript{ileTUV} was similar for both RelA and RelA\textsuperscript{A121E} (Supplementary Fig. 4g–i).
Fig. 4 RelA$^{121E}$ ribosome interaction is analogous to RelA. RT-mutations (deletion and substitutions) in cDNA reads obtained with CRAC analysis of MG1655 encoding relA::HTF or relA$^{121E}$::HTF before and 30 min after isoleucine starvation (here named 0 or 30, respectively). Heatmaps show nucleobase positions with increased error-frequencies caused by RelA$^{+}$RNA crosslinking. Deletions per million, DPM, or Substitutions per million, SPM, are indicated in red and blue respectively. a RT-mutations in the A-site finger (ASF) of 23 S rRNA (nt 880–900). b Close-up on the ASF (PDB: 5IQR) with positions with significant number of RT-mutations. Deletions or substitutions are shown in red and blue, respectively. ZFD and RRM domains of RelA are coloured in pale orange and pale blue. c RT-mutations in helix 15 (H15) of 16 S rRNA (nt 355–375). d Close-up on H15 (PDB: 5IQR) with crosslinking sites. TGS domain of RelA is displayed in pale green. e RT-mutations in the Sarcin–Ricin Loop (SRL) of 23 S rRNA (nt 2650–2670). f Close-up on SRL (PDB: 5IQR) with crosslinking sites. The pseudo-HD of RelA is shown in blue and the position of mutated alanine 121 is highlighted in salmon. RT-mutations from biological replicates see Supplementary Fig. 4a–n.
Fig. 4m–n). Taken together, the crosslinking data indicate that RelA and RelA^{A121E} bind to uncharged tRNA and to the ribosome in a similar manner but mutant protein interacts less efficiently with the ribosome compared to wild-type protein. Importantly, the interactions with ASF and SRL are affected indicating that these interactions might play an important role for SYN domain activation and (p)ppGpp synthesis.

**Alanine substitutions in the H-loop stimulate (p)ppGpp synthesis.** The data presented above provided evidence that the H-loop of the pseudo-HD is important for the regulation of the SYN domain and (p)ppGpp synthesis. Moreover, single or double substitutions in the H-loop decreased ribosome binding and affected for (p)ppGpp synthesis. To investigate further the role of the H-loop, we made site-directed substitutions of four residues of the H-loop into alanine, which would likely affect the structure of the loop. The mutated residues were arginine 117, glutamine 118, lysine 120 and histidine 123 (Mutant is referred to as RelA^{QUAD::HTF}, Fig. 5a).

Surprisingly, the mutant showed increased survival on SMG plates as compared to wild-type (Fig. 5b). The stimulatory effect of the RelA^{QUAD} mutant was independent of the HTF tag, as a similar effect was observed with the untagged protein (Supplementary Fig. 1f). A similar effect was also observed when assaying AT resistance (Supplementary Fig. 1g). In an attempt to explain this surprising effect, (p)ppGpp synthesis was measured by TLC after isoleucine starvation (Fig. 5c–d). Interestingly, while RelA showed an approximately 5-fold increase in (p)ppGpp synthesis, RelA^{QUAD::HTF} only showed about a 3-fold increase in response to isoleucine starvation. The strain expressing RelA^{QUAD::HTF} on the other hand, already had an elevated basal level of (p)ppGpp prior to starvation (about 2-fold higher than the basal level of RelA). The RelA^{QUAD::HTF} protein levels were comparable to wild-type RelA before and after starvation and hence the higher basal level of (p)ppGpp is due to the higher basal synthetase activity of the mutant protein rather than the amount of protein per se. (compare lanes 7–8, Supplementary Fig. 1h). This suggests that the RelA^{QUAD} mutant intrinsically produces more (p)ppGpp than the wild-type RelA. This feature surprisingly did not affect growth on LB plates, but supported better survival and growth on SMG and AT plates. In conclusion, four alanine substitutions in the H-loop of RelA increased basal level of (p)ppGpp and growth on SMG and AT plates. In conclusion, four alanine substitutions in the H-loop of RelA increased basal level of (p)ppGpp and growth on SMG and AT plates. In conclusion, four alanine substitutions in the H-loop of RelA increased basal level of (p)ppGpp and growth on SMG and AT plates. 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**Discussion**

Here, we have established a new fundamental function of the N-terminal inactive pseudo-hydrolase domain (pseudo-HD) of RelA for the activation of the synthetase domain (SYN). By genetic, biochemical and in vivo crosslinking analysis, we show that the pseudo-HD domain is important for regulating RelA synthetase activity. Further, we identify a loop between helix α6 and α7 (Residues 114–130), which is divergent and longer in hydrolase inactive RelA homologues as compared to hydrolase active Rel/SpoT (Fig. 1b). Random mutagenesis in this loop led to the identification of residues, which when mutated, affects RelA synthetase activity (Fig. 2b). Introduction of multiple alanine substitutions in the loop (RelA^{QUAD}) has an stimulatory effect on (p)ppGpp synthesis and increases the basal level before isoleucine starvation (Fig. 5b–d). Surprisingly, a single point mutant RelA^{A121E} in the pseudo-HD severely affects SYN domain function and (p)ppGpp synthesis (Fig. 2c–d).

Recently, it has been shown in the RelTts of *Thermus thermophilus*, that the loop between α6 and α7 changes conformation and moves away from hydrolase active site to make hydrolase active site free for ppGpp hydrolysis and goes towards it to block the hydrolase active site24. This conformational change is mediated by binding of ppGpp to the hydrolase active site of the protein. Additionally, binding of ppGpp to the hydrolase domain directly precludes the binding of GDP/ATP in the synthetase domain to prevent ppGpp hydrolysis and synthesis happening simultaneously24. Similar observations had also been reported for RelBsu of *Streptococcus dysgalactiae*, here the binding of ppGpp in the hydrolase domain does not prevent binding of GTP or GDP in the synthetase domain, but prevents binding of ATP and (p)ppGpp synthesis22,23. Recently it was shown that the TGS domain of RelBsu is involved in the regulation of the synthetase domain25. Upon binding of RelBsu to the ribosome along with uncharged tRNA, the TGS domain moves away from the synthetase domain...
with structural changes in the N-terminal domains including α7 of the hydrolase domain. Moreover, substitutions in α7 (R125E/M127E) were observed to decrease hydrolase activity and increase synthetase activity.23. Thus, in all cases the hydrolase domain seems to control (p)ppGpp synthesis.

In E. coli RelA, the hydrolase domain is inactive and crucial residues for hydrolytic function including the conserved HDxxED motif is absent (Supplementary Fig. 1a). However, our results show that the hydrolase domain still plays a similar role of regulating SYN domain function and that might be the reason for it to be preserved in the RelA even with the extended H-loop between helix α6 and α7.

The single point mutant RelA121E identified in our study is severely affected for (p)ppGpp synthesis (Fig. 2c–d). This mutant was still able to bind to the ribosome and uncharged tRNA in response to amino acid starvation, which is consistent with the fact that the substitution is present in the pseudo-HD, distant from ribosome and tRNA binding domains (Fig. 3a–b). The similar binding pattern with the ribosome and tRNA of this mutant confirms that the mutant protein is structurally similar to wild-type RelA protein and this single point mutant has not affected protein structure drastically. Interestingly, while crosslinking to Helix 15 of 16S rRNA is similar between RelA and RelA121E, crosslinking to the ASF and the SRL is slightly affected (59% and 53%, respectively, Fig. 3b). Thus far, we know that the ZFD and RRM domains are responsible for the binding to ASF; however, the mechanistic details of RelA crosslinking to SRL is still not clear.2,19,20,38. Due to the location of the SRL in the vicinity of the pseudo-HD and the dynamic nature of the N-terminus, we earlier suggested that the pseudo-HD interacts with SRL and could promote RelA activation.2 In addition, crosslinking to the SRL was found to be lower in the RelA121E, the crosslinking pattern is similar to wild-type suggesting that the H-loop is perhaps not responsible for this interaction (Fig. 4c, f). Previously, it has been shown that cleavage between G2661 and A2662 of SRL by Bacillus subtilis RNAse has evolved differently for monofunctional RSH proteins, which has been previously shown that ribosomal protein L11 and the SRL could promote RelA activation2. Although crosslinking to the SRL shows up to 59% and 53%, respectively, Fig. 3b). Thus far, we know that the ZFD and RRM domains are responsible for the binding to ASF; however, the mechanistic details of RelA crosslinking to SRL is still not clear.2,19,20,38. Due to the location of the SRL in the vicinity of the pseudo-HD and the dynamic nature of the N-terminus, we earlier suggested that the pseudo-HD interacts with SRL and could promote RelA activation.2 In addition, crosslinking to the SRL was found to be lower in the RelA121E, the crosslinking pattern is similar to wild-type suggesting that the H-loop is perhaps not responsible for this interaction (Fig. 4c, f). Previous results have shown that cleavage between G2661 and A2662 of SRL by Bacillus subtilis RNAse has evolved differently for monofunctional RSH proteins, which has been previously shown that ribosomal protein L11 and the SRL could promote RelA activation2. Although crosslinking to the SRL shows up to 59% and 53% respectively, Fig. 3b). Thus far, we know that the ZFD and RRM domains are responsible for the binding to ASF; however, the mechanistic details of RelA crosslinking to SRL is still not clear.2,19,20,38. Due to the location of the SRL in the vicinity of the pseudo-HD and the dynamic nature of the N-terminus, we earlier suggested that the pseudo-HD interacts with SRL and could promote RelA activation.2 In addition, crosslinking to the SRL was found to be lower in the RelA121E, the crosslinking pattern is similar to wild-type suggesting that the H-loop is perhaps not responsible for this interaction (Fig. 4c, f).

Methods

Strains and plasmids. Strains constructed and plasmids used in this study are listed in Supplementary Methods. Strains, plasmids and oligonucleotides are listed in Supplementary Table 1.

Media and growth conditions. Escherichia coli K-12 strains were routinely grown in liquid LB complex medium or on solid LB agar medium at 30 or 37 °C. For amino acid starvation experiments the bacterial cells were grown in MOPS (morpholinepropanesulfonic acid) minimal medium at 30 °C or 37 °C supplemented with 0.2% glucose, with all nucleobases (10 µM each) and incubated at 30 °C with shaking. At OD600 > 0.5, cells were diluted 10-fold to an OD600 of ~0.05 and were left to grow with shaking at 30 °C with H3P04 (100 µM/culture). After ~2 generations (OD600 of ~0.2), amino acid starvation was induced by the addition of valine (500 µM/culture). Fifty-micro liter samples were withdrawn before and after 5, 10, 20 and 30 min after addition of valine. The reactions were stopped by the addition of 10 µl of ice-cold 2 M formic acid and centrifuged at maximum speed for 1 h at 4 °C. 10 µl of each reaction mixture was loaded on polyethyleneimine (PEI) cellulose thin layer chromatography (TLC) plates (purchased from GE Healthcare) and separated by chromatography in 1.5 M potassium phosphate at pH 3.4. The TLC plates were revealed by phosphorimaging (GE Healthcare) and analysed using the Imagej software.44. The increase in the level of (p)ppGpp was quantified as the fraction of (p)ppGpp (g)ppGpp measurements. (p)ppGpp measurements during isoleucine starvation were performed as described previously by Michael Cashel.22. Overnight cultures of relevant strains were diluted 100-fold in 5 ml of MOPS minimal medium supplemented with 0.2% glucose and all nucleobases (10 µM each) and incubated at 30 °C with shaking. At OD600 = 0.5, cells were diluted 10-fold to an OD600 of ~0.05 and were left to grow with shaking at 30 °C with H3P04 (100 µM/culture). After ~2 generations (OD600 of ~0.2), amino acid starvation was induced by the addition of valine (500 µM/culture). Fifty-micro liter samples were withdrawn before and after 5, 10, 20 and 30 min after addition of valine. The reactions were stopped by the addition of 10 µl of ice-cold 2 M formic acid and centrifuged at maximum speed for 1 h at 4 °C. 10 µl of each reaction mixture was loaded on polyethyleneimine (PEI) cellulose thin layer chromatography (TLC) plates (purchased from GE Healthcare) and separated by chromatography in 1.5 M potassium phosphate at pH 3.4. The TLC plates were revealed by phosphorimaging (GE Healthcare) and analysed using the Imagej software.44. The increase in the level of (p)ppGpp was quantified as the fraction of (p)ppGpp (g)ppGpp measurements.

Random mutagenesis screening using error-prone PCR. To screen for RelA hydrolase mutants with altered synthetase activity the loop region was amplified from relA using oligos loop-mut-f and loop-mut-r using the DreamTaq polymerase (Thermo) According to Rasila et al.45. Hundred microlitre PCR was prepared containing 10 U DreamTaq polymerase, 10 µM of each dNTP, 0.3 µM of each primer, colony DNA as template and 2–4 µl mutagenesis buffer (4 mM dTTP, 4 mM dCTP, 2.5 mM MnCl2, 27.5 mM MgCl2). The PCR product was purified and electroporated into recombination competent TG1ΔλΔlacI + Cre::ΔrelA R6C::HTF containing pBluescript II SK+ (from Lewin et al.2). After 1 or 2 days of growth on LB agar medium containing 100 µg/ml ampicillin and 1 µg/ml anhydrotetraacycline. Positive Sc–I resistant clones were re-screened on LB plates and MOPS MM SMG plates at 30 °C.

RelA-RNA interactions by UV crosslinking and analysis of cDNAs. Cross-linking and analysis of cDNAs (CRAC) was performed essentially as previously described in Winther et al.2. Supplementary Fig. 3a shows an overview. MG1655 relA:HTF and MG1655 relA121E:HTF were grown overnight (ON) MOPS minimal medium supplemented with 0.2% glucose and all nucleobases (10 µM each) at 30 °C. The ON cultures where then diluted to OD600 = 0.005 into two flask containing 2 L MOPS minimal medium and incubated with shaking at 30 °C. After ~2 generations (OD600 = ~0.2) one culture was UV crosslinked in a W5 crosslinking unit (Van Remmen UV technique) by irradiation with 1800 ml of UV-C for 100 s. The other culture was starved for isoleucine by addition of 500 µg/ml L-Valine for 5 or 30 min before exposure to UV. After UV irradiation the cultures were separated into 1 L aliquots, harvested and the pellet was washed in ice-cold 1XPBS (Phosphate Buffer Solution). Before rapid freezing in liquid nitrogen the pellets were stored at ~80 °C before proceeding with purification. Pellets were dissolved 1 ml Lysis buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% NP-40, 5 mM β-Mercaptoethanol and Complete protease inhibitor) and lysed by vortexing 5 × 1 min with 3 ml 0.5 mm Zirconia beads (Thistle Scientific). Lysates were filtered and centrifuged and incubated with 200 µl anti-FLAG M2 affinity gel (Sigma–Aldrich) for 2 h at 4 °C. The resin was washed twice with Wash buffer (50 mM Tris-HCl pH 7.8, 0.1% NP-40, 5 mM β-Mercaptoethanol and 1 M (high salt) or 150 mM NaCl (low salt), respectively). The resin was resuspended in 600 µl low salt Wash buffer and RelA was cleared from the resin by treatment with 5 µl H2O2 and RNaseA (proven) and incubated for 15 min at 37 °C. Crosslinker was removed and sample (500 µl) was trimmed using 1 µl 0.7U RNaseT2 (Agilent Technologies) for 5 min at 37 °C and stopped by addition of guanidine-HCl to a final...
concentration of 6 M. The trimmed sample was subsequently bound to 100 μL Ni-NTA superflow agarose (Qiagen) overnight at 4 °C in Denaturing buffer (50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 5 mM β-Mercaptoethanol) supplemented with 10 mM Imidazole. The resin was then washed twice in Denaturing buffer and three times in Reaction buffer (50 mM Tris-HCl pH 7.8, 10 mM captoethanol) with 10 mM Imidazole. The resin was then washed twice in Denaturing buffer supplemented with 0.5% NP-40. Barcoded 5 μM of 3′-end mirCat-33 linker (see Supplementary Table 1) was ligated to the RNA using T4 RNA Ligase I in Reaction buffer containing 1U/μL RNasin for 6 h at 25 °C. The reaction was stopped by washing once with Denaturing buffer and three times with Reaction buffer supplemented with 0.5% NP-40. The RNA was then 5′-end phosphorylated using T4 polynucleotide kinase (Thermofisher) and 0.5 μCi/μL [γ-³²P]-ATP for 40 min in Reaction buffer at 37 °C. ATP was added to a final concentration of 1.25 mM and incubation was continued for 20 min. The reaction was stopped by washing once in Denaturing buffer and three times with Reaction buffer supplemented with 0.5% NP-40. Barcoded 5′-end 1.25 mM and incubation was continued for 20 min. The reaction was stopped by washing once in Denaturing buffer and three times with Reaction buffer supplemented with 0.5% NP-40. Barcoded 5′-end ligation of 1.25 μM of 3′-end mirCat-33 linker (see Supplementary Table 1) was ligated to the RNA using T4 RNA Ligase I in Reaction buffer containing 1U/μL RNasin overnight at 16 °C. The resin washed three times in Wash buffer (50 mM Tris-HCl pH 7.8, 50 mM NaCl, 150 mM Imidazole, 0.1% NP-40 and 5 mM β-Mercaptoethanol) and the RenA RNA complex eluted twice with 200 μl elution buffer (50 mM Tris-HCl pH 7.8, 50 mM NaCl, 150 mM Imidazole, 0.1% NP-40 and 5 mM β-Mercaptoethanol). The eluate was then precipitated using trichloroacetic acid and the precipitate dissolved in 1× LDS loading buffer (Life Technologies) before separation on 4–12% NuPAGE gel (Life technologies) in 1× MOPS running buffer (Life technologies). The RNA was then sequenced on a flow cell using T4 RNase H at 37 °C. The libraries were generated by PCR using LA Takara taq polymerase (Clontech) and oligos P5 and PE (see Supplementary Table 1) size selected on a agarose gel and extracted using the MNEulute extraction kit (Qiagen).

The DNA libraries were sequenced on the Illumina MiSeq platform (50 bp single-end reads) and the sequencing output analysed using the pyCRAC software package8. We have previously adapted this approach for RelA2. FastQ single-end reads) and the sequencing output analysed using the pyCRAC software.

We have previously adapted this approach for RelA2. FastQ single-end reads) and the sequencing output analysed using the pyCRAC software.

Previously we have identified regions of significant enrichment after isoleucine starvation. cDNA reads and substitutions introduced in the cDNA reads were counted in selected regions showing significant enrichment after isoleucine starvation. cDNA reads and crosslinking sites were visualized by plots and heatmaps in R.

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Author contributions

K.S.W. conceived and initiated the study. K.S.W. and A.K.S. performed the experiments and analysed the data. K.S.W. and A.K.S. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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