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LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following original publications, referred to by their numerals.

I. Kuzmenko A∗, **Tankov S**∗, English BP∗, Tarassov I, Tenson T, Kamenski P, Elf J,Hauryliuk V. Single molecule tracking fluorescence microscopy in mitochondria reveals highly dynamic but confined movement of Tom40. Scientific Reports. 2011; 1:195

II. English BP, Hauryliuk V, Sanamrad A, **Tankov S**, Dekker NH, Elf J. Single-molecule investigations of the stringent response machinery in living bacterial cells. Proc. Nat. Acad. Sci. U S A. 2011 Aug 2; 108(31): E365–73

III. Shyp V, **Tankov S**, Ermakov A, Kudrin P, English BP, Ehrenberg M, Tenson T, Elf J, Hauryliuk V. Positive allosteric feedback regulation of the stringent response enzyme RelA by its product. EMBO Reports. 2012 Sep; 13(9): 835–9

* Equal contribution

In papers I, I have performed mitochondrial preparations, microscopy and analyzed the data and participated in drafting the manuscript. In paper II, I performed mEos2 tracking, analysis and bacterial growth experiments. In paper III, I have performed part of the biochemical research.

LIST OF ABBREVIATIONS

AA	Amino Acids
ATP	Adenosine Tri-Phosphate
Cryo-EM	cryoelectron microscopy
EF-G	Elongation Factor G
EF-P	Elongation Factor P
EF-Ts	Elongation Factor Thermo stable
EF-Tu	Elongation Factor Thermo unstable
eIF5B	eukaryotic translation Initiation Factor 5B
fMet-tRNA$_i$	N-formyl-methionyl-tRNA$_i$, intiator tRNA
FRAP	Fluorescence recovery after photobleaching
GAP	GTPase-activating protein
GDP	Guanosine Di-Phosphate
GEF	Guanine nucleotide Exchange factors
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
IC	Initiation Complex
IF	Initiation Factor
mRNA	messenger Ribonucleic Acid
MSD	Mean Squared Displacement
ORF	Open Reading Frame
PALM	Photoactivated Localization Microscopy
ppGpp	Guanosine 5´-diphosphate 3´-diphosphate
pppGpp	Guanosine 5´-triphosphate 3´-diphosphate
PTC	Peptidyl Transfer Center
RF1	Release Factor 1
RF2	Release Factor 2
RF3	Release Factor 3
RNAP	RNA Polymerase
RRF	Ribosome Recycling Factor
rRNA	ribosomal Ribonucleic Acid
RSH	Rel/Spo Homolog
SAS	Small Alarmone Synthetase
SD	Shine-Dalgarno sequence
SHX	L-Serine Hydroxamate
SPT	Single-particle tracking
SRL	Sarcin Ricin Loop
STED-FCS	Stimulated Emission Depletion-Fluorescence Correlation Spectroscopy
tRNA	transfer Ribonucleic Acid
UTR	untranslated region
YFP	Yellow Fluorescent Protein

INTRODUCTION

Sensing changes in the environment and rapidly responding to them is the key for bacterial survival. One of the most important regulatory systems, the stringent response, is orchestrated by alarmone nucleotides guanosine pentaphosphate, pppGpp, and guanosine tetraphosphate, ppGpp, collectively named (p)ppGpp. Regulation by alarmone nucleotides is one of the core processes regulating bacterial transcription, translation and replication.

Discovered in 1960s by Cashel and colleagues, these products of GTP and GDP, first called magic spots, rapidly accumulate during amino acid starvation in *Escherichia coli.* Under these conditions deacylated tRNA enters the ribosomal A-site where it is sensed by an enzyme RelA – a representative of so-called RelA/SpoT Homologue (RSH) family of proteins. Despite being discovered almost four decades ago, the molecular mechanism of RelA is still poorly understood. Specifically, the relationship between RelA binding to the ribosome and ppGpp synthesis is a matter of debate.

This thesis contributes to two aspects of our understanding of RelA's mechanism of action. First, using single molecule microscopy, technique developed for studying RelA and the diffusive behavior of mitochondrial channel TOM40 (Paper I) in living cells, I have followed RelA's diffusion in bacteria under conditions of amino acid limitation, which resulted in a formation of so-called 'hopping' model of RelA's catalytic cycle (Paper II). Second, using biochemical system from purified components, I have contributed to the discovery of RelA's activation by its product, ppGpp (Paper III).

REVIEW OF LITERATURE

1. Protein synthesis in bacteria

Proteins take part in almost every process of life including enzymatic catalysis, maintenance of cellular structure, immune response, cell signaling and many others. The primary amino acid (AA) sequence of the protein defines its 3D structure and therefore functions. The AA sequence of a protein is encoded in DNA, which is transcribed into the messenger RNA (mRNA) and then translated into the protein by the ribosomes (Crick 1970). Ribosomes are large and complex molecular machineries composed of both protein (ribosomal proteins, r-proteins) and RNA (ribosomal RNA, rRNA) molecules. Translation of the information from mRNA to proteins by the ribosome is aided by aminoacylated transport RNAs (tRNA) acting as adapters. Details of structure, size and rRNA sequence of the ribosomes differ between bacteria, archaea and eukaryotes, however, the general process of protein synthesis is well conserved among the different domains of life (Fox 2010). In bacteria, the 70S ribosome is composed of small (or 30S) subunit and large (or 50S) subunit (**Figure 1)**. In *Escherichia coli*, the workhorse of the molecular biology, the 50S subunit consists of 5S and 23S rRNA molecules and 33 proteins (L1-L36, with L designating large subunit). The small 30S subunit is composed of single 16S rRNA molecule and 21 proteins (S1-S21, with S designating small subunit) (Czernilofsky et al. 1974).

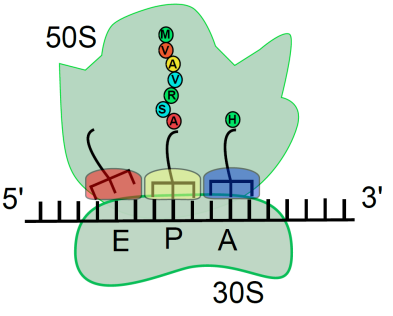

Figure 1. The molecular architecture of bacterial ribosome. The small (30S) and the large (50S) ribosomal subunits form a hetero-dimer during active translation. mRNA contains codons corresponding to the sequence of the AA needed for protein synthesis. The ribosome have three tRNA binding sites, named A (acceptor site, binds incoming AA-tRNA), P (peptidyl, holds the tRNA carrying the growing polypeptide chain) and E (exit, forms the exit path for deacylated tRNA).

The small ribosomal subunit is translating the information from mRNA into AA sequence while the large subunit is conducting the catalysis of the peptide bond formation through the transpeptidation reaction (Carter et al. 2000). The three tRNA binding sites, A-, P-, and E-site act as a conveyor belt sequentially passing the tRNAs through the ribosome.

Protein translation is complex and highly regulated process divided into four steps: initiation, elongation, termination and recycling (**Figure 2**). At each of the steps, the ribosome is assisted by a specific set of protein and RNA molecules.

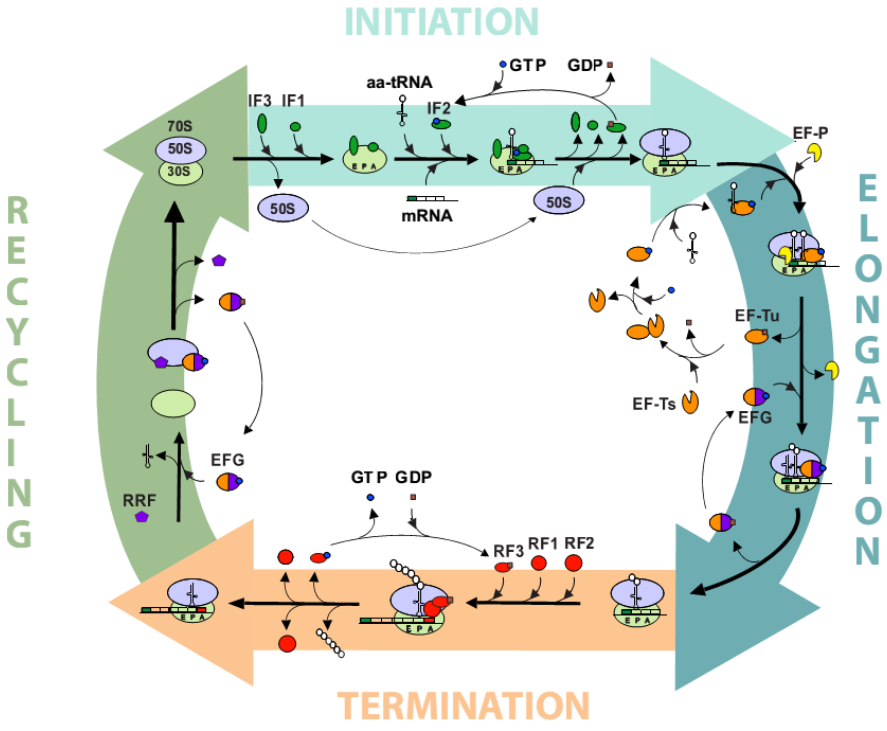

Figure 2. Schematics of the bacterial ribosomal functional cycle. Bacterial translation can be divided into four main steps: initiation (light blue solid arrow), elongation (dark blue solid arrow), termination (light brown solid arrow) and recycling (green solid arrow). Initiation is the first and most conserved stage in bacterial translation and is facilitated by three initiation factors (IFs) (green circles and ellipses) IF1, IF2 and IF3. The 70S complex formed during initiation enters the elongation cycle upon the arrival of the ternary complex (T3) aminoacyl-tRNA:EF-Tu:GTP to the ribosomal P-site. The complex is dissociated with the hydrolysis of guanosine triphosphate (GTP) (blue circles) resulting in EF-Tu (orange ellipse) bound to guanosine diphosphate (GDP) (brown square) leaving the ribosome and further recycled to active EF-Tu:GTP by elongation factor EF-Ts (orange pie shape). EF-P (yellow) is a protein factor that stimulates the accommodation of proline tRNA and consequent transpeptidation and is critical for efficient translation of proteins containing polyproline stretches. The nascent peptide is translocated from A- to P- site by translational GTPase EF-G (orange/purple ellipse) in a GTP-dependent manner. Upon reaching stop codon, translation enters in termination phase involving release factors (RFs) (red circles); RF1 and RF2 recognize the stop codon and cleave off the polypeptide, and GTPase RF3 contributes to processivity and accuracy of the process. After release of the newly synthesized protein, ribosome enters the recycling stage, which involves splitting of 70S ribosome into subunits and preparation of for a new round of initiation facilitated by RRF (purple pentagon) and EFG (orange/purple ellipse).

1.1 Initiation

The accurate recognition of start position – the initiation codon – on the mRNA is the first step in translation initiation that defines the open reading frame, ORF, encoding the protein chain. Therefore, precise initiation is the key to correct translation and is tightly regulated by numerous protein factors. In bacteria, there are considerably fewer components involved in the initiation than in Eukaryotes and Achaea. The mRNA (**Figure 3**), containing information for synthesizing protein is loaded onto ribosome and the initiation codon is recognized by the aminoacylated and formylated initiator tRNA (fMet-$tRNA_i$). The formation of a pre-initiation complex from 30S subunit, mRNA, and fMet-$tRNA_i$ is the first step in bacterial translation. Formation of the pre-initiation complex is regulated by three initiation factors IF1, IF2 and IF3 and is guided by sequence signals encoded in mRNA: the initiation codon AUG and the Shine-Dalgarno (SD) sequence, SD (Gualerzi and Pon 2015).

The SD sequence of canonical mRNAs (**Figure 3**) interacts with the anti-SD sequence of the 16S rRNA to maintain IF3 in the complex (Lee et al. 1996). The efficiency of the SD sequence is strongly dependent on its spacing from the start codon as well as the base-pairing potential with the anti-SD sequence (Ringquist et al. 1992). The SD sequences spacing can vary from 5 to 13 bases, with its optimal distance of 8 to 10 bases for *E. coli* genes (Chen et al. 1994). However, most SD sequences have small deviation from the GGAGG core (Sengupta et al. 2001). Strong SD sequence can compensate a weak start codon and counteract mRNA secondary structure (de Smit and van Duin 1993).

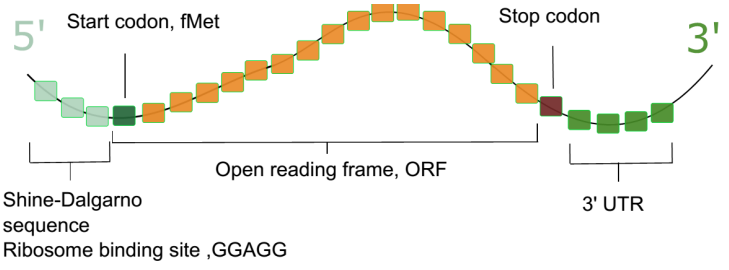

Figure 3. Schematic representation of bacterial mRNA. A typical bacterial mRNA consists of 5' untranslated region (UTR), which includes Shine-Dalgarno sequence, ORF (initiating with a start codon and terminating with one of the stop codons: UAG, UAA and UGA) and 3'-UTR. The Shine-Dalgarno sequence anchors the ribosome on the mRNA positioning the start codon in the P-site.

As mentioned above, translation initiation in bacteria is controlled by three initiation factors: IF1, IF2 and IF3. IF2 is the largest – and, arguably, the most important – initiation factor, since specific contacts between IF2 and fMet-$tRNA_i$ are crucial during the translation initiation. These interactions determine the precision in the selection of the correct initiation site of mRNA and in the establishment of the first peptide bond (La Teana et al. 1996,

Guenneugues et al. 2000). IF2 belongs to translational GTPase protein family that binds and hydrolyzes GTP (Atkinson 2015). The GTPase proteins form a large family of enzymes containing a highly conserved G domain that hydrolyzes GTP to GDP and inorganic phosphate (P_i) (Scheffzek and Ahmadian 2005). This reaction converts the GTPase from its active, GTP bound form, to inactive, GDP bound form and is regulated by GTPase-activating proteins (GAPs) (Ross and Wilkie 2000). The reverse reaction, turning the GTPase 'on' can be catalyzed by guanine nucleotide exchange factors (GEFs) (Cherfils and Zeghouf 2013), which displace the GDP from the GTPase, leading to its recharging with a new GTP molecule (**Figure 4**).

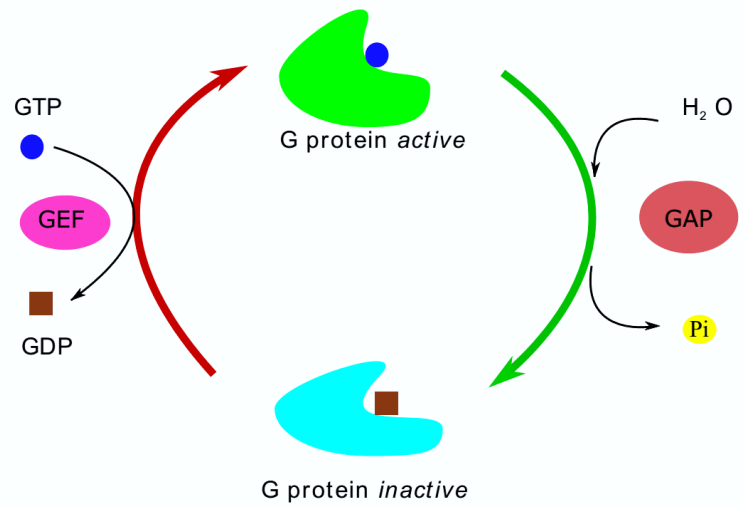

Figure 4. The functional cycle of a GTPase. Active (green) and inactive (light blue) form of the GTPase bound to GTP (dark blue circle) and GDP, respectively. Regulation of the cycle is controlled by guanine nucleotide-exchange factor (GEF) (purple), which catalyze the exchange of GDP for GTP, and GTPase-activating proteins (GAP) (red), which increase the rate of GTP hydrolysis to GDP. For several GTPases involved in translation, including IF2, GEF is not needed and the nucleotide exchange occurs spontaneously.

After associating with the 30S subunit, in a complex with GTP, IF2 binds fMet-$tRNA_i$ and transfers it into hybrid P/E site (Milon et al. 2010). Ribosome subunit association activates IF2 GTPase activity, leading to GTP hydrolysis to GDP and P_i, which, in turn, induces conformational change leading to IF2 release and formation of 70S initiation complex (Antoun et al. 2003). Cryoelectron microscopy (Cryo-EM) studies have revealed the molecular details of IF2 binding to 30S subunits and 70S IC, allowing direct assignment of function to individual domains of the protein (Allen and Frank 2007, Julian et al. 2011, Eiler et al. 2013). The G domain of IF2 is interacting with the

ribosome, contacting functionally important element, Sarcin Ricin Loop, SRL, that is involved in regulation of the GTPase activity. The N-terminal domain contributes to the binding with the 30S subunit while the C-terminal domain interacts with the initiator tRNA, directly contributing to its selection (Krafft et al. 2000, Allen and Frank 2007) (**Figure 5 A, B and C**).

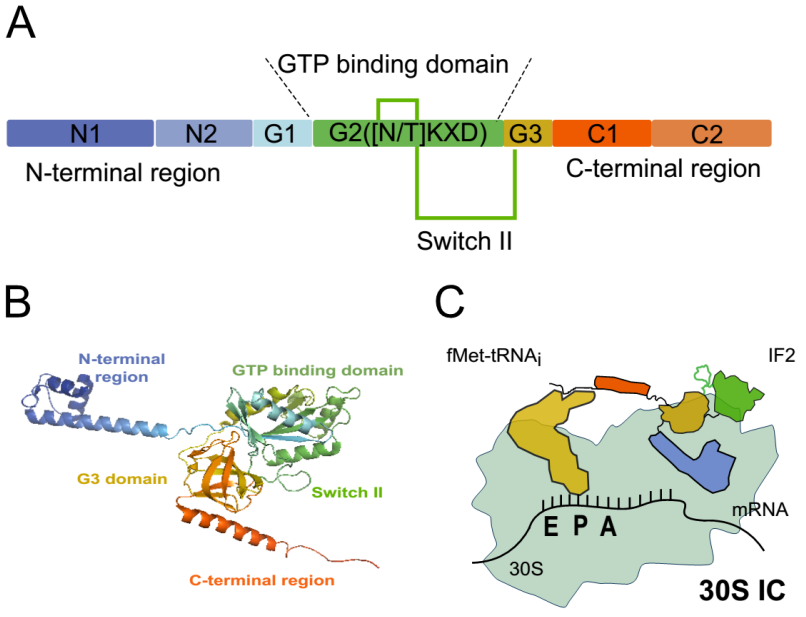

Figure 5. Domain topology of translational GTPase IF2 and the structure of bacterial initiation complex. A) Domain topology of *E.coli* IF2. While in most translational GTPases (trGTPases) the G-domain is situated at the N-terminus, in IF2 it has an additional N-terminal extension. The N-terminal region (**N1**, **N2** and **G1**) contributes to the binding of IF2 to the 30S subunit. The GTP binding domain is followed by the classical domain II (**G3**) conserved amongst all trGTPases. Switch II undergoes conformational changes upon GTP/GDP binding, transitioning the GTPase's from the GDP to the GTP state. C-terminal region consists of domain III (**C1**) and domain IV (**C2**), which interacts with the 3'-end of the tRNA. **B**) Crystal structure of IF2 (adapted from Simonetti et al. 2013). Domains are annotated and color-coded: N-terminal region, blue; GTP binding domain, green; G3 domain, yellow and C-terminal region, orange. Switch II region is annotated and indicated in light green. **C**) Scheme of the late steps of 30S IC formation. The specific recognition of fMet-tRNA$_i$ (yellow) by IF2 C-terminal (orange) contributes to its selection and plays fundamental role during translation initiation in bacteria.

Biochemical and structural studies by cryo-EM (Julian et al. 2011, Simonetti et al. 2013, Sprink et al. 2016) have shown that initiation factors IF1 and IF3 assist IF2 in selection of initiation tRNA and initiation codon (Antoun et al. 2006, 2006, Milon et al. 2010, Pavlov et al. 2011, Milon et al. 2012). IF1 binds to the

decoding part of the A-site blocking initiator tRNA from binding and directing it into the P-site (Carter et al. 2001). Additionally, the factor stimulates ribosome subunit dissociation and IF2 binding affinity (Moazed et al. 1995, Dahlquist and Puglisi 2000). IF3 stimulates 70S dissociation (Subramanian and Davis 1970) and prevents the ribosomal subunit reassociation before correct initiation has been accomplished (Kaempfer 1972). Furthermore, it directs fMet-tRNA$_i$ into the P-site and stimulates the P-site codon-anticodon interactions, thus promoting the formation of the correct 30S IC (Meinnel et al. 1999, Antoun et al. 2006) .

Once 70S initiation complex is assembled and initiator tRNA is accommodated, the initiation factors are released from the ribosome (Antoun et al. 2003) and the ribosome proceeds into elongation (Blaha et al. 2009).

1.2 Elongation

Precise decoding of the mRNA is crucial for protein translational fidelity and stability. Production and aggregation of misfolded proteins can be very toxic for the cell (Bucciantini et al. 2002), therefore there is a solid evolutionary pressure for production of correctly synthesized proteins, especially strong in the case of highly expressed proteins (Drummond et al. 2005).

In the beginning of the elongation, the ribosome is in the post-translocation state with fMet-tRNA$_i$ in the P-site and vacant A-site ready to accept the ternary complex (T3) formed by AA-tRNA and EF-Tu:GTP (Moazed and Noller 1989). Initial contact between the T3 and the ribosome is mediated via the interaction of EF-Tu and the 50S subunit (Schmeing et al. 2009), followed by tRNA recognition of the codon by the anticodon of the tRNA. After AA-tRNA enters A-site, the peptide bond is formed, catalyzed by the ribosome itself (Leung et al. 2011). The initial binding of the complex is dependent on the presence of ribosomal protein L7/L12 suggesting that EF-Tu interaction with L7/L12 endorses ternary complex binding to the ribosome (Kothe et al. 2004). The formation of the peptide bond is characterized with the movement of tRNAs into hybrid A/P and P/E sites and by intersubunit rotation (Agirrezabala et al. 2008, Julian et al. 2008).

The process of transpeptidation is characterized by exceedingly low error frequency in translation (10^{-3}–10^{-4}) (Kurland and Gallant 1996), which is achieved by utilizing a two-step selection process (Rodnina and Wintermeyer 2001). During the first step, the initial selection, a codon-anticodon pair is formed by binding T3 to the ribosome. The correct codon-anticodon pairing leads to a stronger binding: if codon does not match anticodon, the binding affinity of the tRNA remains low and the ternary complex falls off (Ramakrishnan 2002). Exceedingly low intrinsic GTPase activity of EF-Tu is highly induced when tRNA anticodon matches a codon of the mRNA on the ribosome (Sedlak et al. 2002), and the AA-tRNA is selected again during the so-called proofreading step. The GTP hydrolysis induces conformational

change in EF-Tu:GDP complex leading to low AA-tRNA affinity that induces dissociation from the ribosome (Yokosawa et al. 1975). EF-Tu:GDP is further recycled to active EF-Tu:GTP by elongation factor EF-Ts (Wang et al. 1997).

Ribosome-catalyzed transpeptidation has similar kinetics for most of amino acids. An important exception is proline, which has considerably slower transpeptidation kinetics (Pavlov et al. 2009). Recent findings demonstrate that the translation of a specific subset of mRNAs in bacteria requires elongation factor P (EF-P) (Doerfel et al. 2013). EF-P prevents the ribosome from stalling during the synthesis of proteins containing repeated proline residues (Ude et al. 2013, Woolstenhulme et al. 2015). It is shown that EF-P binding site overlaps peptidyl transfer center (PTC), which suggests an important role for EF-P in the modulation of specificity of peptidyltransferase (Blaha et al. 2009).

After transpeptidation the elongation factor G (EF-G) catalyzes so-called translocation of the peptidyl-tRNA into P-site and deacylated tRNA into E-site (Moazed and Noller 1989, Zhou et al. 2014). The mRNA shifts correspondingly in order for a new codon to be presented in the A-site (Spirin 1985). The elongation cycle continues until the full-length protein is synthesized and ribosome reaches one of the stop codons.

1.3 Termination and recycling

When one of the three termination codons (UAA, UAG or UGA) meets the A-site, protein synthesis is stopped and translation enters to termination phase. Proteins known as release factors bind to the ribosome and induce hydrolysis of the ester bond connecting protein with tRNA, allowing the protein to exit the ribosome.

During termination the peptide attached to P-site tRNA is released by the class 1 (RF1 and RF2 in bacteria) assisted by class-2 release factor, trGTPase, RF3 (Song et al. 2000, Zaher and Green 2011, Koutmou et al. 2014). Class 1 factors bind directly to the ribosome, recognize the stop codons in A-site and promote hydrolysis of the ester bond between the polypeptide and tRNA (Brown and Tate 1994). UAG stop codon is recognized by RF1, UGA by RF2 and UAA by both factors (Scolnick et al. 1968).

The role of the translational GTPase RF3 is controversial. It was first shown to promote dissociation of class-1 factors from the ribosome (Freistroffer et al. 1997, Zavialov et al. 2001). However, recent studies suggested that RF3 is involved mainly in the quality control of protein synthesis rather than in the recycling phase (Zaher and Green 2011).

Upon completion of the termination step, post-termination complex consists of 70S ribosome, mRNA and uncharged tRNA in the P-site (Hirokawa et al. 2002). A specialized ribosome recycling factor (RRF) and EF-G disassemble the post-termination complexes and dissociate the ribosome into 30S and 50S subunits (Hirokawa et al. 2005, Zavialov et al. 2005). IF3 replaces the deacylated tRNA, releases the mRNA (Savelsbergh et al. 2009) and prevents subunits

from re-association, thus recycling ribosomes for a new round of translation (Subramanian and Davis 1970, Kaempfer 1972).

2. Alarmone (p)ppGpp and The Stringent Response

2.1 Physiological role of (p)ppGpp in bacteria

The (p)ppGpp plays key role in the activation and regulation of the adaptive mechanisms that bacteria employ in order to accommodate to the adverse conditions (Haseltine and Block 1973, Hauryliuk et al. 2015, Liu et al. 2015). Most of the knowledge about this mechanism is obtained from observations in *E. coli*, however, the enzymes involved are widespread in almost all species of bacteria and plants (Atkinson et al. 2011).

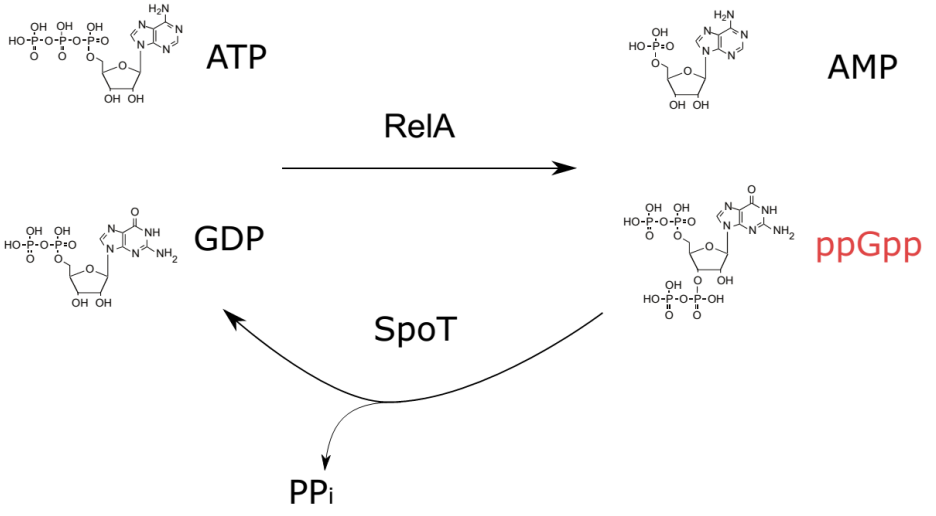

Figure 6. ppGpp synthesis and degradation. Guanosine tetraphosphate (ppGpp) (in red) formation by RelA from ATP and GDP /GTP nucleotides and dephosphorylation to GDP/GTP and inorganic phosphate, PPi, by SpoT.

ppGpp and pppGpp are synthesised by RelA/SpoT Homolog (RSH) enzymes (**Figure 6**) (Atkinson et al. 2011). The large multi-domain proteins RelA and SpoT that gave a name to the protein family RelA-SpoT-Homologue, were the first proteins historically described that are involved in both synthesis and degradation of (p)ppGpp. The two proteins have common evolutional origin from an ancestral bifunctional ribosome-dependent Rel protein (Mittenhuber 2001, Atkinson et al. 2011).

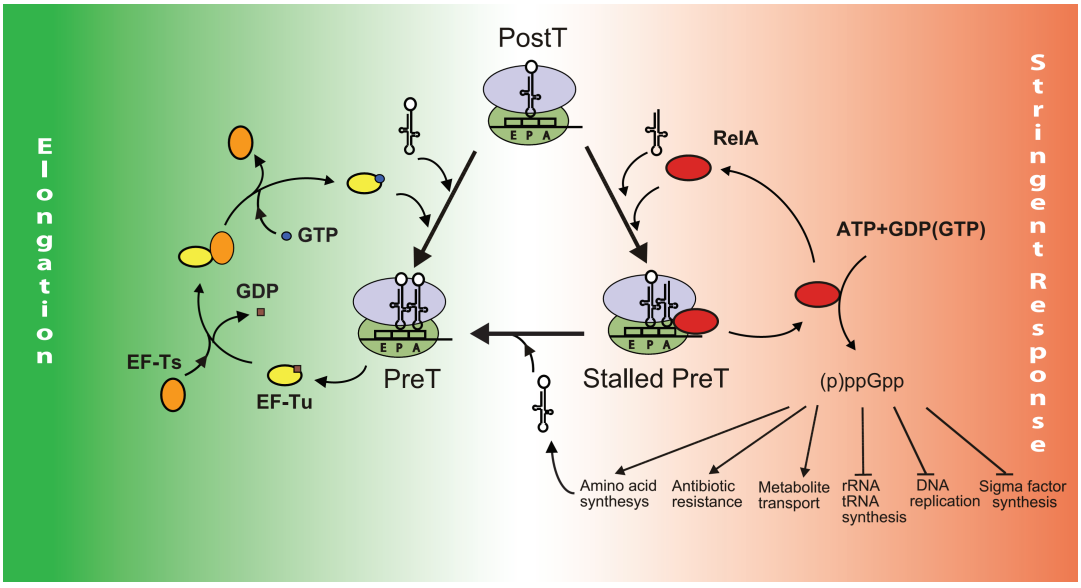

Figure 7. Schematics of (p)ppGpp synthesis and degradation by RSH enzymes. In bacteria, starvation signals can trigger activation of the stringent response via RelA. RelA is activated at the ribosome when translation is halted due to the entry of an uncharged tRNA into the A-site.

RelA has pronounced, ribosome-dependent (p)ppGpp synthesis activity (Haseltine et al. 1972) tightly regulated by the ribosome translational state. RelA is strongly activated by ribosomes containing deacylated tRNA in A-site while active translation holds RelA in enzymatically inactive state (**Figure 7**) (Haseltine et al. 1972, Wendrich et al. 2002). Activation of RelA upon amino acid starvation, accumulation of (p)ppGpp and the following rewiring of bacterial physiology is referred to as 'the stringent response'.

Until recently, the lack of detailed RelA structure limited the understanding of mechanisms behind its binding and activation. It has been shown that RelA binds to the large subunit (Ramagopal and Davis 1974) and it is strongly dependent on the ribosomal protein L11 for activation (Knutsson Jenvert and Holmberg Schiavone 2005). Recent cryo-EM structures of the RelA:ribosome complex shows that the ribosome-bound RelA is stabilizing an unusual tRNA form, with the acceptor arm making contact with RelA far from its normal location in the peptidyl transferase center (Agirrezabala et al. 2013, Arenz et al. 2016, Brown et al. 2016, Loveland et al. 2016). RelA bound to deacylated tRNA containing ribosome adopts distinct confirmation where the C-terminal domain is wrapped around a highly distorted A-site tRNA (Arenz et al. 2016, Brown et al. 2016).

SpoT possess weak (p)ppGpp synthesis activity (Xiao et al. 1991), stimulated by iron and fatty acid limitations (Vinella et al. 2005, Battesti and Bouveret 2006). However, SpoT has much stronger hydrolytic activity towards

(p)ppGpp (An et al. 1979). SpoT may sense many other kinds of starvation (carbon source, iron, phosphate, fatty acid and nitrogen) (Spira et al. 1995, Vinella et al. 2005) and is involved in surface attachment in *E. coli* by regulating ppGpp-mediated biofilm formation (Boehm et al. 2009).

2.2 (p)ppGpp

The main impact of (p)ppGpp production is regulation of transcription through binding and altering the activity of RNA polymerase (RNAP) (Reddy et al. 1995, Ross et al. 2013). In *E. coli* (p)ppGpp and transcription factor DksA directly bind to the RNAP, and thus, down-regulating transcription from the promoters of rRNA and ribosomal protein genes (Murray et al. 2003, Lemke et al. 2011) and enhancing the transcription of amino acid biosynthesis genes (Paul et al. 2005). In *Bacillus subtilis*, (p)ppGpp regulates transcription of rRNA operon promoters indirectly by changing GTP/ATP ratio, hence, regulating transcription via effects on the concentration of initiator nucleotide (Krasny and Gourse 2004).

Besides regulation of the transcription, (p)ppGpp is involved in many others physiological processes, such as regulation of mRNA half-life (Gatewood and Jones 2010), cytoplasmic polyphosphate levels (Kuroda et al. 2001) and DNA replication (Wang et al. 2007). The pleiotropic effects of ppGpp are responsible for its role in antibiotic resistance (Nguyen et al. 2011), biofilm formation (He et al. 2012), formation of persisters (Helaine and Kugelberg 2014) and many others phenotypes. New (p)ppGpp functions are still being discovered – e.g. inhibition of the ribosome assembly (Corrigan et al. 2016).

3. Dynamics of cellular proteins

3.1 Single particle tracking

The tracking of molecules using single particle tracking (SPT) provides information at single molecule level as opposed to bulk measurements providing averaged information about many and many molecules. The wide range of application of SPT includes analyses of cell surface molecules (Baker et al. 2007, Carayon et al. 2014), viral infection of cells (Brandenburg and Zhuang 2007, Sun et al. 2013) and gene expression (Janicki et al. 2004, Coulon et al. 2014, Newhart and Janicki 2014).

The tracking of individual particles can give valuable information about the diffusion-related biological processes. However, in order to be observed, the molecules of interest need to be labeled, tracked and analyzed. The main obstacle during all these steps is the noise coming from different sources, such as background fluorescence, labels dark state or pixelation. Microscope resolution is an important factor to be considered for overcoming this problem, however, certain noise can be generated from other sources.

The most common approach for labeling protein molecules for studying the dynamics of cellular processes by SPT is fusion with fluorescent proteins (Harms et al. 2001, Elf et al. 2007). Fluorescent proteins, FPs, are widely applied for studies of the molecular mechanisms of various molecular and cellular functions inside the live cells. Fluorescently labelled proteins can be genetically encoded and functionally independent of additional cofactors. Transfected cells expressing fluorescent proteins enhance resolving the heterogeneity and spatial organization of the target proteins to which they are bound (Betzig et al. 2006). Although widely used, the labeling with fluorescent proteins has its technical disadvantages. The main problems are fast photobleaching rate, limiting molecule tracking into very short time frames (Yu et al. 2006) and maintaining low expression levels, which allows fluorescent molecules to be detected individually. Many fluorescent proteins, when expressed, can cause false localization patterns and form bright foci due to clustering, and thus, altering the natural diffusion behavior (Landgraf et al. 2012).

The use of photoactivatable fluorescent proteins that transform their spectral properties in response to irradiation with light of a specific wavelength and intensity have added new possibilities to the single molecule localization methods. For example, photoconvertible single-molecule label Dendra2 (Gurskaya et al. 2006) allows photoactivation control on top of the protein expression regulation (Niu and Yu 2008).

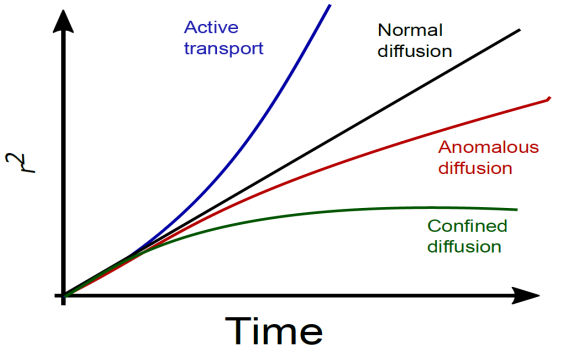

Figure 8. Plots of mean square displacement (MSD) as a function of time for different diffusion modes. Freely diffusing molecules feature an MSD (black line) proportional with time. Molecules whose diffusion is hindered by obstacles (red line) or confined (green line) result in plateauing of the MSD (D<1) curve for longer time intervals. Molecules going with a flow or being actively transported show an upward curvature with time (blue line).

The images acquired by SPT tracking represent individual fluorescent particles on dark background with each frame of the movie representing the position of the particle at certain time point. The particle trajectories are obtained by

extraction of their x-and y-coordinates diffraction from the frames where they are present. The extraction is obtained by 2D-Gaussian function fitting of each particle intensity profile and afterward used for calculating the equivalent trajectories based on a nearest neighbor algorithm (Sbalzarini and Koumoutsakos 2005, Godinez et al. 2009).

The resulting trajectories are most commonly analyzed by calculating of the mean square displacement, MSD, as a function of time (**Figure 8**). MSD provides information about particles motion behavior by representing squared distances between a particle's start and end position for all time-lags within one trajectory (Saxton 1997). The diffusion behavior is interpreted by fitting-in it to one of the standard types of motion: confined normal (Brownian) diffusion, anomalous subdiffusion, and active transport (**Figure 8**). Nevertheless, multiple transitions between different types of diffusion can occur and complicate the analysis.

As opposite to eukaryotic proteins, bacterial ones are usually not confined into compartments in their movement, with a exception of few special cases (Shapiro et al. 2002). The protein mobility can range from free diffusion to confined motion or immobilization. However, membrane proteins diffusion path is restricted to the plasma membrane surface and is locally two dimensional as opposed to the three dimensional movement of cytosolic proteins.

3.2 Diffusion behavior of membrane proteins

Protein dynamics and lateral diffusion in cell membrane is the most important mechanism that shapes the cell interaction with the environment. The dynamics of this process governs membrane-protein complex formation, cellular transport and cell integrity. The use of SPT techniques has revolutionized investigations of protein diffusion in membranes. However, most of the research has been focused on eukaryotic cell membrane proteins where proteins freely diffuse in confined microdomains (Vrljic et al. 2002, Douglass and Vale 2005). Much less, SPT data is available for bacterial membrane proteins. One of the earliest studies on protein diffusion in *E. coli* membrane showed that LamB (maltodextrin transport channel) displays confinement into a region with diameter of 29 nm (Oddershede et al. 2002) or 100–300 nm teetering (Gibbs et al. 2004). Other proteins studied with SPT imaged at 40 Hz, BtuB and OmpF exhibit very slow and confined (BtuB, 0.05 ± 0.01 $\mu m^2 s^{-1}$) or long range (OmpF, 0.006 ± 0.002 $\mu m^2 s^{-1}$) mobility. Similar slow Brownian diffusion was observed for flagella motor protein MotB labeled with green fluorescent protein (GFP) (0.0088 ± 0.0026 $\mu m^2 s^{-1}$) (Leake et al. 2006) and for the membrane-bound histidine kinase PleC (0.012 ± 0.002 $\mu m^2 s^{-1}$) (Deich et al. 2004).

3.3 Cytoplasmic diffusion of proteins

The cytoplasm is confined crowded space where molecules mainly rely on diffusion for interaction. Indeed, measuring parameters like diffusion coefficients and distributions of molecules can answer many questions regarding their behavior and patterns of interaction (Lippincott-Schwartz et al. 2001, Luby-Phelps 2013). The mobility of biomolecules is characterized by many different methods, mainly depending on fluorescent probes and single-molecule approaches.

In order to explore cell microenvironment, SPT of GFP molecules is combined with simulation of Brownian motion. The efficiency of the GFPs fluorophores is strongly dependent on cell micro conditions and particularly on the concentrations of molecular oxygen (Bogdanov et al. 2009). The structure and content of the cytoplasm play an important role in protein diffusion in the cell. The cytoplasm is highly dynamic environment with non-uniform diffusion properties. Various dynamic processes, such as active transport, polymerization of cytoskeletal elements (Shih and Rothfield 2006) or vesicle transport (Vale 2003), together with the fact that cytoplasm is generally not a simple viscous fluid but has rather complex arrangement (Luby-Phelps et al. 1987, Fabry et al. 2001) make cytoplasmic diffusion characterization quite complicated.

Earlier studies have investigated diffusion of proteins in the cytoplasm of *E. coli* employing techniques such as fluorescence recovery after photobleaching (FRAP) that is able to obtain bulk diffusion coefficients (Swaminathan et al. 1997, Partikian et al. 1998, Dayel et al. 1999). Latest developments in high-speed single-molecule microscopy allow individual molecule diffusion imaging in the three-dimensional cytoplasm of the cell (Beausang et al. 2013, Perillo et al. 2015).

According to studies using FRAP (Terry et al. 1995), the diffusion coefficient of fluorescent proteins expressed in *E. coli* cytoplasm is 6–14 $\mu m^2\ s^{-1}$, while initial single molecule experiments using yellow fluorescent protein (YFP) tagged structural protein MreB observed diffusion coefficient in the range of 1.6–1.95 $\mu m^2\ s^{-1}$ (Kim et al. 2006). Recent single-molecule studies using higher sampling rate of 250 Hz showed that mEos2, a freely diffusing photoconvertible GFP variant diffuses in the cytoplasm with 13 $\mu m^2\ s^{-1}$ (English et al. 2011) while another photoconvertible protein Kaede was shown to diffuse homogeneously within 6.2-7.4 $\mu m^2\ s^{-1}$ (Bakshi et al. 2011).

All these examples of protein mobility in bacterial cytoplasm are just a small illustration of the capacity of single-molecule microscopy techniques to resolve essential biologically questions at the level of single molecules.

RESULTS AND DISCUSSION

Aims of the study

The specific **Aims** of this work were:

- To establish an experimental system for tracking single cytoplasmic and membrane protein molecules that enables to follow rapidly moving freely diffusing molecules.
- To establish the enzymatic cycle of RelA in living bacterial cell by following RelA's diffusive behavior during starvation and unperturbed growth.
- To uncover the effects of RelA's product, ppGpp, on RelA's enzymatic activity.

1. Single molecule tracking

Detection of single molecules in living cells is a powerful method that enables to examine biological events at a level inaccessible for the conventional measurements techniques. Single-molecule fluorescence tracking is bringing a new view into cellular processes at unique structural and temporal resolution.

Nowadays, the systems used for *in vivo* tracking are mostly based on photo-activated localization microscopy (PALM) (Betzig et al. 2006, Hess et al. 2006) and stochastic optical reconstruction microscopy (STORM) (Rust et al. 2006). Those super resolution microscopy techniques are able to capture images with a higher resolution than the diffraction limit, but are restricted to observation of considerably slowly diffusing (Kim et al. 2006), membrane bound (Gibbs et al. 2004) or immobile molecules (Elf et al. 2007). In order to optimize these methods, stimulated emission depletion-fluorescence correlation spectroscopy (STED-FCS) conjunction was used (Sahl et al. 2010) resulting in increased temporal resolution but limited spatial array.

In publications I (Kuzmenko et al. 2011) and II (English et al. 2011), we have developed *in vivo* tracking microscopy assay that allowed us to track fast and slowly diffusive cytosolic (stringent factor RelA and free GFP variant mEos2) or membrane bound (mitochondrial membrane channel Tom40) proteins. We combined super-resolution tracking of photoconvertible proteins (Manley et al. 2008, Niu and Yu 2008) with stroboscopic time-lapse imaging (Xie et al. 2006), a method used in strobe photography adding an extra sharpness to the picture taken. This was achieved by laser exposure for short time intervals in which the reporter molecule does not diffuse beyond the diffraction-limited spot. The short laser pulses where synchronized with the frame time of the camera enabling observation of fluorophores during the laser flash and avoiding autofluorescent background of environment, such as the crowded bacterial cytosol or cell membrane (**Figure 9)**.

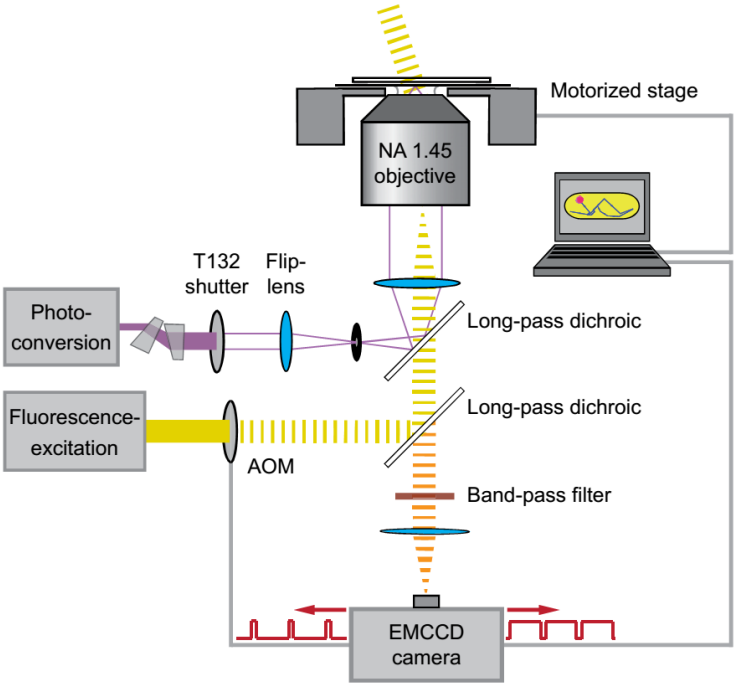

Figure 9. Schematic diagram of the optical setup used in the current work. A violet photoconversion laser (405 nm) and a wide-field yellow excitation laser (555 nm) beam are spatially overlapped and focused onto the sample by flip-lenses. The violet photoconversion laser beam is shuttered by a mechanical shutter and synchronized with an EMCCD camera. (adapted with permission from English et al. 2011.)

1.1 Single-molecule tracking of membrane proteins

The structure and function of bio-membranes and its components has been investigated in details, however, there is very limited information about the dynamics of the cell membrane protein components. One promising model system for membrane single molecule research is the yeast mitochondrion. They can be easily visualized with vital fluorescent dyes, immunofluorescence, or targeted fluorescent proteins possessing low background of fluorescence. In addition, mitochondria also retain strong evolutionary conservation in the biogenesis of membrane proteins making them promising model system for studying membrane transport (Zeth 2010, Ulrich et al. 2014).

Previous research on the mitochondrial membrane protein dynamics is limited to only few components, such as Tom7, displaying heterogeneous diffusive properties within several sub-populations (Sukhorukov et al. 2010).

In publication I (Kuzmenko et al. 2011), we investigated, by means of single-molecule tracking microscopy the diffusion of the main mitochondrial protein import component Tom40. The fluorescently labeled Tom40-Dendra2

complex in the mitochondrial membrane showed highly mobile but confined diffusion properties.

The isolated and immobilized intact *Saccharomyces cerevisiae* mitochondria with Tom40-Dendra construct, were imaged (**Figure 10A**), with frame and exposure time of 5 ms and analyzed in a comparison with immobilized Dendra2 molecules (**Figure 10B**) in order to achieve accuracy and stable vibrational control of the microscopy setup.

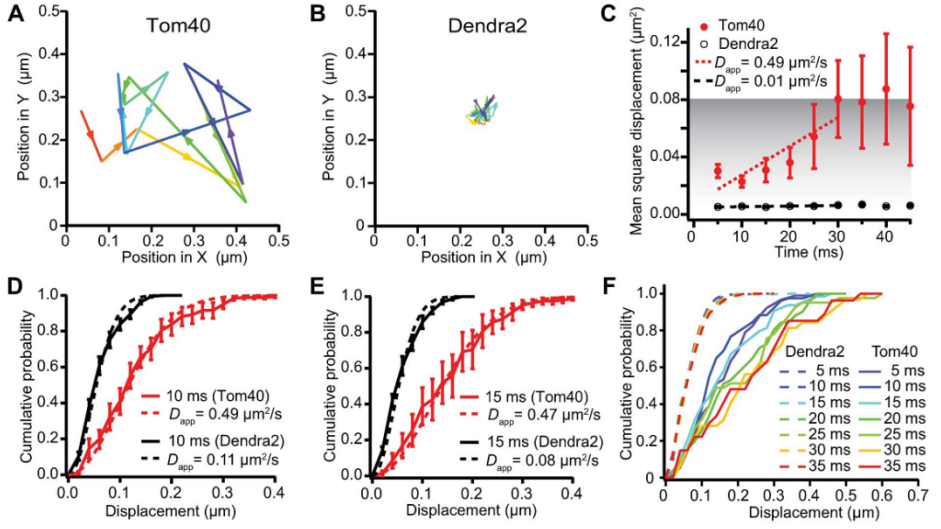

Figure 10. Analysis of Tom40 diffusion as compared with immobilized Dendra2 molecules. A) Experimental single molecule trajectory of Tom40 with a frame time of 5 ms and an exposure time of 5 ms. **B**) Tracking of immobilized Dendra2 protein. One single molecule trajectory with a frame time of 5 ms and an exposure time of 5 ms. **C**) Trajectory-averaged mean square displacements (MSDs) over different time intervals. The error bars represent the experimental standard errors of the means. MSDs from Tom40 (red) and MSDs from immobilized Dendra2 molecules (black). **D**) Trajectory-averaged cumulative distribution functions (CDFs) of displacements over 10 ms for Tom40 (in red) and immobilized Dendra2 (in black). The error bars represent the experimental standard errors of the means. **E**) Trajectory-averaged cumulative distribution functions (CDFs) of displacements over 15 ms for Tom40 (in red) and immobilized Dendra2 (in black). The error bars represent the experimental standard errors of the means. **F**) Step-averaged cumulative distribution functions (CDFs) of displacements over 5–35 ms for Tom40 (solid lines) and immobilized Dendra2 (dashed lines), color-coded as indicated in the insert box. (Copied with permission from Kuzmenko et al. 2011.)

We have shown that Tom40 expresses strikingly different patterns of diffusion (0.5 $\mu m^2 s^{-1}$) (**Figure 10 C, D and E**) when compared with typical eukaryotic membrane proteins (Simons and Sampaio 2011). Tom40 diffuses considerably

freely, but confined within domains similarly to the diffusion patterns of bacterial protein PleC (Deich et al. 2004) or Lck clusters in T-cells (Douglass and Vale 2005).

The nature of this spatial restriction of Tom40 diffusion could be result of the highly heterogeneous nature of the mitochondrial membrane and/or caused by protein-protein interactions within the membrane. These results showed that the single particle tracking (SPT) time-lapse assay that we have developed and implemented, enabled us to quantitatively describe the diffusion properties of membrane proteins such as Tom40.

1.2 Single-molecule tracking of freely diffusing proteins

A molecular mechanism of the stringent response induction has been proposed and summarized as the so-called hopping model (Wendrich et al. 2002). The model suggests that during the stringent response, deacylated tRNA blocks ribosome A-site, RelA binds to the stalled ribosome, adopts catalytically active conformation and synthesizes one molecule of (p)ppGpp. The conversion of ATP and GTP leads to (p)ppGpp production resulting in conformational changes in RelA that lowers the affinity to the ribosome. The dissociation of RelA from the ribosome is followed by 'hopping' to another ribosome. Thus, RelA performs general scanning of the cells translational system (Wendrich et al. 2002).

In publication II (English et al. 2011), we directly tested and evaluated the hopping model *in vivo* by employing our stroboscopic single molecule tracking microscopy method. We examined the individual diffusion characteristics of single RelA molecules throughout the (p)ppGpp synthesis cycle.

We tracked two reference molecules: the small cytosolic freely diffusing photoconvertible GFP variant mEos2 (McKinney et al. 2009) as a reference for free unbound RelA and a GFP photoconvertible variant Dendra2 (Gurskaya et al. 2006) tagged ribosomes as a comparison to ribosome-bound RelA (**Figure 11A**). Both mEos2 and Dendra2 are monomeric photoconvertible proteins that fold efficiently at 37° C and successfully label targets that are intolerant of fusion to fluorescent protein dimers and tandem dimers. These fluorescent tags produce less clustering artifacts than other fluorescent proteins, although not ideally mimicking the wild type (Landgraf et al. 2012).

Nonactivated 'green' Dendra2 possesses excitation maximum at 490 nm and emission maximum at 507 nm. Similarly, mEos2 has green absorbance peak at 506 nm and green emission peak at 516 nm when inactivated. After irradiated with UV light, Dendra2 and mEos2 photoconvert to their red state with excitation-emission maximum 553/557 nm for Dendra2 and 571/581 for mEos2 (Chudakov et al. 2007).

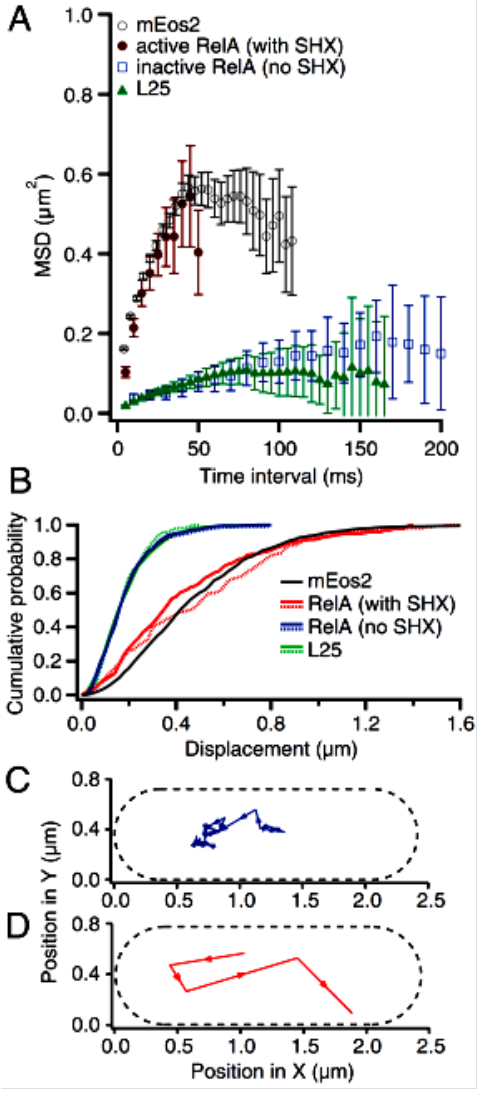

Figure 11. Diffusion of ribosomes, RelA and a small fluorescent protein mEos2 in actively growing cells and during the stringent response. **A**) Mean square displacements (MSDs) from ribosomal protein L25 (in green) and inactive RelA (in blue) are indistinguishable during *E. coli* exponential growth. Experimentally induced stringent response by addition L-Serine hydroxamate changes dramatically the RelA diffusion (in brown) resulting in similar diffusion behavior to mEos2 (in grey). The error bars represent the experimental standard errors of the means. **B**) Cumulative distribution functions (CDFs) of displacements of inactive RelA (in solid blue) and L25 (in dashed-green) with 20-ms frame time showing very similar diffusion behavior. The apparent diffusion coefficient of RelA when cells are starved increases more than eightfold (red and dashed-red curves) and is very similar to the CDF of mEos2 (in gray).
C) One experimentally obtained single-molecule RelA trajectory with a frame time of 20 ms and an exposure time of 2 ms during cells exponential phase. **D**) One experimentally obtained single-molecule RelA trajectory with a frame time of 20 ms and an exposure time of 2 ms when cells during experimentally induced stringent response using L-SHX. (adapted with permission from English et al. 2011.)

Comprehensive analysis of the single mEos2 trajectories showed very fast evenly distributed diffusion of the molecules, screening the whole cell cytosol (**Figure 11B**).

By analyzing the local apparent diffusion coefficients of small molecule subpopulations located in different cell sections within 4 ms, we observed some spatial variation in apparent diffusion coefficient ranging from 8 to 16 $\mu m^2 s^{-1}$. These variations in the apparent diffusion coefficient were correlating with bacterial cell shape and faster diffusion of the molecules in the less confined middle section compared with the restricted diffusion next to the cell walls.

Additionally, an experimental comparison of MSD curve of mEos2 and MSD curves from simulated normal diffusion trajectories showed very similar diffusion patterns of mEos2 compared with the random motion **(Figure 11A)**.

Sharply distinguishable MSD curve of the Dendra2 labeled ribosomes compared with mEos2 showed around 25 times slower diffusion of ribosomes. The apparent diffusion of the fluorescently labeled ribosomes used as a reference for ribosome-bound RelA displayed much higher confinement observed as plateauing in MSD curves compared with the mEos2 one **(Figure 11B)**.

This confinement could be a result of localization of the translational process in certain cell areas (Lewis et al. 2000) or ribosome-mRNA tethering in translational complex (Montero Llopis et al. 2010) as most of the ribosomes are actively translating (Scott et al. 2010).

Taking into account the two control experiments, we performed in vivo tracking of RelA fused with Dendra2 at C-terminus. The mean MSD curves and cumulative distribution functions of displacements showed statistical parity with the ribosomal ones indicating that RelA exhibits similar diffusive behavior as the ribosomes (**Figure 11C**). The results are clearly supporting the idea that during non-starving conditions, RelA is tightly bound to the ribosomes. Expectedly, when we tracked and analyzed RelA diffusion behavior under starved condition induced by L-Serine Hydroxamate (SHX) (competitive inhibitor of seryl-tRNA synthetase), we observed intensive shift in RelA diffusion behavior. The diffusion pattern that RelA displayed during stringent response becomes very similar to the one that mEos2 had in our reference experiments (**Figure 11D**). RelA freely diffuses through the whole volume of the bacterial cell while in its active ribosome-free state. Additionally, we showed that RelA dissociates from the ribosomes and diffuses freely during heat-shock similarly to SHX-induced stringent response.

These results are clearly correlating with the main aspects of the hopping model (Wendrich et al. 2002). However, we do not detect RelA rapid shifting between its ribosome-bound and free state that is predicted by the hopping model. Recently, similar single molecule study reported different RelA diffusion patterns (Li et al. 2016) with stronger ribosome binding after induced starvation. In addition, much less freely diffusing RelA molecules in both normal and starved conditions were detected (Li et al. 2016). Although the study has additional advantages, such as double starving conditions (cells grown in AA free medium and inclusion of SHX), less activation laser power for shorter periods and using three different labeling schemes (RelA-YFP, RelA-mEos2 and RelA-Dendra2), the discrepancy of the results between both studies have no explanation. However, Poly(L-lysine) cell adhesion to the coverslip for imaging, used by Li et al., 2016 is very efficient technic but can disrupt the proton-motive force (Katsu et al. 1984, Strahl and Hamoen 2010) and strongly affect protein localization (Colville et al. 2010) in *E. coli*.

Moreover, we observe that all RelA molecules remained dissociated from the ribosome for hundreds of milliseconds, which suggests different (p)ppGpp synthesizing mechanism from the standard hopping model. Thus, an extended

hopping model is proposed, where many (p)ppGpp molecules are produced upon dissociation of enzymatically active RelA from the ribosome. This new model can be rationalized in the framework of the existing biochemical data for RelA (Mechold et al. 2002) and is further explained by positive feedback loop acting at the enzymatic level (Shyp et al. 2012).

2. Allosteric activation of RelA by (p)ppGpp

The accumulation of (p)ppGpp during the stringent response leads to rRNA and ribosomal protein genes transcription inhibition. Simultaneously (p)ppGpp activates transcription from the promoters of amino acid biosynthesis genes (Paul et al. 2005). The nature of such regulation demands rapid accumulation of (p)ppGpp synthesized by RelA for the activation of the stringent response. Enzymatic feedback inhibition and feedback activation are widely used as pathway regulation by the cell with the latter being extremely rear process. Previous observations of the ppGpp production during time course (Payoe and Fahlman 2011) showed deviations from linearity in earlier time points due to a lag effect suggesting different mechanism of regulation than negative feedback auto-inhibition.

In publication III (Shyp et al. 2012), we investigate the nature of the lag effect using in vitro stringent response system similar to that used in Jones et al. 2008. We showed that production of (p)ppGpp is responsible for enhancement of the RelA enzymatic activity by positive feedback loop acting at the enzymatic level. The stimulatory effect is specific for ppGpp, and other nucleotides do not influence the RelA specific activation.

The mechanism of RelA product-mediated activation is strictly specific for (p)ppGpp and it is strongly dependent on the ribosomal protein L11. The activation effect of (p)ppGpp is not altered in the presence of other strong RelA activators, such as A-site tRNA suggesting different mechanisms of influence (**Figure 12 A, B, C and D**).

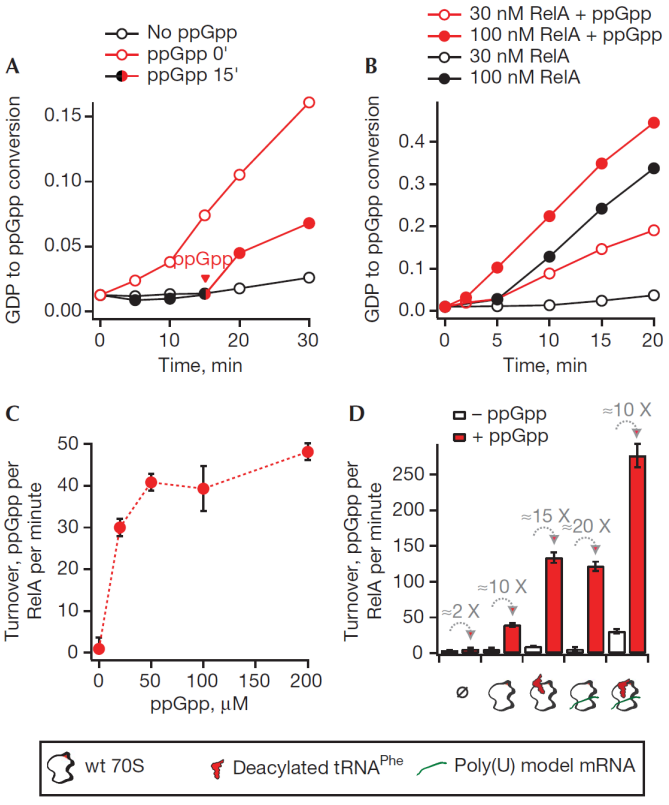

Figure 12. Activation of 70S-dependent synthetic activity of RelA by its product, (p)ppGpp. A) Time course of 70S-dependent ppGpp synthesis with the addition of (p)ppGpp at 0 min (hollow red circles) or at 15 min (solid circles, black and red) and the absence of (p)ppGpp (hollow black circles) **B**) Time course of 70S-dependent (p)ppGpp synthesis by RelA in the absence (black circles) and presence (red circles) of (p)ppGpp with using 30 nM (hollow circles) and 100 nM (solid circles) RelA. **C**) 70S-dependent RelA synthetic activity as a correlation of (p)ppGpp concentration. **D**) 70S ribosomes, poly (U) and deacylated $tRNA^{Phe}$ effect on (p)ppGpp synthesis in the presence (solid red bars) and absence (hollow bars) of (p)ppGpp. RelA regulation by its product allows fast accumulation of (p)ppGpp, and thus, rapid modulation of the transcription (adapted with permission from Shyp et al. 2012).

The direct allosteric regulation of RelA by (p)ppGpp is the first described example of an enzyme regulated through direct positive feedback control by its product. In addition to the long (p)ppGpp synthetases, some bacteria contain considerably smaller enzymes with fewer regulatory domains. Recently, the product activation was shown also for some of these small alarmone synthetases (SAS) (Gaca et al. 2015, Steinchen et al. 2015). The physiological relevance of RSH activation by its product, as well as the molecular mechanisms of this regulation are yet to be uncovered.

CONCLUSIONS

1. By combining super-resolution tracking of photoconvertible proteins with high-speed stroboscopic time-lapse imaging, we have set up a single particle tracking system for measuring the diffusion of membrane proteins, cytoplasmic proteins and macromolecular complexes.
2. The RelA molecules are bound to the ribosomes longer under unstarved conditions as compared to starvation. This suggests that activation of the enzyme is accompanied with dissociation from the ribosome.
3. RelA is allosterically activated by its reaction product, (p)ppGpp. This suggests a mechanism for very rapid triggering of stringent response.
4. The mitochondrial transport channel Tom40 does not exhibit free diffusion in the mitochondrial membrane. On the contrary, Tom40 is diffusing in outer mitochondrial membrane in a highly mobile but confined manner.

SUMMARY IN ESTONIAN

Juhukõnnid translatsioonis

Poomisvastus on võtmetähtsusega adaptiivsete mehhanismide regulatsioonil, mis aitavad bakteritel ebasoodsaid keskkonnatingimusi üle elada. Soolekepikeses (*Escherichia coli*) on selles protsessis oluliseks ensüümiks RelA, mis vastusena aminohappenäljale sünteesib signaalmolekuli (p)ppGpp. See signaalmolekul mõjutab transkriptsiooni, translatsiooni ja rakkude jagunemist.

Meie töötasime välja ühe molekuli jälgimise mikroskoopia metoodika, mis võimaldab mõõta molekulide difusiooni rakus. Kasutasime seda metoodikat erineva kiirusega liikuvate molekulide kirjeldamiseks. Rakus vabalt difundeeruva valgu näiteks oli fluorestseeruv valk mEos2. Hoopis teistsuguste omadustega valguks osutus mitokondri membraanivalk Tom40, mille liikumine on ühte asukohta piiratud. RelA puhul täheldasime nii vabu, kiirelt difundeeruvaid molekule kui ka ribosoomile seondunud ja seetõttu aeglaselt liikuvaid molekule.

Kombineerides ühe molekuli jälgimise tulemusi biokeemiliste andmetega, pakume välja RelA valgu töötsükli mudeli. Kuhjuv (p)ppGpp põhjustab samuti RelA aktivatsiooni. Sellisel viisil tekib positiivse tagasisidestusega regulatsioonisüsteem ja signaalmolekuli kontsentratsioon tõuseb kiiresti.

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