transARREST summary

During protein synthesis by the ribosome, the nascent polypeptide travels through the exit tunnel, a long cavity connecting the active site where peptide bonds are formed to the intracellular milieu. Although this was previously deemed a passive process, it is now established that certain peptide sequences interact with the ribosomal RNA and proteins lining the walls of the exit tunnel to pause or arrest protein synthesis. In many cases, translational arrest also depends on the presence of a small molecule – such as an amino acid or an antibiotic – that is sensed by ribosomes engaged in the synthesis of a specific arrest peptide.

In order to gain a deeper insight into this process, we focused on two complementary objectives:

1. To understand the mechanisms through which nascent chains regulate the ribosome

In order to fulfill this objective, we sought to obtain high-resolution X-ray structures of various stalled ribosome nascent chain complexes (RNCs) and perform their biochemical characterization. At the time when the project was undertaken, strategies for preparing RNCs relied on cell-free expression systems and produced samples of varying quality and stoichiometry, precluding their analysis by X-ray crystallography. To address this issue, we sought to prepare peptidyl-tRNAs directly by attaching synthetic peptides onto tRNAs expressed *in vivo*, using small RNA enzymes known as flexizymes. Peptidyl-tRNAs obtained in this manner can be mixed with the ribosome to form RNCs, thus sidestepping some of the problems stemming from ribosomal peptide synthesis. Work in this area focused on two distinct biological systems: (i) translational arrest in the presence of macrolide antibiotics by peptides carrying short +X(+) arrest motifs, and (ii) polyproline-dependent translational arrest and stalled ribosome rescue by elongation factor EF-P. Although high-quality RNCs could be obtained for both systems, the resolution revolution sweeping the cryo-electron microscopy (cryo-EM) field encouraged us to use this technique over X-ray crystallography as our method of choice for structural characterization. Ultimately, we obtained a high resolution cryo-EM reconstruction of a bacterial ribosome undergoing drug-dependent translational arrest at a +X(+) motif, explaining how these ubiquitous tri-amino acid sequences block ribosomes in the presence of widely-used antibiotics, such as erythromycin. Importantly, the biological integrity of the RNCs assembled *in vitro* could be established using biochemical assays. This work forms the basis of an upcoming publication.

In addition, our crystallographic analyses of peptide-mediated translational inhibition took us into an unexpected direction, as we demonstrated that proline-rich antimicrobial peptides (PrAMPs) produced by the host immune response of insects and mammals block translation by binding to the exit tunnel of the bacterial ribosome (Seefeldt *et al.* (2016) *Nucleic Acids Res* 44, 2429; Seefeldt *et al.* (2015) *Nat Struct Mol Biol* 22, 470) (Figure 1). The overlapping binding sites for arrest peptides, PrAMPs and antibiotics that target the large ribosomal subunit suggest that an increased understanding of these three types of ribosomal ligand could help us design improved translation inhibitors. These might, in turn, form the basis for novel antimicrobials with greater efficacy against multidrug-resistant bacterial pathogens.

2. To explore the functional diversity of nascent chain-mediated phenomena

Translation inhibition by arrest peptides is critically dependent on their amino acid sequence, but often requires an additional low molecular weight ligand, such as a drug or a metabolite, to be sensed by the RNC. As a result, arrest peptides are used for metabolite-dependent gene regulation in both prokaryotes and eukaryotes (Seip & Innis (2016) *J Mol Biol* 428, 2217) (Figure

2). Biological processes that are regulated by arrest peptides in bacteria include the induction of the *erm* resistance genes by macrolide antibiotics (e.g. erythromycin), the sensing of soluble tryptophan by a ribosome-associated TnaC peptide, targeting of the expression of the SecA pre-protein translocase to the cell membrane by the nascent SecM polypeptide, the expression of the YidC2 membrane insertase by the MifM peptide and the regulation of SecDF2 in low-salinity environments by the arrest peptide VemP.

Biochemical and structural studies have shown that interactions between nascent peptides and the ribosome that induce translational arrest do so by impairing tRNA accommodation, peptide bond formation or peptide release. However, the arrest code dictating whether a given nascent peptide is prone to inhibiting its own synthesis has yet to be elucidated, the range of metabolites that can be sensed by the nascent peptide is unknown and the molecular bases of the arrest mechanism itself are only partially understood. In order to address these questions, we developed inverse toeprinting, a new method to map the position of ribosomes arrested on messenger RNAs during *in vitro* translation (Seip *et al.* (2018) bioRχiv, doi: https://doi.org/10.1101/298794). This approach directly links the translational arrest phenotype to the genotype that causes it, enabling us to simultaneously monitor the stalling potential of $>10^{11}$ peptides in a high-throughput fashion. Unlike ribosome profiling, it protects the entire coding region upstream of a stalled ribosome, making it possible to work with transcripts of unknown sequence.

We used inverse toeprinting to characterize the pausing landscape of free and drug-bound bacterial ribosomes engaged in translation of a random transcript library. The high-throughput nature of inverse toeprinting resulted in a comprehensive list of arrest motifs along with a quantitative measure of their pause strength, in good agreement with prior *in vivo* data. In addition, we have begun to re-evaluate the primary sequence determinants underlying drug-dependent translational arrest by the Erm leader peptides. These comprise several families of arrest peptides involved in the acquisition of resistance against macrolide-lincosamide-streptogramin-B antibiotics. By integrating the structure and sequence data obtained from these studies, we seek to decipher the rules underlying peptide-mediated translational arrest and to systematically assess its biological extent and distribution in nature. In addition, our method can further be adapted to study other sequence-dependent translational processes.

To conclude, the *transARREST* project helped us establish two key tools for the analysis of peptide-mediated translational arrest. On the one hand, we showed how the flexizyme technology could be used to prepare stalled RNCs for structural studies. In term, this will make it possible to study peptides featuring non-proteinogenic amino acids or even synthetic peptide-foldamer hybrids. On the other hand, we developed a new, high-throughput tool to systematically assess the functional diversity and biological distribution of arrest peptides in nature. An unforeseen outcome of the project was the realization that certain naturally occurring antimicrobial peptides target the exit tunnel of the ribosomal exit tunnel, highlighting key similarities between arrest peptides, PrAMPs and several classes of antibiotics. In term, these similarities might be exploited to design new, improved antimicrobials.