SUPPLEMENTARY INFORMATION

Current project status and preliminary data for each of the two proposed structures are outlined below:

1. Substrate recognition by the aminoglycoside-resistance 16S rRNA (m^7G1405) methyltransferases. With the Dunham Lab (see Biosketch), we previously determined X-ray crystal structures of the pathogen-associated methyltransferase (MT) NpmA and the Myxobacterial MT Kmr bound to the 30S subunit (PMCID PMC4035980 and West *et al.*, in preparation). These enzymes modify 16S rRNA nucleotide A1408 to form m^1A1408 and confer resistance to a diverse range of aminoglycosides. These studies showed that NpmA and Kmr exploit similar features of 30S for substrate docking but employ unique molecular mechanisms to flip A1408 into their active site for modification. Our current focus is on the more clinically-relevant aminoglycoside-resistance rRNA MT subfamily (enzymes ArmA and RmtA through H, identified in multiple human and animal pathogens). These enzymes modify 30S on an adjacent nucleotide in helix 44 (h44), to incorporate m^7G1405 modification and confer exceptionally high resistance to **ALL** 4,6-deoxystreptamine aminoglycosides (e.g. gentamicin, amikacin and plazomicin). Our recent work on RmtC (1) strikingly showed that to access the G1405 target nucleotide, these enzymes appear to significantly disrupt features of the 30S subunit as assessed by the large movement of the head domain. We have recently collected and processed cryo-EM images of 30S-RmtC complex stabilized using the NM6 SAM analog which clearly demonstrate the feasibility of high-resolution structure determination by this method (**Fig. 1**). Our current map has allowed fitting of the RmtC crystal structure into density and confirmation that the enzyme's NTD (N1 and N2 subdomains, **Fig. 1B**) play important roles in 30S binding, including recognition of the same conserved rRNA tertiary surface bound by NpmA and Kmr. *High-resolution structure determination using the NCCAT Titan Krios will be essential for determining all molecular details of substrate recognition and to allow rebuilding of the distorted 30S structure and flipped G1405 base*.

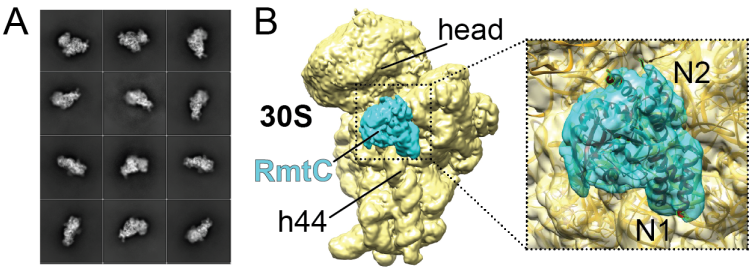

Fig. 1. Preliminary cryo-EM analysis of 30S-RmtC complex. A. 30S-RmtC complex 2D class averages. **B.** Example 3D reconstruction with additional density (cyan) in the expected location of the RmtC (dashed box; ~4.6Å overall resolution).

2. Substrate recognition by the dual specificity (30S/50S) M. tuberculosis (Mtb) 2'-O-MT TlyA. The ribosome-targeting tuberactinomycin antibiotic capreomycin is an essential antibiotic used as a second line treatment against *Mtb*, the causative agent of tuberculosis. Capreomycin binding to the 70S ribosome, and thus its anti-mycobacterial activity, is *dependent* upon 16S and 23S rRNA 2'-O-ribose methylation by the enzyme TlyA. We previously determined the structure of the *Mtb* C-terminal MT domain and identified a potential role for the short interdomain linker in controlling enzyme activity on its two structurally distinct substrates (2). We also hypothesize that the TlyA NTD has distinct but overlapping surfaces that contribute to recognition of 30S or 50S; our current focus is determining the molecular basis for this dual substrate specificity in TlyA beginning with high-resolution structure determination of the *M. smegmatis* 50S-TlyA-NM6 SAM analog complex. We have performed preliminary cryo-EM analysis (**Fig. 2**) clearly demonstrating feasibility of determining this structure. *High-resolution structure determination using the NCCAT Titan Krios will be essential for de novo building of the TlyA NTD and remodeling of 23S rRNA surrounding the C1920 target.* [Note: Once comparable preliminary data are acquired, we will submit an additional proposal to determine the 30S-TlyA complex structure to complete our understanding of dual substrate recognition by TlyA.]

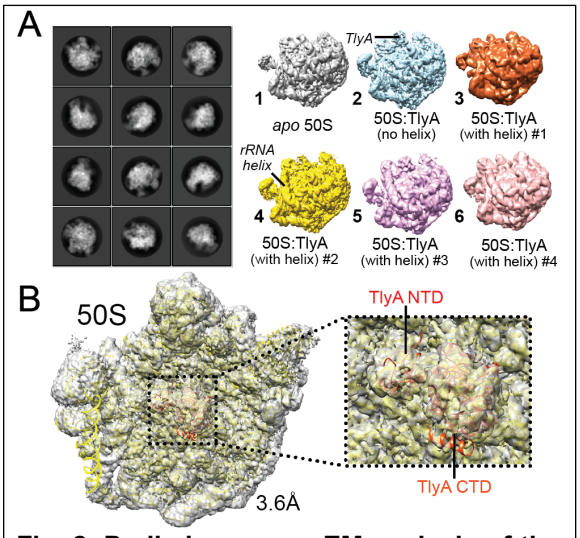

Fig. 2. Preliminary cryo-EM analysis of the 50S-TlyA complex. A. Sample (12 of ~20) 2D class averages of 50S-MT (*left*) and 3D classification identifying *apo* 50S and multiple MT-bound particle classes (*right*). **B.** 50S-TlyA maps (class 2) with fit *M. smegmatis* 50S structure (PDB 5O60) and TlyA.

References: **1.** Nosrati, M., *et al.* (2019). *J. Biol. Chem.* **294**, 17642-17653; **2.** Witek, M.A. *et al.* (2017). *J. Biol. Chem.* **292**(5), 1977-1987.