Sub-Project 1: rRNA modifications and antibiotic resistance (Conn). We have previously expressed/purified target proteins for all three components of this sub-project; protocols for 30S subunit purification are well established and we have an on-going collaboration (Comstock, WFU) to provide the SAM analog ("NM6") that is central to our strategy to trap rRNA methyltransferases (MTs) on the 30S subunit. We have also prepared functional complexes and/or biochemically tested several targets (thereby establishing robust protocols for all other planned structures in this Sub-project): **1a)** *E. coli* 30S complexes with three mechanistically diverse m^1A1408 MTs; **1b**) three pathogen-derived m^7G1405 MTs bound to authentic 30S subunits; and **1c**) *E. coli* 30S complexes with the intrinsic MTs RsmE and RsmH. Examples of our strategy to ensure high quality specimen for data collection at NCCAT are shown in **Fig. 1,** including use of *rsm* deletion strains to prepare unmodified 30S that are substrates for the corresponding MT (**Fig.1A**), and use of NM6 to enable high-resolution structure determination (**Fig.1B**).

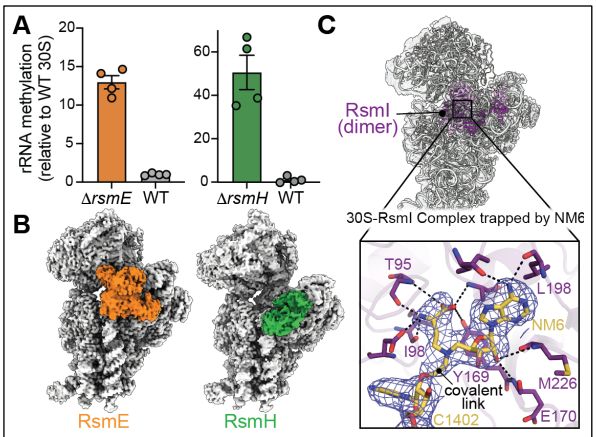

Fig. 1. Preliminary data for Sub-Project 1. **A**, Biochemical and preliminary cryoEM analysis using data from the Emory core for 30S-RsmE and 30S-RsmH (**1c**). **B**, Example of rRNA MT trapping using NM6 shown for 30S-RsmI (~2.4Å) using data collected at NCCAT on a prior period of BAG support.

Sub-Project 2: Novel ribosome-targeting drugs (Conn and Dunham). We will address three key questions in this Sub-project: **2a**) *How do some aminoglycosides adjust their interactions with the ribosome to maintain activity in the presence of resistance rRNA modifications (see 1a,b, above)?* We developed a computational-experimental framework to enable prediction of "evasion" capacity and to guide future aminoglycoside redesign (new analogs will be synthesized by the Crich lab at UGA). Our first target is the 70S (containing the m^1A1408 modification) in complex with micronomicin (a top predicted evader), and we anticipate pursuing structures of at least 1-2 top new analogs from the Crich lab; **2b**) *How do trans-translation inhibitor MBX-4132 and KKL-1005 impair non-stop bacterial ribosomes*? We have preliminary data that these new inhibitors affect both actively growing bacteria and persistent bacteria, unlike all known antibiotics. To understand the molecular basis of this inhibition, we have determined a structure of MBX-4132 bound to *M. tuberculosis (Mtb)* nonstop 70S ribosomes (**Fig. 2A**). We propose to solve one other ribosome structure with additional translation factors bound. We also propose to solve the structure of inhibitor KKL-1005 bound to an elongating 70S ribosome, as KKL-1005 has a distinct mode of action compared to MBX-4132; **2c**) *How do small molecules result in stop codon readthrough on the eukaryotic ribosome?* In collaboration with the Cooperman lab (UPenn), we have used a defined biochemical assembly system for eukaryotic ribosomes to show preliminary data of these ribosomes undergoing stop codon readthrough induced by an inhibitor (**Fig. 2B**).

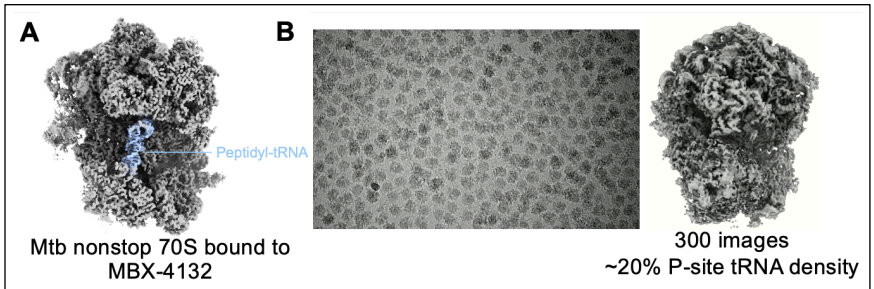

Fig. 2. Preliminary data for Sub-Project 2. **A**, 3D reconstruction of *Mtb* nonstop ribosome, and **B**, images and 3D classification of eukaryotic *Artemia salina* 80S carrying a five amino acid chain and a stop codon readthrough inducing small molecule.

Sub-Project 3: RNA chemical modifications (Dunham). In collaboration with the Koutmou lab, we are studying how specific mRNA chemical modifications increase both mRNA stability and translation. We are specifically interested in inosine, 2'-F and linked mRNAs. All materials have been cloned, purified, assembled into functional ribosome complexes. The Koutmou lab (U.Michigan) has performed detailed kinetic assays to determine at what step these mRNA modifications affect protein synthesis. Preliminary screening data for *E. coli* 70S ribosomes bound to an IUG mRNA in the A site trapped with EF-Tu-$tRNA^{Val}$-GDPNP ternary complex being delivered is shown in **Fig. 3**. Five additional complexes will be assembled and structures determined.

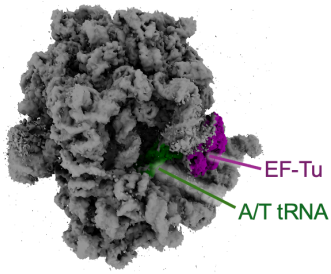

Fig. 3. Preliminary data for Sub-Project 3.