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14. ABSTRACT

The universally conserved nucleotide (A2058) of 23S rRNA in all bacterial ribosomes, when methylated (m^6A2058), causes cross-resistance to multiple families of therapeutically important antibiotics. The abundance and essentiality of ribosomes make them an attractive target for the detection of resistant pathogens based on the unique m^6A2058 signature. The goal of this study is to develop immunoreagents and synthetic binders that can be used as a rapid diagnostic tool for antibiotic resistant bacteria. They can also be used as a capturing tool to isolate homogenous populations of m^6A2058-ribosomes for structural and biochemical determination, a critical step to delineate the molecular mechanisms of new ribosome-targeting antibiotics. In this 18-month project, our success in the first phase (first 12 months, Aims 1-2) has offered a strong proof-of-principle to further improve the immunoreagents that specifically recognize the resistant ribosomes bearing the m^6A2058 modification. However, extending the oligonucleotide helper did not improve the specificity and binding of the antibody-oligo conjugate, possibly because the additional antisense regions are inaccessible to the A2058 nucleotide inside the fully assemble ribosomes. We generated anti-m^6A2058 antibody and successfully used it for immunoprecipitation to perform selective ribosome profiling. In the final 6 months, we performed an in vitro phage display screen (Aim 3) to identify several synthetic peptide binders of m^6A2058 rRNA, one of these peptides was labeled with fluorescent dye, allowing in situ discrimination of m^6A2058 rRNA from unmethylated rRNA.

15. SUBJECT TERMS
RNA methylation, ribosome, antibiotic resistance, macrolide

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1. INTRODUCTION

Dimethylation of a universally conserved adenine, A2058, in bacterial rRNA causes cross-resistance against all three critically important families of antibiotics (**m**acrolides, **l**incosamides, and **s**treptogramins (MLS))(1). A2058 dimethylation (hereafter called m^6A2058) occludes MLS from the ribosome, thereby allowing normal protein biosynthesis and bacterial growth (2). The RNA methylation enzyme *ermB* is widespread in almost all ESKAPE (***E**nterococcus faecium, **S**taphylococcus aureuus, **K**lebsiella pneumonia, **A**cinetobacter baumannii, **P**seudomonas aeruginosa, and **E**nterobacter species*) pathogens, which are the leading causes of hospital-acquired infections (HAI). Erm-mediated MLS resistance often represent up to 60-98% of the pathogenic MLS-resistant clinical isolates, a percentage that surpasses MLS resistance caused by drug inactivation and efflux mechanisms combined.

Overcoming *ermB*-mediated MLS resistance has been hampered by the inability to isolate homogeneous population of m^6A2058-ribosomes for structural and biophysical kinetic studies of MLS binding. In addition, detection of ErmB-resistant strains in clinical settings often requires blood culture followed by PCR amplification of the *erm* genes or by antibiotic susceptibility tests, both of which are time consuming. The latter also suffers from sensitivity issues. Ribosomes represent more than one-third of the bacterial biomass, and approximately two-thirds of ribosomes are composed of rRNA. In exponentially growing bacteria, rRNA are present at ~50,000 copies per cell. This project seeks to develop a bifunctional tool capable of pulling down m^6A2058 ribosomes from crude bacterial lysates with the ability to detect the resistant ribosomes in blood without conventional microbial culture. To this end, we were able to obtain antibody-RNA conjugate and natural antibody that exhibit high specificity to m^6A2058 RNA. We also obtained several synthetic peptide binders of m^6A2058 RNA from an in vitro phage display screen.

2. KEYWORDS

RNA methylation, macrolide, antibiotic resistance, ribosome, m^6A, ESKAPE pathogen, translational regulation, infectious diseases, bacterial pathogenesis.

3. ACCOMPLISHMENTS

3.1. What were the major goals of the project?

We proposed to use a three-pronged approach to develop antibody and/or aptamer that specifically binds to $m^6{}_2$A2058 ribosome. Specific goals are:

Aim 1. To engineer and antibody-oligo conjugate specific for m^6A2058 ribosomes.
Aim 2. To generate natural antibodies using an RNA-carrier conjugate.
Aim 3. To generate synthetic peptide binders using phage display.

3.2. What was accomplished under these goals?

Aim 1. To engineer and antibody-oligo conjugate specific for $m^6{}_2$A2058 ribosomes.

We have significantly improved the specificity of a commercially available monoclonal antibody (Synaptic systems) that cross-reacts with m^6-adenosine (m^6A). First, we performed enzyme-linked immunosorbent assay (ELISA) experiment to quantitate the amount of m^6A methylation in the total RNA isolated from the *Staphylococcus aureus ermB*-deficient (*ermB*$^-$), *ermB*-proficient (*ermB*$^+$), and a catalytically inactive Y103A allele ((*ermB*^{Y103A}). As shown in Figure 1A-1B, we found that RNA from the *ermB*$^+$ strain is > 3 times more methylated than the *ermB*$^-$ and *ermB*^{Y103A} strains, presumably due to specific methylation of the A2058 nucleotide in the 23S rRNA; whereas the 12-13% methylation in the *ermB*$^-$ and *ermB*^{Y103A} strains were attributed to background m^6A in the other five non-A2058 sites. These data indicate that the monoclonal antibody has the ability to enrich the RNAs bearing the m^6A2058 modification but cannot

discriminate m^{6}A2058 from other m^{6}A-RNA molecules, e.g. m^{6}A1518, m^{6}A1519 in the 16S rRNA and m^{6}A1618, m^{6}A2030, and m^{6}A2503 in the 23S rRNA.

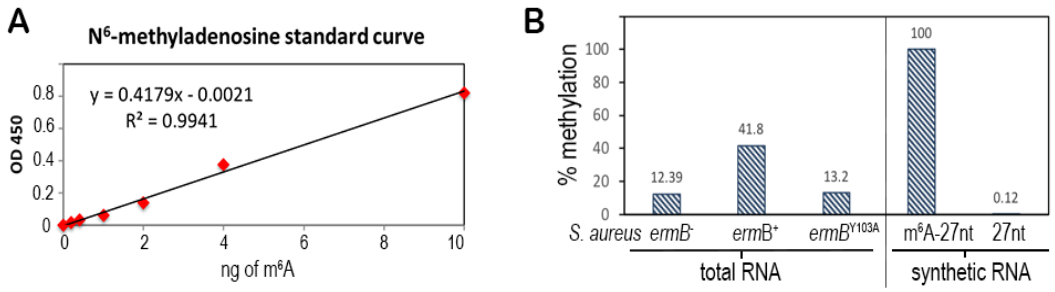

Figure 1. Detection of m^{6}A2058 modified RNA by ELISA. **(A)** Standard curve using the N^6-methyladenosine (m^{6}A) as the reference. **(B)** Degree of A2058 N^6-methylation upon ErmB expression. Two hundred nano-grams of total RNA isolated from the *Staphylococcus aureus ermB*-deficient (*ermB*$^-$), *ermB*-proficient (*ermB*$^+$), and a catalytically inactive Y103A ((*ermB*^{Y103A}) strains were used in each reaction. Unmodified or m^{6}A2058-modified synthetic RNAs of 27-nt was used as a negative and positive control, respectively. RNA targets were immobilized to a 96-well microplate and subsequently captured with an HRP-conjugated anti-m^{6}A (Synaptic LLC). The amount of m^{6}A modification was determined using the standard curve in panel (A). The percentage of m^{6}A is calculated relative to the m^{6}A-27 nt control.

To improve the specificity of the antibody against m^{6}A2058, we conjugated the antibody with a 12-mer anti-sense oligonucleotide against either the upstream (5'-GCGACAGGACGG) or the downstream region (5'-AAGACCCCGUGG) of m^{6}A2058 by the Solulink's HyNic/4FB bioconjugation linkage system (TriLink). This system allows the removal of the unconjugated HyNic-antibody and 4FB-oligonucleotides without laborious purification steps. We tested the specificity of the antibody-oligo adducts by North-Western hybridization. Total RNA from *S. aureus* lacking an RNA methyltransferase gene *ermB* (Δ*ermB*) or containing a catalytically inactive *ermB*^{Y103A} and the wild type *ermB* were resolved on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with the antibody-oligo conjugates (Figure 2). We found that the conjugate harboring the downstream antisense oligo successfully reacts to the m^{6}A2058 in the 23S rRNA but cannot discriminate m^{6}A epigenetic marks from the three m^{6}A sites on 16S rRNA that are catalyzed by the housekeeping rRNA methyltransferase.

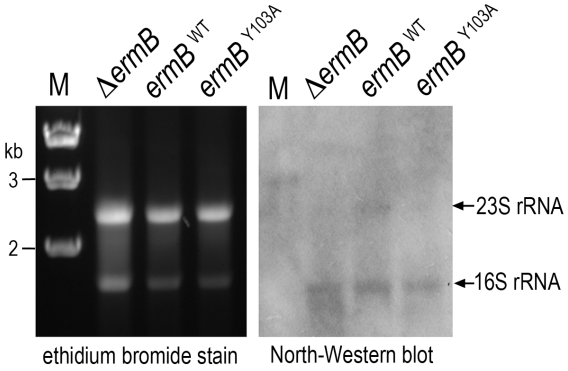

Figure 2. North-western blot confirming the specificity of the antibody-oligo conjugate. (Left) Ethidium bromide stained denaturing gel showing the 23S rRNA and 16S rRNA of *S. aureus* strains. Two-micrograms of DNAse-treated RNAs were loaded per lane. **(Right)** Recognition of the antibody-oligo conjugate (1:50 dilutions) specifically with the 23S RNA in the *ermB*- expressing strain, but not the catalytically dead mutant Y103A or a strain lacking *ermB*. The antibody conjugate also cross-reacts with the m^{6}A marks in the 16S rRNA.

Since our last reporting, we had extended the antisense oligo helper(s) to 20 nucleotides in an attempt to better differentiate various m^{6}A marks based on their flanking sequences. We found that extending the length of the oligo helper(s) significantly hinders antibody conjugation efficiency, and after purification less than 10% of the conjugates were obtained. Additional North-Western blots using this limited material demonstrated that increasing the lengths of oligo did not improve the discriminatory ability of the conjugate, rather it also reduced its binding affinity to the m^{6}A2058-23S rRNA, supporting a previous studies reporting that the distal flanking regions of A2058 are mostly inaccessible to oligonucleotide probes (3).

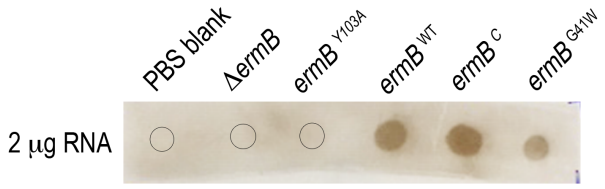

Figure 3. Dot blot demonstrating the specificity of anti-rabbit anti-m^{6}A-RNA. Purified total RNAs from different S. aureus strains were hybridized with 1:500 dilutions of antisera. The antibody does not recognize RNA from a strain lacking *ermB* (Δ*ermB*) and catalytically inactive ermB^{Y103A} but strongly reacts with that of strains carrying the wild-type *ermB*, a constituitively expressed *ermB* (*ermB*^{C}) and a benign mutation in *ermB* (*ermB*^{G41W}).

Aim 2. To generate natural antibodies using an RNA-carrier conjugate.

We had conjugated a 27 nt synthetic RNA bearing the m^{6}A2058 modification with a high-molecular weight carrier weight carrier **k**eyhole **l**impet **h**emocyanin (KLH) using Imject™ Mariculture KLH conjugation kit (Thermo Scientific). We covalently linked to covalently link the 5' amine-containing m^{6}A-RNA (IDT DNA) via the amine-reactive NHS-ester, followed by size exclusion chromatography to remove uncoupled RNA or KLH (4). This conjugate was used to raised antibodies in rabbits, guinea pigs and rats. We found this RNA conjugate was only able to elicit proper immune response in rabbits but not in other animals (see "notes on animal use"). Our dot blot analysis using purified RNA from different *S. aureus* backgrounds showed that the antibody generated from rabbit hosts is highly specific to *ermB*-producing strains but not to the strains with unmodified-A2058 RNA (Figure 3).

Notes on the use of animals: No fund from this award has been used for animal work for the entire funding period. Antibody production was an ongoing work before the notice of award in 2018. A commercial vendor (Josman, LLC) conducted the experiments and the service fees were paid from the principal investigator's Saint Louis University faculty startup fund.

In addition, we had purified the antibody and immunoprecipitated m^{6}A2058-ribosomes from *S. aureus* lysates to perform ribosome profiling (Ribo-seq). The enrichment of m^{6}A2058 ribosomes allowed us to specifically recover mRNA footprints protected by the m^{6}A2058 ribosomes but not the unmodified ribosomes. By applying the high-precision next-generation sequencing based Ribo-seq, we identified many transcripts that are inefficiently translated by the m^{6}A2058 ribosomes (relative to the unmodified ribosomes), many of these genes encode virulence factors (Figure 4.). Selected examples are genes that encode surface protein SdrE, themonuclease Nuc, and clumping factor ClfA. Of note, we observed an

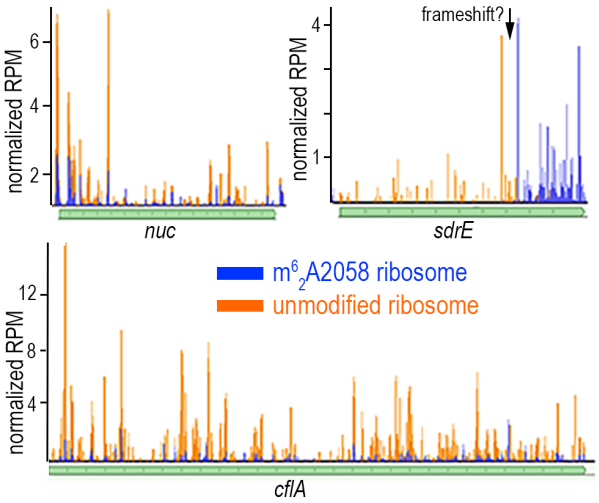

Fig. 4. Selected ribosome density plots showing down-regulated translation in *S. aureus* harboring m^{6}A2058 ribosome. The y-axis indicates normalized ribosome density in reads per million (RPM).

unusual ribosome density distribution in *sdrE* indicative of possible frameshifting. We are currently investigating how and why m^6A2058 ribosomes are prone to translational errors and slower translational elongation.

Aim 3. To generate synthetic peptide binders using phage display.

In the final 6 months of this project, we used the commercial Ph.D.-7™ Phage Display libraries >1×10^{15} diversity) (New England BioLabs) to select Fabs that recognize the m^6A-RNA following standardized procedures used for the selection of RNA-binding proteins and synthetic antibodies (5). Biotin-tagged m^6A-RNA (TriLink, IDTDNA) was immobilized on neutravidin-coated plates. The libraries was panned for four rounds for biotinylated m^6A-RNA after pre-incubation with 100 pmol 5' biotinylated DNA oligos of random sequence to eliminate false-positive binders. After each round of washing and selection with yeast tRNA competitors, recovered phages were amplified with the M13 helper phage. The affinity of PEG-purified phages to m^6A-RNA was measured with ELISA using biotinylated m^6A-RNA that has been immobilized on neutravidin-coated Maxisorp plates. Positive clones were sequenced. The library was composed of random heptapeptides fused to the surface exposed minor coat protein of M13 phage. After four rounds of selection with non-specific elution. Specific elution favored two peptide candidates, RQVAKHQ and TYPFFHR. We custom synthesized the Alexa Fluor dye-labeled peptides and performed fluorescence in situ hybridization (FISH) to examine the specificity of these peptides to the A2058 methylated and unmethylated rRNA inside fixed *S. aureus* cells. We found that Alexa Fluor 488-RQVAKHQ peptide was able to detect m^6A2058-rRNA in *S. aureus* carrying a constitutively expressed ErmB RNA methyltransferase (strain *ermBL*(R7stop)-*ermB*), whereas a strain with a catalytically inactive ErmB (strain *ermBL-ermB*(Y103A)) did not interact with the heptapeptide (Figure 5). The results offer a proof-of-principle that small synthetic peptides are capable of discriminate base modification. However, due to time constraint we had not exhaustively test if the heptapeptide could recognize m^6A2058 ribosome as efficiently as the m^6A2058 rRNA in living cells.

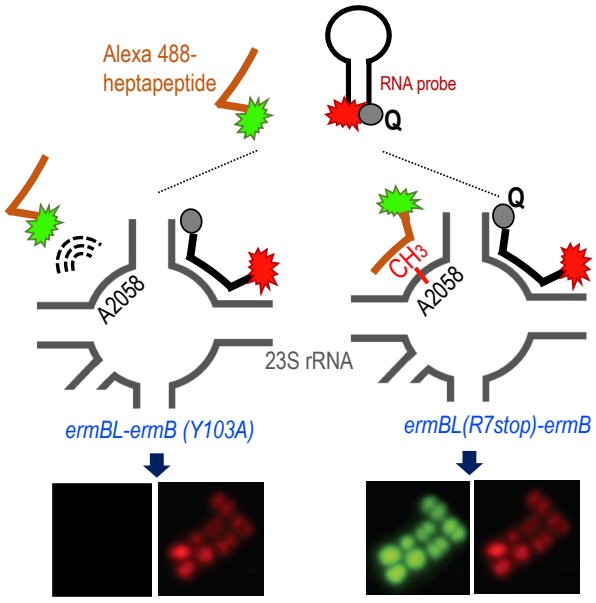

Fig. 5. Microscopic detection of m^6A2058 modification using fluorescently labeled-heptapeptide. FISH was performed in *S. aureus* cells overexpressing ErmB or carrying a catalytically dead ErmB. An RNA molecular beacon labeled with Alexa Fluor 688 (red) serves as the internal calibrator to account for rRNA concentrations. The molecular beacon binds to the remote region of the 23S rRNA.

Summary: In general, we have met the key milestones in the SOW. Although additional experiments are necessary to improve the quality and quantity of the developed reagents. We obtained highly specific immunoreagents (antibody-RNA conjugate and antibody) and synthetic peptide(s) against the m^6A2058-rRNA. On the other hand, we also encountered significant barriers in terms of improving the specificity and binding affinity of the antibody-RNA conjugate. Moreover, due to time limitation, we had not yet examined the sensitivity limits of the heptapeptide candidates. An attempt to improve the binding affinity of these heptapeptides by the originally proposed **s**ystematic **e**volution of **l**igands by **e**xponential **e**nrichment (SELEX) (6) has also been restricted by the recent COVID-19 pandemic.

3.3. What opportunities for training and professional development has the project provided?

This project has supported a B.S. degree research technician (Ms. Kathryn Shields) and has partially funded two postdoctoral fellows (Dr. Arnab Basu, Dr. Anna Liponska). Dr. Basu had assisted Ms. Shields in performing experiments and providing guidance in data collection and interpretation. Dr. Basu has gained new knowledge in ribosome modification and enzymology. Dr. Basu had returned to his home country (India) to pursue an independent position. Ms. Shields was not familiar with Staphylococcal genetics and protein and RNA chemistry prior to joining the team. She has since then broadened her technical and conceptual skills in these areas through her efforts in creating and confirming an antibody that specifically binds to the m^6A2058 ribosomes. Ms. Shields has a manuscript in the pipeline, in which she receives writing guidance from the PI. Dr. Anna Liponska helped with the phage display experiments and constructing several fluorescence reporters to validate Aim 3. All trainees had presented their work at the national and international conferences.

4. IMPACT

4.1. What was the impact on the development of the principle discipline(s) of the project?

There is an unmet need for highly specific antibodies for resistant m^6A2058-ribosomes. Success in the initial phase (Aims 1-2) of this long-term project has offered a solid proof-of-principle to develop the immunoreagents required for the detection of other RNA modifications at single nucleotide resolution. The immediate impact of this proposal includes the mechanistic validation of the new macrolides. The m^6A2058 modified regions are evolutionarily conserved across all resistant bacterial species; thus, the antibody-based diagnostic kits will be able to detect one or more bacterial pathogens that link the resistance profiles to the abundance of m^6A2058-ribosomes. These tools will also have a long-lasting impact on patient care by providing a sensitive, specific and adaptable assay for pathogen detection, thereby allowing timely treatment decisions.

4.2. What was the impact on other disciplines?

The availability of the proposed reagents will significantly advance both applied (clinical diagnostics) and basic RNA and ribosome biology as a whole. m^6A2058-RNA specific antibody and synthetic peptides are useful molecular tools for the enrichment of m^6A2058 ribosomes (Aim 2) and quantitative detection of m^6A marks in bacteria (Aim 3).

4.3. What was the impact on technology transfer?

The developed reagents potentially are patentable for commercial use in the future.

4.4. What was the impact on society beyond science and technology?

Presentations of the findings produced from this project raised public awareness about antimicrobial resistance and its counter strategies.

5. PRODUCTS

5.1. Publications, conference papers, and presentations.

Publications

Basu, A., Shields, K.E. and Yap, M.-N. F. (2020) The hibernating 100S complex is a target of ribosome recycling factor and elongation factor G in Staphylococcus aureus. *Journal of Biological Chemistry. 295: 6053-6063.*

Shields, K., Liponska, A., and Yap, M.-N. F. (2020) Selective translation of epitranscriptomic marked ribosomes. *major revision. (unable to perform the requested experiments due to lab shutdown during the COVID-19 pandemic)*

Invited seminars

- Yap, M.-N. F. (Feb 2020) "Ribosome hibernation: what, why and how". Department of Microbiology and Immunology, University of Kansas Medical Center, Kansas city, KS, USA.
- Yap, M.-N. F. (Jan 2020) "Ribosome hibernation: what, why and how". Chicago RNA Club. University of Chicago, Chicago, IL, USA.
- Yap, M.-N. F. (Nov 2019) Department of Microbiology and Immunology, Loyola University Chicago Health Science Division, Maywood, IL, USA.
- M.-N. F. Yap (March 2019). "Ribosome silencing as a bacterial survival strategy". Department of Microbiology. The Ohio State University, Columbus, OH, USA.
- M.-N. F. Yap (April 2019). "Stress management by ribosome silencing". Center for Infectious Diseases, Tsinghua University School of Medicine, Beijing, China.
- M.-N. F. Yap (Jan 2019)." Ribosome silencing in bacterial pathogenesis". Bacteriology Laboratory, Wadsworth Center, Albany, NY, USA.

Conference Plenary talks

- M.-N. F. Yap (Jan 2019) "Regulation of ribosome silencing". Ribosome Structure and Function 2019, Merida, Mexico.
- Yap, M.-N. F. (July 2019) The 20th International Conference on Bacilli and Gram-Positive Bacteria, Washington, D.C. USA

Conference abstracts

- Shields, K, and Yap, M.-N.F. (2019) "Consequences of multidrug resistant ribosomes". EMBO Protein synthesis and translational control. Heidelberg, Germany.
- Shields, K, and Yap, M.-N.F. (2019) "Functional consequences of methylated ribosomes". Rustbelt RNA meeting, Case Western Reserve University. Cleveland, OH, USA
- Basu, A., Shields, K., and Yap, M.-N. F. (2018) 25th Annual Microbial Pathogenesis Conference. "Ribosome inactivation in Staphylococcal pathogenesis". University of Iowa, Iowa City, IA, USA.
- Basu, A., and Yap, M.-N. F. (2018) Gordon conference in Microbial Stress Response. "Thermal and nutritional control of ribosome hibernation". Mount Holyoke College, MA, USA.

5.2. Website(s) or other internet site(s)

Nothing to report.

5.3. Technologies or techniques.

Nothing to report.

5.4. Inventions, patent application, and/or licenses

Nothing to report.

5.5. Other products.

Nothing to report.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7.1. what individuals have worked on the project?

Name	M.-N. Frances Yap, Ph.D.
Project role	Principle Investigator
Effort (months worked)	1.8
Contribution	Dr. Yap was responsible for the overall coordination and supervision of this study. This includes experimental design, training, analyzing data and preparing for dissemination of the results.
Funding support	This grant, NIH GM121359, Saint Louis University, Edward Mallinckrodt, Jr Foundation. Northwestern University Faculty Startup Fund.

Name	Arnab Basu, Ph.D.
Project role	Postdoctoral Fellow
Effort (months worked)	2.8
Contribution	Dr. Basu trained Ms. Shields in her first 3 months of employment. Dr. Basu provided technical guidance, conceptual input and data interpretation.
Funding support	This grant, NIH GM121359

Name	Kathryn Shields, B.Sc.
Project role	Research Technician
Effort (months worked)	12
Contribution	Ms. Shields performed work in identifying, modifying and testing the specificity of antibodies that recognizes m^6A epitranscriptomic marks
Funding support	This grant

Name	Anna Liponska, Ph.D.
Project role	Postdoc Fellow
Effort (months worked)	6
Contribution	Dr. Liponska worked on Aim 3 to engineer ErmB expression strains, helping with phage display and validation of probes.
Funding support	This grant, Northwestern University Faculty Startup Fund

6.2. Has there been a change in the active other support of the PI(s) or key personnel since the last reporting period?

Yes. Dr. Basu and Ms Shields left the laboratory for other positions. Some of the work were done by a new postdoc Dr. Anna Liponska.

6.3. What other organizations were involved as partners?

Saint Louis University was the only performing site in the first 12 months of this project. All other experiments in the remaining 6 months were conducted at the Northwestern University.

7. SPECIAL REPORTING REQUIREMENTS. Not applicable

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