Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Deanna Dailey January 27, 2021

Chemical profiling of Inosine using a Click-Compatible Acrylamide and EndoV Protein

by

Deanna Dailey

Dr. Jennifer Heemstra
Adviser

Department of Chemistry

Dr. Jennifer Heemstra

Adviser

Dr. Monika Raj

Committee Member

Dr. Rachel Hall-Clifford

Committee Member

2021

Chemical profiling of Inosine using a Click-Compatible Acrylamide and EndoV Protein

By

Deanna Dailey

Dr. Jennifer Heemstra

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Chemistry

2021

Abstract

Chemical Profiling of Inosine using a Click-Compatible Acrylamide and EndoV Protein
By Deanna Dailey

RNA undergoes extensive post-transcriptional modifications that have a range of biological effects. Inosine is a ubiquitous modification that effects coding and non-coding functions in several types of RNA. Altered Franklin-Crick base-pairing leads to modified coding in mRNA and also changes splicing patterns. This modification participates in cellular stress and immune responses. However, the field is limited by the lack of sensitive and selective editing detection methods. Current methods use a Michael addition to introduce adducts for high throughput sequencing techniques, however these methods often cannot distinguish inosine apart from a guanosine base, within error of PCR. These methods are time-intensive and costly as well. Herein, we synthesized an acrylamide derivative with click chemistry functionalization for enrichment and detection of inosine. This provides a cheaper, quicker way to probe editing rates on certain RNA substrates. Additionally, protein identification of these edits is a promising new direction of A-to-I edit determination. Current researchers have had success utilizing eEndoV protein to recognize and enrich inosine-containing RNA. Herein, we further explore the use of EndoV, and other homologs to probe A-to-I editing sites.

While this technology has the power to personalize disease treatment, there are some ethical concerns that might arise from this type of research. We as a Global Health Community must ensure that everyone receives equal access to this technology regardless of race or ethnicity, location, or cost. Additionally, we have to safeguard against using RNA studies and genomic data to define certain groups as less healthy, weaker, or less deserving.

Chemical profiling of Inosine using a Click-Compatible Acrylamide and EndoV Protein

By

Deanna Dailey

Dr. Jennifer Heemstra

Advisor

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Chemistry

2021

Acknowledgements

My thanks go to Dr. Jen Heemstra, Dr. Rachel Hall-Clifford, and Dr. Monika Raj, my committee members, who took time out of their busy schedules to have many Zoom calls to discuss this work and other areas of life. In addition to my committee members, the graduate students I worked with, Steve Knutson and Ansley Felix, offered sage advice and guidance on this project, and to whom I am grateful. I would also like to thank current and former members of the Heemstra Lab, Megan Korn, and other graduate and undergraduate students for their friendship and support. Even though they are not at Emory anymore, I wish to thank the entire Weinert Lab, Dr. Emily Weinert, graduate students Yasha Duggal and Shannon Rivera, and undergraduate students Leo Briggs, Carmen Metzler, and Hugh Dong for teaching and encouraging me in my first year of research. Many thanks to the Emory Writing Center tutor, Carissa Martin for their help in polishing this work. I also wish to thank my family and friends for their patience, and support durning this time, especially my father and sister, who were my sanity, my roommate Carissa Martin for their encouragement and stress management techniques and Victoria Hart for her listening ear and many “hell yes’s”

Table of Contents

Table of Contents for Figures and Tables

Chapter 1: Introduction

The blueprint for life comes from genetic material in the form of deoxyribonucleic acid (DNA). DNA is transcribed to ribonucleic acid (RNA) which is then translated to amino acids which are the building blocks of proteins. DNA and RNA are both ribose sugar to which a phosphate group and a nitrogenous base are attached.[1] There are four canonical nitrogenous bases in DNA, two purines (adenine A and guanine G) and two pyrimidines (thymine T and cytosine C). Instead of thymine, RNA has uracil (U).[1] Hydrogen bonds are base-specific and help guide recognition of the complementary strands. Adenine has a double hydrogen bond with thymine, and cytosine has a triple hydrogen bond with guanine. Edits involving insertion, deletion, and substitution of nucleic bases can change the reading frame of proteins, add a premature stop codon, and change the sequence of amino acids.[2-3]

In addition to these common bases, there are numerous naturally occurring and man-made nucleobases (Figure 1). These non-canonical bases signal different reactions within the cell and are used to study translation and protein synthesis. They are used and studied in drug development and treatment. One of the most common edits to form non-canonical bases is via methylation. mRNA methylation is required for certain transcription, nuclear export, translation and degradation processes.[3] For example, 5-methyl-deoxycytidine (mdC, or 5-methyl-cytidine, m^5C, in RNA) (Figure 1), the first non-canonical base discovered, acts as a regulatory epigenetic element effectively silencing the following gene,[2,4] when found in the promoter sequence. Within the past 30 years, more bases have been discovered predominately in brain and embryonic stem cells. It is hypothesized that studying these non-canonical bases can explain epigenetic plasticity and tissue differentiation. This methylation has many different functions within a cell. It can aid in RNA stabilization, affect the binding of RNA binding proteins,[3] and alter RNA secondary structure.

Additionally, methylation is reversible, allowing the cell to regulate gene expression. Understanding how bases are altered and interact in the cell allow researchers to design specific druggable proteins.[5] These non-canonical bases are used in time-release protein drugs and mRNA vaccines. They contribute to the stability and effectiveness of these therapies.

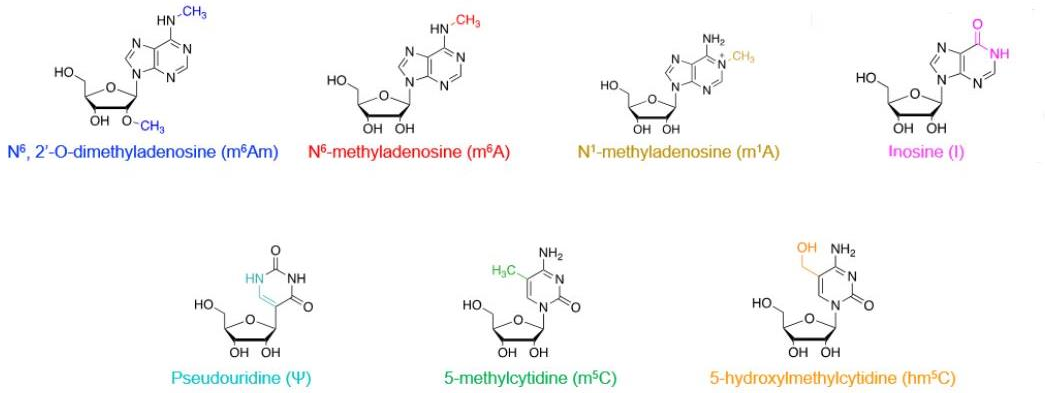


Figure 1: Common modified ribonucleic bases. Inosine, the focus of this paper, is seen in the top right, in pink.

1.1: A-to-I RNA editing

Non-canonical bases are either naturally edited into the transcriptome, or they are biochemically edited in a lab setting. One of the most studied and common natural genetic edits of RNA in animals is adenosine-to-inosine (A-to-I) editing.[2] This chemical change alters the hydrogen bonding of the complementary base pairs. Adenosine binds with uracil in RNA, while inosine preferentially binds with cytosine. When sequenced with RNA-seq, A-to-I editing sites appear as an adenosine to a guanosine[2] change. A-to-I editing is catalyzed by an enzyme called adenosine deaminase acting on RNA (ADAR).[2] The adenosine is deaminated by a hydrolytic attack by a Zn^{2+} activated water molecule.[6] The nucleophilic attack on the oxygen of the water at the C6 location releases ammonia as a side product (Figure 2). ADAR is a highly conserved enzyme found in most multi-cellular organisms.[2] Three ADARs in particular are of great

importance to the mammalian genome: ADAR1, ADAR2 (also known as ADARB1), and ADAR3 (also known as ADARB2).[2] ADAR1 is double-stranded RNA-specific adenosine deaminase, and ADAR2 and ADAR3 are dsRNA-specific editases. All three of these ADARs have a dsRNA-binding domain, in the α-β-β-α configuration.[6] Both ADAR1 and ADAR2 are commonly expressed in cells, however; ADAR1 is expressed at higher rates, and it catalyzes the majority of the known edits. ADAR3 and its binding and functionality are still poorly understood.

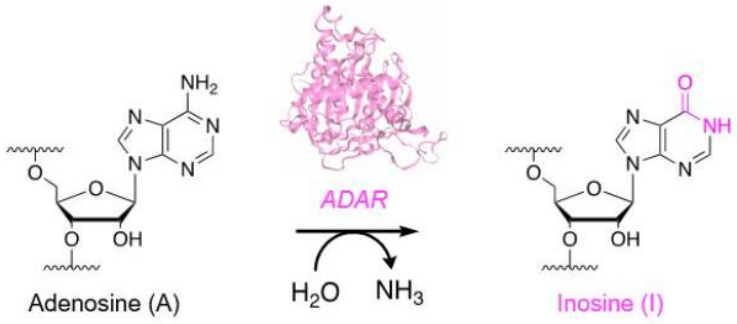


Figure 2: ADAR catalyzing the deamination of adenosine to form inosine. This changes the complementary base pair from uracil to cytosine.

Based on current research, A-to-I edits are found all across the entire transcriptome in both coding and non-coding regions. A-to-I editing is required for embryogenesis, neurological functions, and innate cellular immunity.[7] In other cell types, higher rates of A-to-I editing might lead to autoimmune diseases and neurological disorders, as well as several types of cancers.[7] High rates of editing in other cell types have been linked to epilepsy, amyotrophic lateral sclerosis, glioblastoma, schizophrenia, autism, and Alzheimer's disease. Because the effects of RNA editing are so vast, there is a hope that this pathway can be therapeutically targeted. Due to difficulties identifying A-to-I editing locations, researchers have yet to find all locations and understand the varying editing rates and results.

1.2: Detecting RNA Edits: Sequencing

There are ~5 million identified A-to-I sites in the transcriptome.[8] However, it is difficult and expensive to discover and quantify these edits using standard sequencing methods. Through a process of reverse transcription (the process of converting RNA back into DNA) and differently colored fluorescently tagged nucleic bases, sequencing allows the researcher to "read" what the template strand says by "writing" a strand of DNA.[2,3,8] It is for this reason that an A-to-I editing site appears as an A→G transition durning sequencing. Inosine occurs in a relatively low quantity compared to the whole transcriptome and is expressed at low copy reads. In addition, editing rates differ based on tissue types and cell stages,[8] so RNA-seq data varies greatly.[9] This variability makes it difficult to differentiate whether a A→G site is a legitimate editing site or a random sequencing error.[8] While there have been some statistical and technological improvements to genomic sequencing, being able to quantify and determine A-to-I sites across the entire transcriptome is still very challenging. In addition to random sequencing errors, determining A-to-I sites would require large quantities of RNA substrates,[10] complementary DNA (cDNA) strands, money, time, and specialized equipment for sequencing. Usually, researchers and medical personnel are not able to conduct these types of tests in house. It is important, therefore, to continue researching these systems to understand why and how A-to-I editing occurs and how chemical biologists might be able to leverage these systems for druggable therapies. Recently, the Heemstra lab and many others have discovered other ways to probe A-to-I editing that will push scientific community closer to the goal.[8]

1.3: Detecting RNA Edits: Chemical Labeling

As mentioned above, RNA-seq methods are costly, time and resource consuming, and require specialized sequencing equipment and bioinformatic analysis.[10] While there have been methods developed that detect inosine in smaller substrates, these methods typically utilize radioactive materials that require specialized training, handling,[10] and waste removal. Another method for detecting adenosine deamination is through the introduction of fluorogenic thiolated-adenosine or alkyne-modified analogues into RNA substrates. While these methods are not radioactive, they require intensive phosphonamidite monomer synthesis and can cause structural changes to the RNA that would impact ADAR binding and targeting.

Because of these challenges, easier chemical detection of inosine methods were hypothesized to negate some of the aforementioned problems. One such method, developed by Suzuki and coworkers, utilized the reactivity of inosine to a Michael acceptor, acrylonitrile[11] (Figure 3a). Termed "inosine chemical erasing sequencing" (ICE-seq), this reaction alkylates inosine and will terminate reverse transcription. This process improved the accuracy of Sanger sequencing in detecting A-to-I edit sites. However, ICE-seq was not as sensitive as desired and also required both and RNA and matching cDNA sequence for each assay.[10] Acrylonitrile was then derivatized to allow for biotinylation and enrichment of edited RNA sequences. While this is promising, derivatization requires lengthy and multiple synthesis and purification steps.

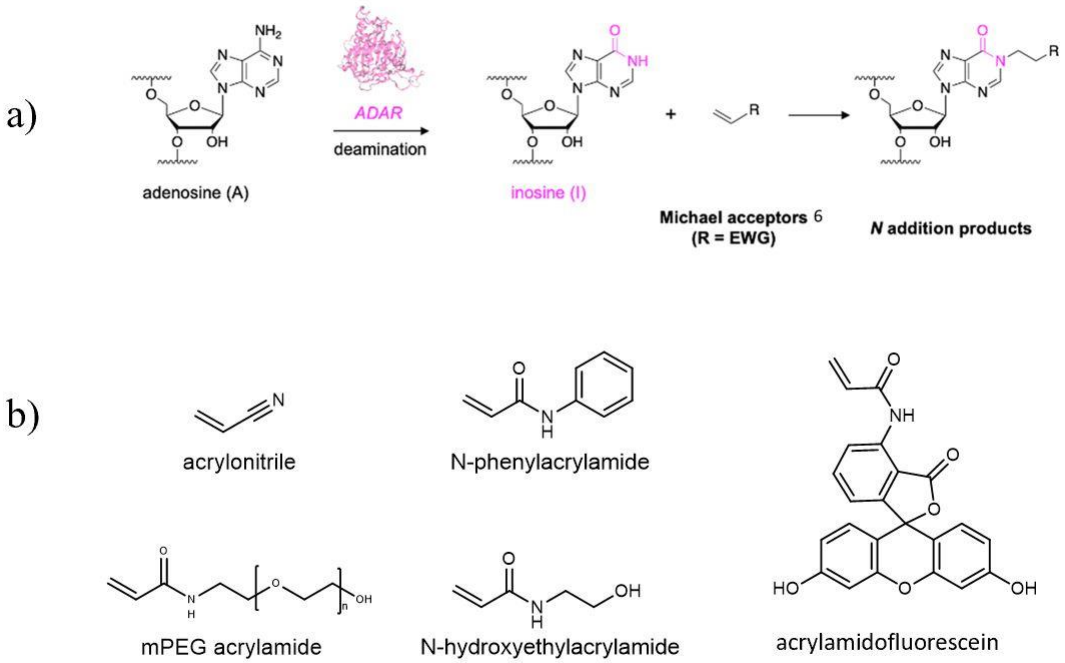


Figure 3: a) Chemical labeling of inosine by Michael acceptors, yielding N1 addition products, halting reverse transcription or attaching functional moieties. b) Different Michael acceptors that have been used to label inosine.

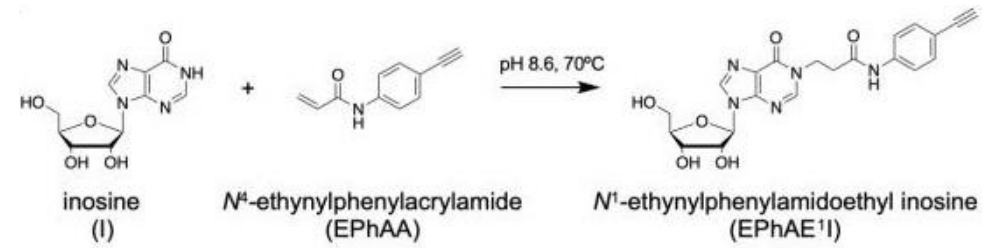
Michael addition is a nucleophilic addition of a nucleophile to an unsaturated carbonyl compound.[12] This chemical reaction is very useful in creating carbon-carbon bonds. When an alkane with electron withdrawing groups (for example, nitroalkane) reacts with an alkene attached to an electron group (for example, methylene) in the presence of a base, the alkene deprotonates the alkane. This process is thermodynamically controlled. Michael additions are widely found in the literature and are invaluable in drug design.

Previous work in the Heemstra Lab demonstrated a fluorescent detection method using an acrylamidofluorescein reagent. While this worked, it limited labeling to fluorescein molecules.[13] However, it was found that acrylonitrile had a higher reaction effiency.[13] That being said, molecules like acrylamide are both easy to synthesize and generalize with different moieties due

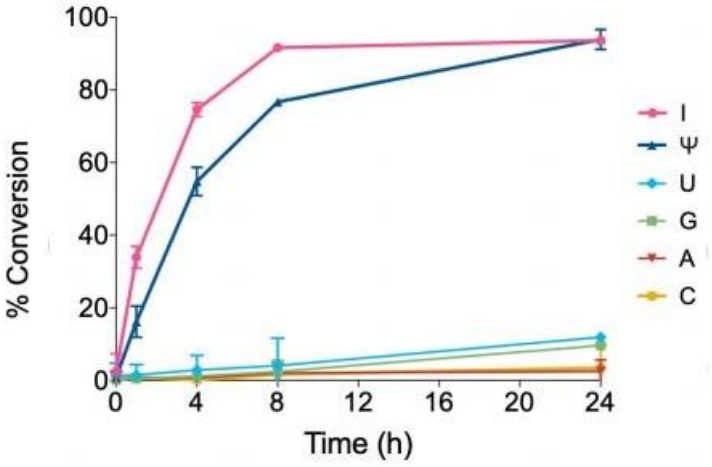
to copper-catalyzed azide-alkyne cycloaddition (CuAAC) or "click" chemistry (described below). This made it a good target to develop an inosine probe. Acrylamide and in particular, *N*-phenylacrylamide, reacted highly with inosine. This is most likely due to significant electron withdrawing properties, similar to acrylonitrile, allowing it to be a good Michael acceptor. *N-(*4-ethynylphenyl)acrylamide (EPhAA) was found by the Heemstra Lab to have good reactivity and a simple synthesis with decent yield. EPhAA was used in this previous study, as well as research discussed in this paper. The Heemstra lab also considered adding an azide group on the end of the molecule but decided on installing an alkyne moiety as it allows for click chemistry.

Click chemistry is equally invaluable in drug design. These reactions are easy to perform, have high yields, do not form racemic mixtures, and are "green," meaning the reactants and by-products are "generally harmless" and "by-products can be removed by nonchromatographic methods."[12] Products are also easy to isolate and are usually stable under physiological conditions. For these benefits, designing a molecular inosine tag that can undergo click chemistry would be very useful, as fluorophores, linkers, and other moieties can be added based on experimental need. The Heemstra lab proved this method, expounded upon in this work.

However, just like other chemical labeling methods discussed above, EPhAA will un-specifically react with other nucleobases in addition to inosine.[8] Previous work in the Heemstra lab discovered that EPhAA would react with pseudouridine (Ψ) and uracil (U) (Figure 4). This is due to the acidic nitrogens in each of the bases that EPhAA reacts with. Additionally, EPhAA could possibly label other bases if left to react for too long. Care will need to be taken to limit the chances of off-target binding, such as optimizing incubation time and designing oligos that have limited U and Ψ bases to limit off target labeling sites.



a



b

Figure 4: a) Reaction scheme for ribonucleoside labeling. b) Reactivity of EPhAA with ribonucleosides.

1.4: Detecting RNA edits: Protein Recognition

Protein recognition is a more recent path of detecting A-to-I edits; one that is promising for its adaptation of naturally occurring enzymes. One such enzyme is endonuclease V (EndoV). This protein is conserved throughout all living organisms, with slight differences in substrates between bacterial and eukaryotic EndoV. Bacterial EndoV binds more readily to inosine-containing DNA, while human EndoV seems to prefer inosine-containing ssRNA.[14] When EndoV was first isolated in 1977, researchers determined that it had DNA cleavage properties in the presence of Mn^{2+}, Mg^{2+}, or at pH of 9.5[15]. Twenty years later, it was shown that EndoV actually recognizes inosine and cleaves at the second phosphodiester bond[14-16].

The binding pocket of EndoV is very similar to RNase H1 enzymes and Argonaute (Argo) proteins. RNase H1 active sites are homologous to the active site of Argo proteins found in the RNAi pathway and are both DNA and RNA endonucleases. Interestingly, eukaryotic Argo proteins are exclusively involved in RNA-guided RNA cleavage, similarly to human EndoV. All three of these proteins share a highly conserved sequence of carboxylates, Asp-Glu-Asp (DED) and occasionally a fourth residue, most often another Asp (DEDD), but sometimes Asn or His (Figure 5).This homology is indicative of the importance of these residues in DNA and RNA binding and cleavage.

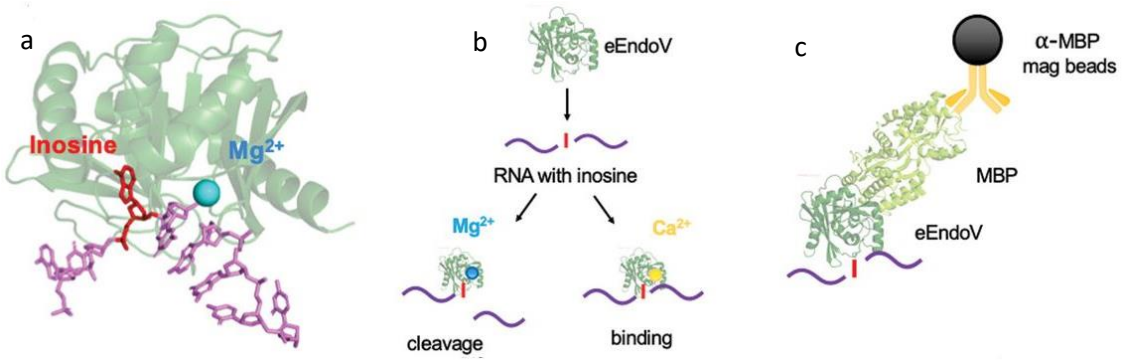


Figure 5: a) Crystal structure of EndoV (PDB 2W35) bound to a single-stranded DNA substrate (purple) with inosine (red) and a Mg^{2+} ion (blue). b) EndoV supplemented with Mg^{2+} results in cleavage of inosine containing transcripts, while Ca^{2+} promotes binding. c) General concept of immunoprecipitation using E. coli EndoV (eEndoV)-maltose binding protein (MBP) fusion and anti-MBP magnetic beads.[17]

In previous work, it was discovered that the cleaving mechanism can be halted in the presence of Ca^{2+} instead of Mg^{2+} (Figure 5).[8] This allowed for EndoV to be used as an inosine-recognizing protein for dsRNA and inosine containing RNA sequence enrichment prior to sequencing. Human EndoV looked promising because of its affinity and adaptability in humans. However, its full substrate preferences are still not fully understood, and other possible substrate binding might compete and cause problems in future testing. *E. coli* EndoV (eEndoV) binds to inosine in a highly specific manner in dsRNA[17-18]. Previous work by Steve Knutson and the

Heemstra Lab devised and proved a new approach using eEndoV to pulldown and enrich inosine-containing RNA sequences before RNA-seq.[8,17]

As discussed, there are many ways to detect A-to-I edit locations, all of varying ease and effectiveness. RNA-seq methods can be expensive, time consuming, and prone to random sequencing error. Chemical labeling of inosine bases by acrylamide derivatives can help detect and/or isolate inosine containing RNA. However, chemical labeling can lead to off target labeling of other nucleotides. Further work would need to limit these activities and optimize the chemical labeling process. Additionally, protein labeling is a recent and promising line of research as it utilizes nature and natural processes to detect and separate inosine containing RNA. Cloning eEndoV and its homologs could increase the knowledge of RNA editing and be leveraged to create a novel detection method or druggable therapy.

Chapter 2: Methods

2.1: HER1 RNA A vs HER1 RNA I Labeling

This experiment was conducted in duplicate. First, a 500 mM EPhAA in 1:1 ethanol: triethylammonium acetate buffer was made, and was pH adjusted to 8.6. This was aliquoted into 2 PCR tubes. 500 pmol of either HER1 RNA A or HER1 RNA I were added to the 100 µL of the EPhAA solution and incubated at 70◦C. At specific time points, 5 µL of the reaction mixture was diluted 1:10 in nuclease free water, and then ethanol precipitated. This purified RNA was reconstituted in nuclease free water and click labeled using the Click-&-Go Plus Labeling Kit and 1 µL of Cy 5 picolyl azide (5mM) in DMSO (Click Chemistry Tools, Scottsdale, AZ). Samples were left to react at room temperature for 1 hour, and then ethanol precipitated. The purified RNA was diluted 1:1 with RNA loading dye. To denature the hairpin structure, the mixture was incubated at 70◦C for 10 minutes. RNA was resolved on a 10% denaturing polyacrylamide gel and stained in TBE SYBR Gold solution for 20 minutes. It was imaged on a GE Amersham Typhoon RBG scanner with Cy5, SYBR Gold, and FITC filters. Densitometric quantifications of bands was performed using ImageJ software.

2.2: Sensitivity of EPhAA Labeling Procedure

This experiment was conducted in duplicate. First, a 500 mM EPhAA in 1:1 ethanol : triethylammonium acetate buffer was made, and was pH adjusted to 8.6. This was aliquoted into 6 PCR tubes. 25 pmol, 2.5 pmol, 250 fmol, 25 fmol, 2.5 fmol or 250 amol of HER1 RNA I were added to the 50 µL of the EPhAA solution and incubated for 6 hours at 70◦C. After the incubation, 5 µL of the reaction mixture was diluted 1:10 in nuclease free water, and then ethanol precipitated. This purified RNA was reconstituted in nuclease free water and click labeled using the Click-&-Go Plus Labeling Kit and 1 µL of Cy 5 picolyl azide (5mM) in DMSO (Click Chemistry Tools,

Scottsdale, AZ). Samples were left to react at room temperature for 1 hour, and then ethanol precipitated. The purified RNA was diluted 1:1 with RNA loading dye. To denature the hairpin structure, the mixture was incubated at 70◦C for 10 minutes. RNA was resolved on a 10% denaturing polyacrylamide gel and stained in TBE SYBR Gold solution for 20 minutes. It was imaged on a GE Amersham Typhoon RBG scanner with Cy5, SYBR Gold, and FITC filters. Densitometric quantifications of bands was performed using ImageJ software.

2.3: Mock HER1 RNA A Editing and Labeling

This experiment was conducted in duplicate. Varying mixtures of HER1 RNA A and HER 1 RNA I were prepared as follows.

Table #1: Mock A-to-I Editing RNA Concentrations

Editing Rate (%)	HER1 RNA A (pmol)	HER1 RNA I (pmol)
100	0	100
75	25	75
50	50	50
25	75	25
15	85	15
10	90	10
5	95	5
2.5	97.5	2.5
0	100	0

Then a 500 mM EPhAA in 1:1 ethanol : triethylammonium acetate buffer was made, and was pH adjusted to 8.6. This was aliquoted into the RNA mixtures and incubated at 70◦C. After 6 hours, samples were diluted 1:10 in nuclease free water and then ethanol precipitated. This purified RNA was reconstituted in nuclease free water and click labeled using the Click-&-Go Plus Labeling Kit and 1 μL of Cy 5 picolyl azide (5mM) in DMSO (Click Chemistry Tools, Scottsdale, AZ). Samples were left to react at room temperature for 1 hour and then ethanol precipitated. The purified RNA was diluted 1:1 with RNA loading dye. To denature the hairpin structure, the mixture

was incubated at 70◦C for 10 minutes. RNA was resolved on a 10% denaturing polyacrylamide gel and stained in TBE SYBR Gold solution for 20 minutes. It was imaged on a GE Amersham Typhoon RBG scanner with Cy5, SYBR Gold, and FITC filters. Densitometric quantifications of bands was performed using ImageJ software.

2.4: Synthesizing and Determination of Efficiency of new EPhAA

Unless otherwise noted, all starting materials were obtained from Sigma Aldrich Corporation (St. Louis, MO) and were used without further purification. Silica gel 60 (230-400 mesh) column chromatography was carried out. ^{1}H and ^{13}C NMR 23 chemical shifts are expressed in parts per million (δ) and obtained on a Varian INOVA 400 spectrometer in the Emory University NMR Research Center. Mass spectrum was obtained on an Agilent 6230 TOF LC/MS.

1.2 eq acrylic acid (1.41 mL, 20.49 mmol) was added to a solution of 4-ethynylaniline (2.0 g, 17.1 mmol), 0.2 eq hydroxybenzotriazole (462.1 mg, 3.42 mmol), and 1.2 eq 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (3.934 g, 20.52 mmol) in anhydrous pyridine (30 mL) under N_2 and was left stirring overnight at room temperature. Reaction progress and consumption of 4-ethynylaniline was confirmed by TLC in 1:1 hexanes: ethyl acetate. The crude reaction mixture was diluted in 50 mL ethyl acetate and washed sequentially with water and brine. The aqueous layer was back-extracted twice with ethyl acetate, and the collected organic layer was dried with $MgSO_4$ and filtered. The organic layer was concentrated under reduced pressure and purified by column chromatography (1:1 hexanes: ethyl acetate). The purified product was concentrated under reduced pressure and dried *in vacuo* to yield 1.26 g (42%) of a salmon-colored powder. ^{1}H NMR (400 MHz, DMSO-d6) δ 10.29 (s, 1H), 7.65 (d, J = 8.5 Hz, 2H), 7.40 (d, J = 8.3 Hz, 2H), 6.40 (dd, J = 17.6, 10.0 Hz, 1H), 6.29 – 6.19 (m, 1H), 5.78 – 5.70 (m, 1H), 4.06 (s, 1H). ^{13}C NMR (400

MHz, DMSO-d6) δ 163.73, 139.97, 133.23, 132.85, 132.78, 132.04, 127.87, 24 119.60, 116.81, 83.96, 80.44. HRMS m/z (ESI) calculated for $C_{11}H_{10}NO$ $(M^{+}H)^{+}$ 172.0762 and found at 172.0637.

To test if this new batch was as efficient as the last batch, the HER1 RNA A vs HER 1 RNA I Labeling procedure was followed using both the old batch of EPhAA and the new batch of EPhAa.

2.5: pETM-41-eEndoV Digest and Cloning

All reagents were acquired from New England BioLabs (Ipswich, MA), unless otherwise mentioned. First, pETM-41-EcPPK (Where did the plasmid come from) (Figure 6) underwent a double digest to remove the EcPPK gene. Double digestion reactions were set up, one for the vector, pETM-41-EcPPK, and one for the insert. For the vector digestion, 1X CutSmart buffer, nuclease-free water, 250 ng of pETM-41-EcPPK, 1µL of NcoI, and 1µL of BamHI were added to a microcentrifuge tube. For the vector digestion, 1X CutSmart buffer, nuclease-free water, 250 ng of pETM-41-EcPPK, 1µL of NcoI, and 1µL of BamHI were added to a microcentrifuge tube. Both reactions were incubated at 37◦C for 15 min.

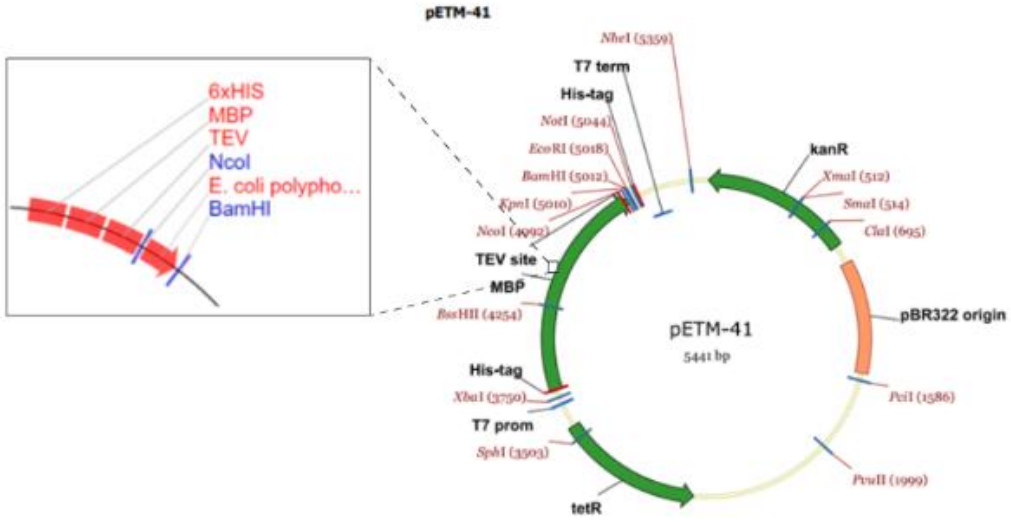


Figure 6: pETM-41 plasmid that was used to clone eEndoV

While the double digests were incubating, a 1% agarose gel was prepared with Sybr Safe (APExBIO, Houston, TX). The vector (pETM-41 plasmid) reaction was then mixed with 6X loading dye and loaded onto the gel. 1 kb DNA Ladder was loaded on the gel for comparison. The gel was run at 110V for an hour and imaged using Amersham Typhoon. The band around 5 kb was cut from the gel and purified using Monarch Gel Purification Kit. The amount of recovered vector was determined by NanoDrop. At the same time, the insert (eEndoV gene) digest was purified with Monarch PCR and DNA Cleanup Kit using the 5:1 buffer to sample ratio. The amount of recovered insert was determined by NanoDrop.

Three ligation reaction were set up. The first was a control reaction of just the vector without the insert. 2 µL of 10X T4 Buffer, 50 ng of vector, and 1µL of T4 Ligase were combined in a microcentrifuge tube. Nuclease free water was added to total volume of 25.2 µL. The second reaction was another control of the vector and insert without T4 DNA Ligase. 2 µL of 10X T4 Buffer, 50 ng of vector, and 19 ng of the insert were combined in a microcentrifuge tube. Nuclease free water was added to total volume of 25.2 µL. The final reaction was the vector, insert, and T4 DNA Ligase. 2 µL of 10X T4 Buffer, 50 ng of vector, 19 ng of the insert, and 1µL of T4 Ligase were combined in a microcentrifuge tube. These

Once the ligation reactions were complete, 5 µL of ligation reaction were added to the respective labeled tube of cells. Without mixing, the cells were placed on ice for 30 min, heated at 42◦C for 30 seconds, then placed back on ice for 5 min. After 5 min, 950 µL of 10 β/Stable Outgrowth Media (SOC) was pipetted into each cell mixture and left shaking at 37◦C for 45 min. At the same time, 6 LB+kan plates were also placed into the incubator to warm to 37◦C. After incubation, the cells were spread onto the warmed plates. Plate 1 was a negative control; no cells were spread onto this plate. Plate 2 was another negative control; 50 µL of untransformed cells

were spread onto plate. Plate 3 was spread with 50 μL of cells with the vector but no insert. Plate 4 was spread 50 μL with cells containing the vector and insert without T4 Ligase. Plate 5 was spread with 100 μL of cells with the vector, insert, and T4 Ligase. Plate 6 was spread with 50 μL of cells with the vector, insert, and T4 Ligase. All plates were incubated overnight at 37◦C. Minipreps were prepared, and the plasmid set off for sequencing.

Chapter 3: Results and Discussion

Previous work in the Heemstra Lab showed that EPhAA will un-specifically label inosine, (see Figure 4)[5] uridine, and pseudouridine. Additionally, at long time points, EPhAA will label guanine. Therefore, further testing to determine the efficiency of EPhAA on native substrates is needed.

3.1: HER1 RNA A vs HER1 RNA I Labeling

A known substrate of ADAR1 was used to test the efficiency of EPhAA. The substrate, known as HER1 and shown in Figure 7a, is a 33-nucleotide oligomer with a hairpin structure. The A-C mismatch destabilizes the starting structure and allows for A-to-I editing, while stabilizing the edited sequence as I binds to C.[10] This substrate was chosen for its known editing rates, and hairpin structure. Additionally, it had the fewest non-essential uridines of other known ADAR1 substrates, making it a good target for this method. In order to limit off target labeling, some of the uracils in the HER1 substrate were edited out, producing a similar RNA oligomer as seen in Figure 7b.

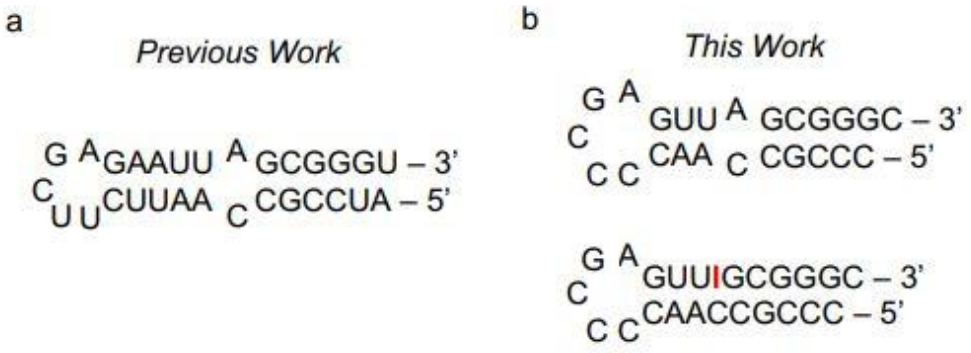


Figure 7: a) The original HER1 mRNA transcript described by Wang et al[9]. b) Redesigned HER1 used in this work for EPhAA labeling experiments. The two versions are: adenosine in the 25th position (HER1 RNA A) and the other "edited" version with inosine in the 25th position (HER1 RNA I)

To determine how long this substrate should incubate in EPhAA for, HER1 RNA A and HER1 RNA I substrates were incubated over the course of 48 hours. At intervals of 2 hours, the reaction was quenched, and copper click labeled.

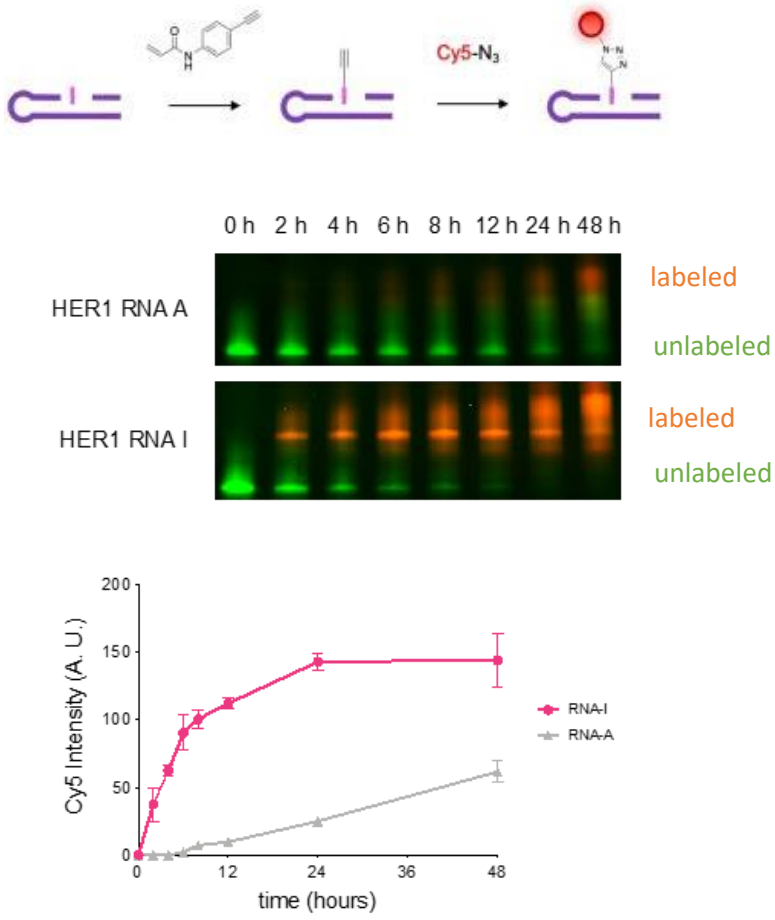


Figure 8:a) scheme of EPhAA labeling of RNA I. b) PAGE gel displaying labeling efficiency of EPhAA at different time points. C) Intensity plot of EPhAA labeling of RNA I.

As shown in Figure 8, the 6-hour time point offered the largest ratio of labeled RNA I to RNA A. At the longer time points, the off-target labeling of other bases like uracil, guanine, and cystine with EPhAA started rising in the background and giving false edited reads. The lack of clear unedited bands in the RNA A gel shows steady degradation over time. To limit false editing reads, 6 hours incubation time was used in all further experiments.

3.2: Sensitivity of EPhAA Labeling Procedure

Once the incubation length was determined, the selectivity of the EPhAA was tested. Through a series of 10-fold serial dilutions, starting with 25 pmol, 250 amol HER1 RNA I was achieved. All solutions of the varying concentrations were ran on a PAGE gel, to determine the limit of detection of the gel (seen in Figure 9).

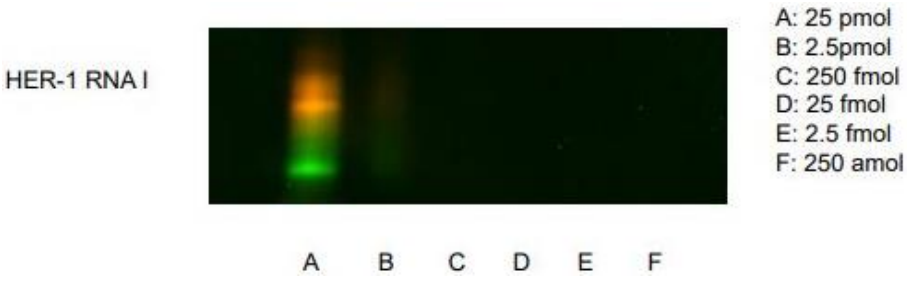


Figure 9: PAGE gel displaying the limit of detection of EPhAA labeled HER1 RNA I.

As seen in Figure 9, approximately 2.5 pmol of HER1 RNA I can be detected on a PAGE gel assay. In future experiments, actual concentration of RNA substrate can be extrapolated based off this data.

3.3: Mock HER1 RNA A Editing and Labeling

Keeping the total concentration constant, different concentrations of HER1 RNA I were spiked in according to Table 2. These varying ratios correspond to different editing rates of 0%, 2.5%, 5%, 10%, 15%, 25%, 50%, 75%, 100%. As expected, the Cy5 Intensity showed a linear relationship with a R^2 value of 0.95 as seen in Figure 10.

Table 2: Mock A-to-I Editing RNA Concentrations		
Editing Rate (%)	HER1 RNA A (pmol)	HER1 RNA I (pmol)
100	0	100
75	25	75
50	50	50
25	75	25
15	85	15
10	90	10
5	95	5
2.5	97.5	2.5
0	100	0

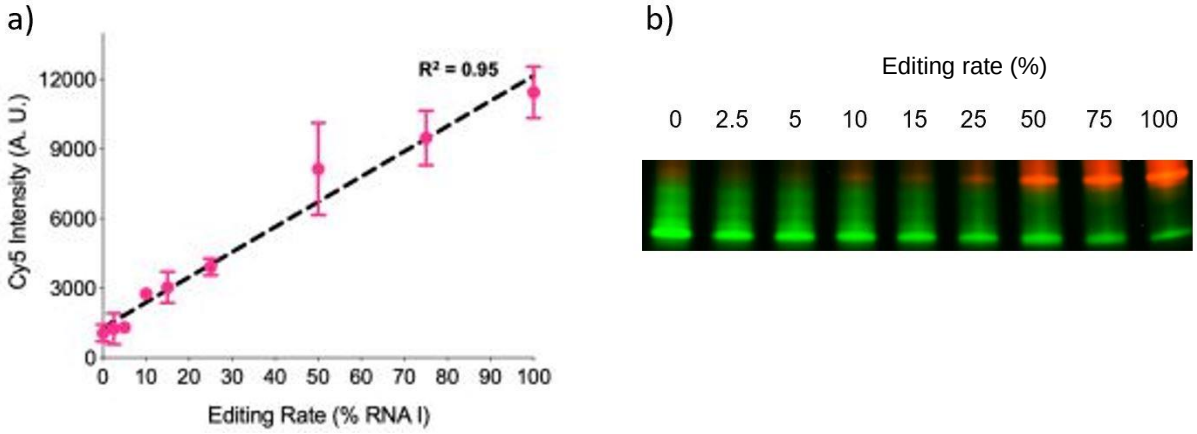


Figure 10: Cy-5 intensity plot versus theoretical editing rate with the linear line of best fit and R^2 value shown. A PAGE gel depicting increased labeling as HER1 RNA I: HER1 RNA A ratio increases.

3.4: Synthesis and Determination of Efficiency of new EPhAA

More EPhAA needed to be synthesized to continue experimentation. It was synthesized and characterized using the procedure found in the methods section. To prove that the new batch of EPhAA was as effective as the first batch, both HER1 RNA A and HER1 RNA I were incubated with both the new and old EPhAA for 6 hours, Cy 5 labeled, and gel imaged. The reactivity of the new batch was comparable based of the intensity of the gel seen in Figure 11.

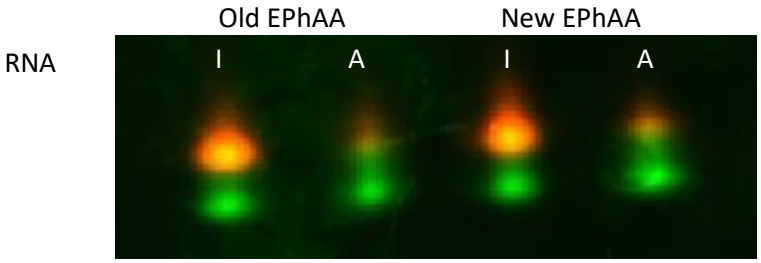


Figure 11: PAGE gel comparing the reactivity of the old batch of EPhAA to the new batch.

3.5: pETM Digestion and Cloning

The eEndoV enzyme needed to be cloned by *E. coli* to continue testing EndoV systems. A new plasmid, pETM-41, with a His-tag, maltose binding protein (MBP), and cleavage sites was used. Hopefully, this plasmid will facilitate the cloning and production of the eEndoV protein. The plasmid was isolated and *E. coli* transformed.

A double digest was performed on the vector plasmid and the gene insert. Both the insert and plasmid were purified. A purified band of about 5 kb, corresponding to the digested plasmid was seen (Figure 12). There was growth seen on the LB+kan plates. The received sequencing data showed that nine out of 10 sequences were without error. There was one point mutation of a C to an A at site 5070 which changed the amino acid from Asp to Glu.

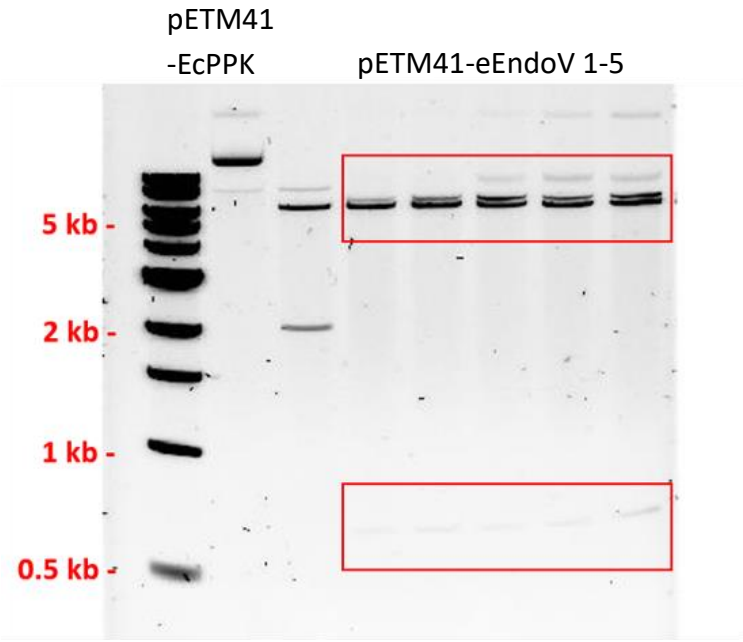


Figure 12: Agarose gel separation of pETM41-EcPPK plasmid. The top red box is the plasmid without the EcPPK gene. These bands were cut out and then ligated with eEndoV gene. The bands in the bottom box are the excised EcPPK gene.

Chapter 4: Ethics

4.1 Innovation and Ethics

As with any new idea or technology, the advances in RNA technology create both exciting prospects for application and an imperative to think critically about how and in what context this technology can be ethically applied. In the unique cases of biomedical and genetic technology, extra thought needs to go to making it accessible and applicable to a broad range of people with respect to class, race, location, socioeconomic status, culture, and other social determinants of health. Privacy concerns would also need to be taken into consideration, and rules need to be in place to determine what genetic data is collected, stored, and who has access to it. Additionally, principles of public health equity require that easy and life-saving technology be accessible to the poor, rural, or marginalized members of a community. All these were considerations of The Human Genome project, and much can be learned about how and why the people and organizations involved made the decisions they did.[1] These considerations are especially important in the American context, given the vast diversity of education levels, race and ethnicity, gender identity, sexuality, and geographic location of the population.

The following section walks through a series of key questions on ethical and equity concerns surrounding genetic and biomedical research. Genetics researchers hope to eventually use this technology as an early detection method for cancers and other chronic illness or even produce a druggable therapy based on biochemical principles of genetic editing. In order to ensure that everyone is treated equitably and fairly, ethical questions of autonomy and informed consent regardless of race, sex, identity, socioeconomic status, and culture need to be discussed. Hopefully, ethical conversations will establish a universal set of ethical guidelines regarding genetic testing and treatment plans that transcends culture, time, and place.

4.2 How will this genetic information be used?

This genetic research, and especially A-to-I editing, has the potential to be used as an early diagnostic tool or a druggable therapy. If diseases and cancers are caught earlier, treatment is less invasive, cheaper, and the patient's quality of life improves. This research was conducted with the intent of creating a quick and accessible genetic editing detection method. Yet, genetic editing can actually infringe on human rights. Theories of autonomy[4,5] and informed consent ripple out to other areas like health insurance, secure employment, and familial relations. We as public health members need to ensure that results of genetic testing are not used to say that a certain population is more likely to have negative health effects because of genetics since that would lead to population-based discrimination[6] as seen with COVID-19 and African American populations.

Genetics-based therapies are a recent scientific discovery. Therefore, there is very little written about the ethics of genetics-based therapies. In the special case of RNA therapeutics, advanced risk and reward analysis must be conducted. We as a scientific community need to agree to what is considered risks and rewards so that the ethics of each hypothesized treatment can be evaluated. This would ensure theories like informed consent, autonomy, and do no harm are followed.[6] Additionally, negative health effects like off-target editing, toxicity, and potency must be negated as much as possible.[4,6]

4.3 How can (and should) RNA research promote equity?

Research projects, labs, and papers have a large role in understanding and creating a more equitable world for those who are often marginalized. Research into the health effects of marginalized communities has spurred conversation in the public forum on systemic structural

violence. Additionally, the research explained above can improve access to care and early detection for certain chronic illnesses and cancers to minority individuals.

However, research is often complicit in excluding certain groups of people. For example, representation in research is seriously lacking.[7] Research is missing the voices of women, BIPOC individuals, and it ignores thoughts and ideas from these individuals. Also, scientific research is only accessible to certain people. Research is conducted, discussed, and written about in a very specific language that is unknown to the lay public. Scientific literacy is very low in America,[8] and people are not literate in genetics, in particular. This makes it difficult for those without advanced degrees and years of formal education to read and understand current research and/or how it impacts them. Those without access to the resource of higher education automatically get left behind.

Limited literacy levels do not just impact those who do not understand. People in power or public office who cannot read and understand scientific discourse make policy decisions ignoring information or only factoring in misinformation. This is evident in the United States' handling of the COVID-19 pandemic. As is often the case, this lack of understanding by policy makers impacts groups already disadvantaged by society at higher rates than others. Even if Americans were to become more scientifically literate and be able to read and understand current research in genetics, vaccines, or stem cells (for example), most of the articles are hidden behind pay walls that limit access. Only those who are affiliated with an institution like a university or scientific company might have access to these articles. The general public has to rely on news articles to accurately relay information or spend a good amount of time finding free articles online that might not be reputable. Occasionally there are great podcasts exploring scientific research, like the ones from The Genetic Literacy Project,[9] but searching for them is difficult.

Another way people of minority background are impacted because of research, especially medical research, is through the study process. Study participation requires extra time off work for more clinical visits, or the study criteria is set in such a way that the majority of participants are cis-gendered white men. Also, many studies take place on college campuses, limiting these participants to mostly college educated, younger members of the population. Marginalized individuals do not benefit from research, but often bear the bulk of participation- without consent. Recently, trial participation has become a main form of biomedicine for vulnerable populations.[10] This means that vulnerable individuals like the deathly ill and low-income individuals turn to clinical trials to receive treatment, monitoring, medicine, or money.[10] Many medical procedures and therapies come from the unconsented and unjust treatment of slaves.[11] Even in the 20th century events like the Tuskegee Syphilis Trial and Nazi experimentation on interned Jews show that marginalized communities are still bearing the un-consented bulk of medical trials.

4.4: How can (and should) the healthcare industry support equity?

Just like research institutions, medicine is not as diverse as the general population. Patients, therefore, do not see themselves equally represented in the medical field. This is a problem in and of itself that also contributes to other issues of racism, sexism, and general bigotry. Everyone has biases, and limited representation in medical fields worsen this effect. This causes distrust among patients. Generally, populations with more distrust of the medical field are those populations with less representation, for example, racial minorities, LGBTQ+ individuals, and women. Additionally, lack of representation and diversity can also decrease cross-cultural dialogue. As mentioned above, each community and culture would have a different viewpoint on what is ethical, equitable, or what should be done in a situation. When doctors fail to understand the motives, feelings, and thoughts of their patients of other cultures, they undervalue their patients' symptoms.

This leads to patients not following prescribed regimens, more distrust, and failure to seek help if condition worsens. Cross-cultural dialogue, and intentional interaction would increase stakeholder engagement from the general public to new diagnostic tests and therapies like what the future of this research holds.[12] By using respected members of a community, be that elders, chiefs, religious leaders, to explain medical practices (after some training) to their own community, the ease which people feel at doctor's offices would increase.[13]

Ease of access to care is another equity concern about these new research ideas. Lower income and rural individuals have a significantly harder time making doctor's appointments,[3] like urban specialists. They may not be able to take off work and travel to the nearest specialist or pay for a hotel and childcare if they have a young family. Lower income individuals often have to choose between medicine and food. This leads them to delay care-seeking until their pain and illness becomes more threatening. At this point, the disease becomes more expensive, invasive, and difficult to treat. Even if this research is able to be adapted to a point of care detection method, it is useless if people do not have access to it either by location or corporate control. A potential fix to this is employing trained professionals from rural, low resource, and marginalized communities to practice in their own communities.[3] We must also push for equitable regulations on gene testing companies to avoid price gouging.[14]

4.5: Considering next steps

I am proud to be a member of a lab that actively raises the voices of BIPOC, first-generation, and other under-represented groups. We discuss what it takes to be anti-racist and are encouraged by group members to constantly check our own biases and work on becoming more equitable. We often have discussions about making sure our lab environment is antiracist, and a welcoming community for everyone regardless of race, ethnicity, religion, sex, gender, or

sexuality. BIPOC members of my lab have shown me what it takes to be committed to building equitable excellence.

We as public health and public health-adjacent professionals need to de-colonize medicine, research, and public health by creating a new public health paradigm focused on combating the inequities that limit human flourishing.[15] While governments and organizations can set rules, there needs to be buy-in from members and benefactors of treatment. Just like members of my lab have to agree that raising voices of those marginalized by general society and research in particular, members of a community have to agree to the change. If there is not significant buy-in from members, the steps a group or organization takes will not be as effective. It is the responsibility of everyone within a system, regardless of identity, to call out oppression. In addition, researchers need to consider the possible negative ways their research can impact people of different cultures.[12] Structural injustices will not be solved overnight. Through continual pressure, we can ensure that future generations live in a more equitable society, one that welcomes and values our inherent differences. We can look forward to a time where everyone receives quality medical attention regardless of race, sexuality, gender, or economic status, if we take steps towards equity today.

Chapter 5: Conclusion and Future Research

Both chemically labeling and protein labeling proved to be successful methods for inosine-containing RNA identification. The Heemstra Lab as well as other groups were able to design methods and systems that will selectively bind to inosine to aid in the detection of A-to-I editing sites. Additionally, future work will hopefully improve this technology for use in medicine as a point of care diagnostic tool or druggable therapy. The accessibility of this research aims to circumvent some of the unequitable factors of medicine. However, researchers and medical professionals need to continue to ask good questions of the ethics and equity of the research being conducted. This way, good science and equitable therapies will continue to be discovered and implemented, making the future bright for everyone.

At the time of writing this, the Heemstra Lab continue to work on optimizing the EndoV enzyme for binding affinity and specificity. The EndoV protein was cloned using the pETM-41 plasmid. This hopefully increased correct replication and protein synthesis. Once the cloning issues are resolved, directed mutagenesis will be executed on the EndoV protein to determine if specificity and binding affinity can be enhanced. Increased affinity could aid in the creation of an A-to-I detection method, and further the understanding of EndoV and ADAR mechanisms.

Chapter 6: Resources:

6.1 Chemistry Portion:

(1) DNA | Definition, Discovery, Function, Bases, Facts, & Structure https://www.britannica.com/science/DNA (accessed Feb 21, 2021).

(2) Eisenberg, E.; Levanon, E. Y. A-to-I RNA Editing - Immune Protector and Transcriptome Diversifier. Nat Rev Genet 2018, 19 (8), 473–490. https://doi.org/10.1038/s41576-018-0006-1.

(3) Jonatha M. Gott; Ronald B. Emeson. Functions and Mechanisms of RNA Editing. Annu. Rev. Genet. 2000, 34, 499–531.

(4) Carell, T.; Kurz, M. Q.; Müller, M.; Rossa, M.; Spada, F. Non-Canonical Bases in the Genome: The Regulatory Information Layer in DNA. Angewandte Chemie International Edition 2018, 57 (16), 4296–4312. https://doi.org/10.1002/anie.201708228.

(5) He, P. C.; He, C. M6A RNA Methylation: From Mechanisms to Therapeutic Potential. The EMBO Journal 2021, 40 (3), e105977. https://doi.org/10.15252/embj.2020105977.

(6) Nishikura, K. A-to-I Editing of Coding and Non-Coding RNAs by ADARs. Nature Reviews Molecular Cell Biology 2016, 17 (2), 83–96. https://doi.org/10.1038/nrm.2015.4.

(7) GRANT13073847. Proposal to the National Institutes of Health. 16 Apr. 2020. Typescript. (accessed Feb 21, 2021).

(8) Knutson, S. D.; Arthur, R. A.; Johnston, H. R.; Heemstra, J. M. Selective Enrichment of A-to-I Edited Transcripts from Cellular RNA Using Endonuclease V. J. Am. Chem. Soc. 2020, 142 (11), 5241–5251. https://doi.org/10.1021/jacs.9b13406.

(9) Wang, Y.; Park, S.; Beal, P. A. Selective Recognition of RNA Substrates by ADAR Deaminase Domains. Biochemistry 2018, 57 (10), 1640–1651. https://doi.org/10.1021/acs.biochem.7b01100.

(10) Knutson, S. D.; Korn, M. M.; Johnson, R. P.; Monteleone, L. R.; **Dailey, D. M**.; Swenson, C. S.; Beal, P. A.; Heemstra, J. M. Chemical Profiling of A-to-I RNA Editing Using a Click-Compatible Phenylacrylamide. Chemistry – A European Journal 2020, 26 (44), 9874–9878. https://doi.org/10.1002/chem.202001667.

(11) Suzuki, T.; Ueda, H.; Okada, S.; Sakurai, M. Transcriptome-Wide Identification of Adenosine-to-Inosine Editing Using the ICE-Seq Method. Nature Protocols 2015, 10 (5), 715–732. https://doi.org/10.1038/nprot.2015.037.

(12) Nair, D. P.; Podgórski, M.; Chatani, S.; Gong, T.; Xi, W.; Fenoli, C. R.; Bowman, C. N. The Thiol-Michael Addition Click Reaction: A Powerful and Widely Used Tool in Materials Chemistry. Chem. Mater. 2014, 26 (1), 724–744. https://doi.org/10.1021/cm402180t.

(13) Knutson, S. D.; Ayele, T. M.; Heemstra, J. M. Chemical Labeling and Affinity Capture of Inosine-Containing RNAs Using Acrylamidofluorescein. Bioconjugate Chem. 2018, 29 (9), 2899–2903. https://doi.org/10.1021/acs.bioconjchem.8b00541.

(14) Zhang, Z.; Hao, Z.; Wang, Z.; Li, Q.; Xie, W. Structure of Human Endonuclease V as an Inosine-Specific Ribonuclease. Acta Crystallogr D Biol Crystallogr 2014, 70 (Pt 9), 2286–2294. https://doi.org/10.1107/S139900471401356X.

(15) Wu, J.; Samara, N. L.; Kuraoka, I.; Yang, W. Evolution of Inosine-Specific Endonuclease V from Bacterial DNase to Eukaryotic RNase. Mol Cell 2019, 76 (1), 44-56.e3. https://doi.org/10.1016/j.molcel.2019.06.046.

(16) Vik, E. S.; Nawaz, M. S.; Andersen, P. S.; Fladeby, C.; Bjørås, M.; Dalhus, B.; Alseth, I. Endonuclease V Cleaves at Inosines in RNA. Nature Communications 4. https://doi.org/10.1038/ncomms3271.

(17) EndoVIPER-seq for Improved Detection of A-to-I Editing Sites in Cellular RNA - Knutson - 2020 - Current Protocols in Chemical Biology - Wiley Online Library https://currentprotocols.onlinelibrary.wiley.com/doi/full/10.1002/cpch.82 (accessed Feb 28, 2021).

(18) García-Caballero, D.; Pérez-Moreno, G.; Estévez, A. M.; Ruíz-Pérez, L. M.; Vidal, A. E.; González-Pacanowska, D. Insights into the Role of Endonuclease V in RNA Metabolism in Trypanosoma Brucei. Scientific Reports 2017, 7 (1), 8505. https://doi.org/10.1038/s41598-017-08910-1.

(19) Fladeby, C.; Vik, E. S.; Laerdahl, J. K.; Neurauter, C. G.; Heggelund, J. E.; Thorgaard, E.; Strøm-Andersen, P.; Bjørås, M.; Dalhus, B.; Alseth, I. The Human Homolog of Escherichia Coli Endonuclease V Is a Nucleolar Protein with Affinity for Branched DNA Structures. PLoS ONE 2012, 7 (11). https://doi.org/10.1371/journal.pone.0047466.

(20) Liang, P.; Xie, X.; Zhi, S.; Sun, H.; Zhang, X.; Chen, Y.; Chen, Y.; Xiong, Y.; Ma, W.; Liu, D.; Huang, J.; Songyang, Z. Genome-Wide Profiling of Adenine Base Editor Specificity by EndoV-Seq. Nature Communications 2019, 10 (1), 67. https://doi.org/10.1038/s41467-018-07988-z.

(21) Alseth, I.; Dalhus, B.; Bjørås, M. Inosine in DNA and RNA. Current Opinion in Genetics & Development 2014, 26, 116–123. https://doi.org/10.1016/j.gde.2014.07.008.

(22) Figure 2: Incision activities for human ENDOV on RNA depends on a... https://www.researchgate.net/figure/Incision-activities-for-human-ENDOV-on-RNA-depends-on-a-ribonucleotide-3-to-inosine-The_fig3_255177741 (accessed Dec 1, 2020).

6.2 Ethics Portion:

(1) Cook-Deegan, R. M. *The Gene Wars: Science, Politics, and the Human Genome*; W. W. Norton & Company, 1994.

(2) Iserson, K. Principles of Biomedical Ethics. *Emergency Medicine Clinics of North America* **1999**, *17*, 283-+. https://doi.org/10.1016/S0733-8627(05)70060-2.

(3) Greg Stapleton; Peter Schröder-Bäck; Ulrich Laaser; Agnes Meershoek; Daniela Popa. Global Health Ethics: An Introduction to Prominent Theories and Their Relevant Topics. *Global Health Action* **2014**, *7* (1). https://doi.org/10.3402/gha.v7.23569.

(4) Ebbesen, M.; Jensen, T. G.; Andersen, S.; Pedersen, F. S. Ethical Perspectives on RNA Interference Therapeutics. *Int. J. Med. Sci.* **2008**, 159–168. https://doi.org/10.7150/ijms.5.159.

(5) Kathi C. Huddleston PhD, RN, CNS, CCRC. Ethics: The Challenge of Ethical, Legal, and Social Implications (ELSI) in Genomic Nursing. *OJIN* **2013**, *19* (1). https://doi.org/10.3912/OJIN.Vol19No01EthCol01.

(6) What are the Ethical Concerns of Genome Editing? https://www.genome.gov/about-genomics/policy-issues/Genome-Editing/ethical-concerns (accessed Feb 10, 2021).

(7) van Knippenberg, D.; Mell, J. N. Past, Present, and Potential Future of Team Diversity Research: From Compositional Diversity to Emergent Diversity. Organizational Behavior and Human Decision Processes 2016, 136, 135–145. https://doi.org/10.1016/j.obhdp.2016.05.007.

(8) Henry I. Miller. Scientifically Illiterate America https://www.hoover.org/research/scientifically-illiterate-america (accessed Mar 13, 2021).

(9) Genetic Literacy Project https://geneticliteracyproject.org (accessed Mar 14, 2021).

(10) Petryna, A. Introduction: Experimental Fields. In *When Experiments Travel: Clinical Trials and the Global Search for Human Subjects;* Princeton University Press, 2009; pp 1–9.

(11) Nuriddin, A.; Mooney, G.; White, A. I. R. Reckoning with Histories of Medical Racism and Violence in the USA. *The Lancet* **2020**, *396* (10256), 949–951. https://doi.org/10.1016/S0140-6736(20)32032-8.

(12) Lavery, J. V. Building an Evidence Base for Stakeholder Engagement. Science 2018, 361 (6402), 554–556. https://doi.org/10.1126/science.aat8429.

(13) Prioritizing Equity video series: Trustworthiness & Vaccines https://www.ama-assn.org/delivering-care/health-equity/prioritizing-equity-video-series-trustworthiness-vaccines (accessed Feb 10, 2021).

(14) Cook-Deegan, R.; Conley, J. M.; Evans, J. P.; Vorhaus, D. The next Controversy in Genetic Testing: Clinical Data as Trade Secrets? *European Journal of Human Genetics* **2013**, *21* (6), 585–588. https://doi.org/10.1038/ejhg.2012.217.

(15) Benatar, S.; Daibes, I.; Tomsons, S. Inter-Philosophies Dialogue: Creating a Paradigm for Global Health Ethics. *Kennedy Institute of Ethics Journal* **2016**, *26*, 323–346. https://doi.org/10.1353/ken.2016.0027.