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An Investigation of Protein Arginine Methyltransferase 1 Substrate Recognition Through Ribonucleoprotein Modifications

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AN INVESTIGATION OF PROTEIN ARGININE METHYLTRANSFERASE 1 SUBSTRATE RECOGNITION THROUGH RIBONUCLEOPROTEIN MODIFICATIONS

by

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Approved:

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ABSTRACT

Just as humans need to communicate with each other, our cells have a series of processes that allows them to communicate from one to another. Protein Arginine Methyltransferase I is an enzyme that helps mediate cellular communication by selectively binding to target proteins and performing chemistry that has broad-reaching downstream effects. However, the mechanisms that underly PRMT1 selectivity have yet to be elucidated. This study uses alterations to a series of known targets, called ribonucleoproteins, to identify some of the characteristics that govern recognition by the PRMT1 enzyme. I have made significant process in the creation, expression, and purification of these targets that can be used to investigate binding to PRMT1.

ACKNOWLEDGEMENTS

My biggest thanks to my incredible and irreplaceable mentor, Dr. Joanie Hevel for being an advocate and providing the space and support to let me spend time in lab. Thank you for putting up with our shenanigans! Additionally, I would like to thank the superstar undergraduates and graduate students in the Hevel Lab, Emeline Haroldsen, Sofiia Hakh, and Mason Hovinga, whom have provided the most fun and positive work atmosphere I have ever experienced. Lastly, a special thanks goes out to my primary lab partner and amazing researcher and friend Vincent Rossi, without whom I could not have done a fraction of the research I have done, won any of the awards that we had, or presented as many times as we have. I am so lucky!

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INTRODUCTION

After the transcription and translation of DNA to form proteins, the proteins are often post-translationally modified to meet a biochemical need for protein functionality. Protein Arginine Methyltransferases (PRMTs) are enzymes that catalyze the post-translational methylation of an arginine side chain in targeted proteins. As a consequence, methylation changes the shape and hydrogen-bonding preferences of this modified protein (Bedford & Clarke, 2009), regulating its ability to bind to other proteins and nucleic acids—the hallmark of communication within the cell. These methylation events impact a wide variety of essential processes within the cell and body, including DNA transcription and repair, immune response, and the function of tumor suppressor genes, among others (Morales et al., 2016). Aberrant PRMT function has been associated with numerous disease states, such as hypertension, chronic lung and kidney diseases, and cancer (Morales et al., 2016; Shishkova et al., 2017). An understanding of the mechanisms and functions of these PRMT enzymes contributes to therapeutic potential in numerous anti-disease and pharmaceutical areas.

For proper communication, arginine methyltransferases first need to bind and recognize their protein targets. Among the slew of proteins in the human body, certain proteins receive the cellular signals conferred by PRMTs, and some do not (**Figure I**). However, the patterns that underlie target recognition by PRMT1 are elusive. Thus far, it has been noted in the literature that the majority of protein methylation sites occur along disordered regions, typically followed by a glycine or RGG domain (Bedford & Clarke, 2009). This is not conclusive, though, as methylation sites have also been predicted on alpha helices (Haroldsen, 2023). Unlike many enzymes that catalyze post-translational modifications, protein arginine methyltransferases do not utilize consensus sequences, further complicating the matter (Morales et al., 2016). Thus, the aim of this

paper is to investigate potential strategies and begin to evaluate the mechanisms that confer target recognition by PRMT1.

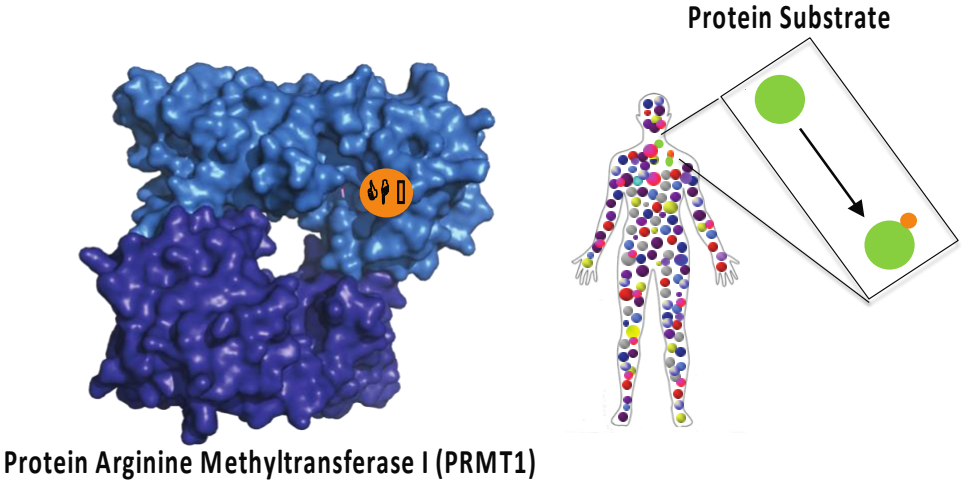

Figure I. Why does PRMT1 (left) methylate (orange circle) some proteins in the body (right) (green circle) and not others (all other colored circles)?

Just as using a "model system" in biology allows researchers to investigate aspects of an organism that we may not directly be able to understand, we can use known targets of PRMT1 to begin to uncover the patterns that govern their recognition. Ribonucleoproteins are well-known PRMT targets that play a variety of essential roles within the cell once methylated. hnRNP A1, Npl3, and hnRNPK are all ribonucleoproteins studied previously in the Hevel Lab. They share some common features: RNA-recognition motifs (RRMs) that are structured motifs composed of a beta sheet and two alpha helices, and a large disordered region with little structure (**Figure IIa**). Preliminary data in the Hevel Lab has identified that despite these structural similarities, many ribonucleoproteins have drastically different binding affinities to PRMT1 (**Figure IIb**).

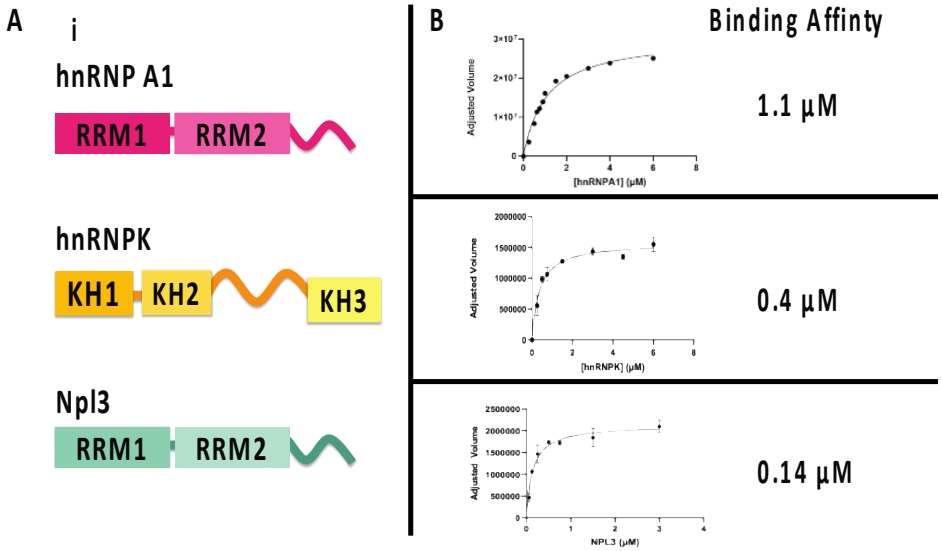

Figure II. a) Structural similarities within ribonucleoprotein targets of PRMT1: hnRNP A1 (pink), hnRNPK (yellow), and Npl3 (turquoise) show conserved RNA recognition motifs (RRMs) and disordered regions. b) Binding curves and apparent dissociation constants (K_D)of these protein targets differ greatly from protein to protein, with Npl3 binding 10-fold more tightly than hnRNP A1.

By making modifications to these targets (deletions, replications, and mutations) and visualizing binding through a fluorescent tag, we can narrow in on the target characteristics that allow recognition by PRMT1. In this work we wanted to question if the binding of the ribonucleoprotein targets by PRMT1 was controlled entirely through the disordered region or if the RRMs also played a role in binding. Additionally, we wanted to know if the numerous positively charged arginine residues in the targets were essential for binding, or if a different residue that still had a positive charge would bind equally well to PRMT1. **(Figure III).** In order to perform these experiments, I designed and created DNA plasmids that encode several ribonucleoprotein target variants, expressed those proteins and started to develop protein purification protocols.

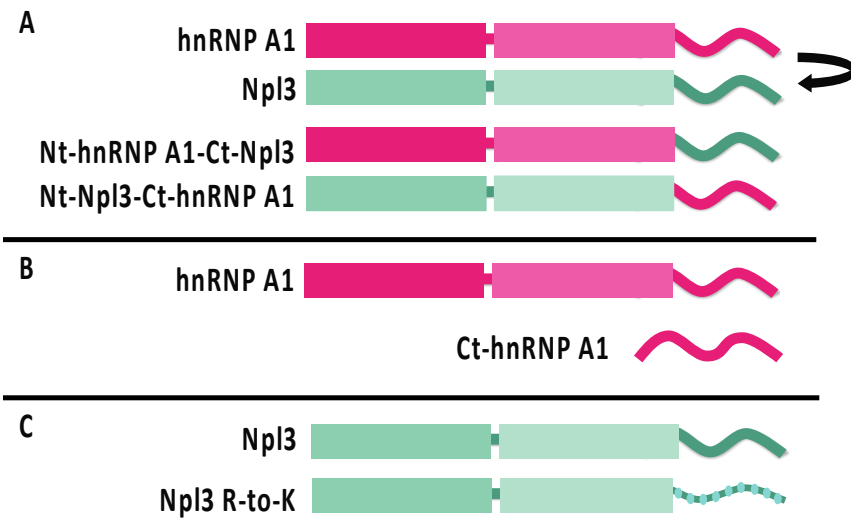

Figure III. Modifications of ribonucleoprotein substrates highlighted in this paper.

a) C-terminal swap of hnRNP A1 and Npl3 b) N-terminal truncation of hnRNP A1 and c) Mutation of all seventeen methylatable arginines to lysines of Npl3

MATERIALS AND METHODS

Identification of a Fluorescent Tag

To assess binding to PRMT1, we needed a fluorescent tag that illuminated brightly at low concentrations, with a molecular weight large enough to see a change in gel shift in Native PAGE upon binding PRMT1. Upon investigating a series of fluorescent tags using the open-source database for fluorescent proteins, FPbase, mNeonGreen was chosen to express on the N-terminus of our protein substrates. A relatively new fluorescent protein, mNeonGreen is a GFP-like beta-barrel protein originally derived from *Branchiostoma lanceolatum*, a shallow-water invertebrate (Shaner et al., 2013). Knowing that we likely needed to alter this DNA sequence for expression in *E. coli*, we reached out to the Den Blaauwen lab in Amsterdam, who kindly shared with us an optimized sequence that expressed successfully in these bacteria (Mertens & den Blaauwen, 2022).

synthetic genes encoding the variants were ordered. Using publicly available sequences from large open-source databases (UniProt) and the structural information available from crystal structures that have been solved of these constructs, I identified the C-terminal and N-terminal regions of hnRNP AI and Npl3 and swapped them. I added a C-terminal Hexa-His tag to allow for easier affinity purification with Nickel, and encoded a series of restriction sites at both termini to clone into the *E. coli* mNeonGreen vector mentioned above. Synthetic genes harboring the above characteristics to create the chimeric variants were commercially ordered.

Once we received and replicated enough plasmid, I cloned the chimeric variant DNA into the mNeonGreen vector. Using restriction enzymes that cut a specific small portion of DNA, I performed a double digest that cleaved the DNA at both ends of the desired vector and insert. To evaluate the efficacy of this step of the experiment, I ran the DNA fragments in a 8% agarose gel (**Figure IV**). From there, I extracted the fragments from the gel and used a gel extraction kit to separate them from the agarose and used a DNA ligase to ligate the fragments together.

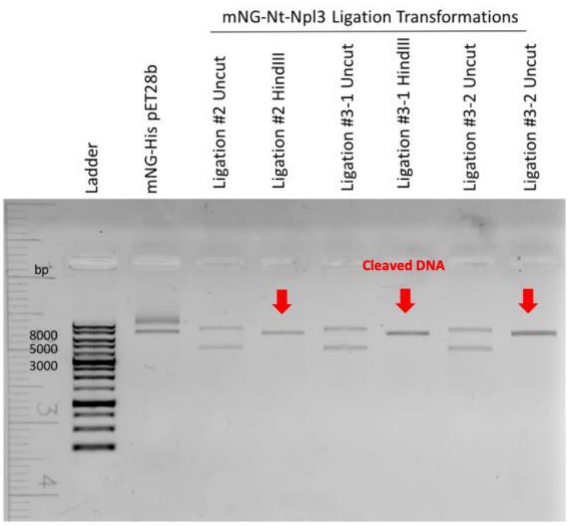

Figure IV. Restriction enzyme digest fragments of mNG vector and Nt-Npl3 construct run on a 0.8% agarose gel.

Colony Polymerase Chain Reaction (PCR)

We needed a way to evaluate the success of the ligation mentioned in the previous step, so we transformed the fragments from the ligations above into DH5Alpha *E. coli* cells. We then used primers specific to regions of DNA flanking the inserted pieces, T7 and T7 Reverse, and performed PCR using the individual colonies as template DNA (**Figure V**). We could then estimate whether the desired portions of DNA had been introduced into the plasmid. The DNA from the colonies with bands at the correct molecular weight was then sent for sequencing using whole plasmid sequencing from Plasmidsaurus. To confirm that the sequenced construct matched our desired construct, we aligned them using Benchling, where the constructs successfully were inserted into the mNeonGreen *E. coli* expression vector **(Figure VI)**.

Figure V. Ligated mNG-Nt-hnRNPA1 constructs amplified using colony PCR and ran on a 0.8% agarose gel. The arrow indicates the desired amplified insert.

Figure VI. Sequence alignment of cloned chimeric construct with expected chimeric construct showed 100% similarity

Expression and Purification of Recombinant Proteins

The mNeonGreen-tagged chimeric ribonucleoprotein target Nt-hnRNP A1-Ct-Npl3 was expressed in BL21 (DE3) cells and purified using nickel affinity chromatography. Through a single purification, I was able to gain mostly (>90%) pure protein, though there was significant nucleic acid contamination, which minimizes its useability in future studies. This was not completed with the Nt-Npl3 construct due to the aforementioned difficulties, but this does show, however, that there is a clear path forward toward its use in binding and gel shift experiments.

RESULTS

RNA Recognition Motifs are not essential for substrate recognition of PRMT1 to hnRNP A1

While not part of this project, these data are closely related and deserve mention in this section. My incredible partner throughout many experiments, Vincent Rossi created a truncated version of mNeon Green- hnRNP A1 with and without the RRMs (**Figure VII**). Even with the structured regions completely removed from the protein, it was still able to bind PRMT1 with similar affinity. This alludes to the potential importance of the disordered C-terminal portion of the protein substrate in binding, and led us to further investigate the C termini of hnRNP A1 and Npl3 through the two C-terminal chimeric species (Rossi, 2023).

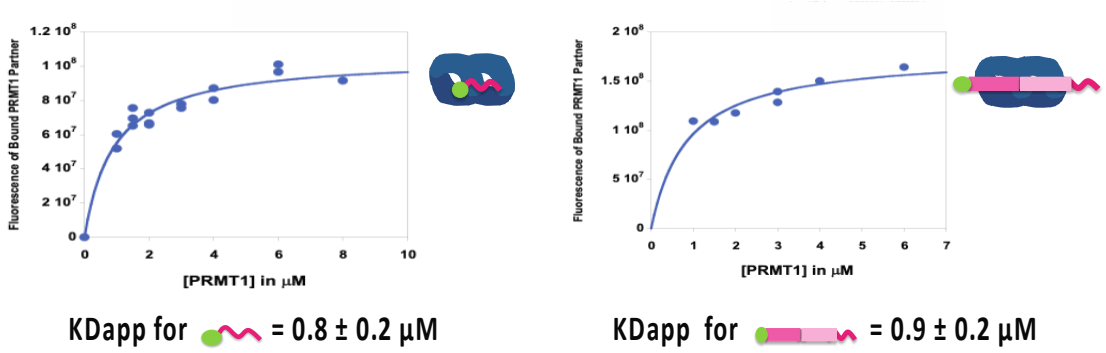

Figure VII. Binding curves as assessed through Native PAGE by Vincent Rossi show a similar Kd apparent for hnRNP A1 with PRMT1 with (right) and without (left) RNA-Recognition Motifs

Chimeric proteins can be cloned and recombinantly expressed in E. coli with a fluorescent tag

The above figures demonstrate the selective cloning and further purification of mNeonGreen labelled ribonucleoprotein substrates that have been successfully cloned, expressed, and purified in *E. coli.* While the process of purification still has yet to be optimized, this demonstrates that

successful *in vitro* use of these constructs is possible, and guidelines have been created for future students who will finish this project.

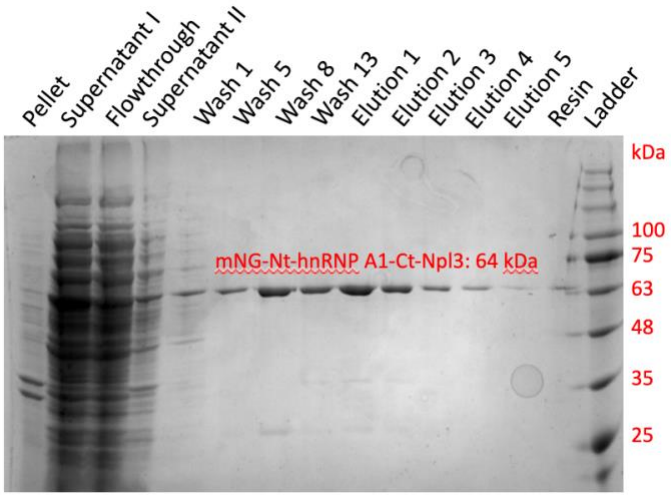

Figure VII. A 12% polyacrylamide SDS-PAGE gel stained with Coomassie blue gel stain visualizing the process of purification of the mNG-Nt-hnRNP A1 construct

A seventeen methyl-lysine AAA species failed to express in E. coli

Following a similar cloning method as mentioned above, I cloned an Npl3 variant containing seventeen arginine to lysine mutations into the mNeonGreen plasmid to assess the role of the arginine in binding PRMT1. We designed the construct with the lysine codon AAA, but when we tried to recombinantly express and purify it in *E. coli*, we were unable to see any expression as assessed through anti-His western blots. When we ordered constructs using AAG as a lysine codon, with help from a summer undergraduate intern, Cassidy Recker, we were able to see expression and were able to purify the construct.

DISCUSSION

This work provides a series of tools and insights into the feasibility of using ribonucleoprotein chimeras as targets for assessing affinity to PRMT1. It was established that mNeonGreen is a bright fluorescent tag that can be used to successfully visualize interaction between PRMT1 through Native PAGE, even at concentrations in the nanomolar range. This provided a tool novel to the Hevel Lab that can now use in other experiments requiring protein detection.

In binding experiments using a truncated version of hnRNP A1 and Native PAGE, my partner Vincent Rossi discovered that removal of the RNA-Recognition Motifs of hnRNP A1 did not result in a significant change in binding affinity to PRMT1, suggesting that all recognition elements in the target are found in the disordered C-terminus of the target. This was unsurprising to me, as the literature has suggested that PRMT1 binds largely at disordered regions, due to the shape of the active site of PRMT1 but is essential to justify modifications to other aspects of the ribonucleoproteins that might confer recognition to PRMT1.

Through cloning using restriction enzymes and assessment using colony PCR, I successfully cloned, expressed, and purified fluorescently tagged C-terminal chimeric versions of ribonucleoproteins hnRNP A1 and Npl3. This sets the groundwork for future students to assay these targets and their association with PRMT1 quickly and efficiently while minimizing the amount of troubleshooting that is all too common in protein purification.

Lastly, I discovered that a seventeen methylatable arginine to lysine mutant (AAA codon) construct of Npl3 does not express in *E. coli*, but expresses when those codons were mutated to an AAG lysine codon instead. Upon some deeper research, we discovered that poly(A) constructs

have been known to act as transcriptional attenuators in *E. coli* (Arthur et al., 2015). It has been shown in the literature that ribosomes slide on lysine-rich homopolymeric A stretches resulting in differential protein production, as well as slows down translation by the ribosome (Koutmou et al., 2015)(Charneski & Hurst, 2013). This account also differed from what has been seen in the literature because our R-K mutations were interspaced, rather than one after another as reported above. These data are essential not only for future Hevel students looking to design and purify recombinant proteins, but also have the potential for a case study or piece of data to a larger manuscript, as it could be useful to the biochemical community to avoid expression issues like ours.

While we haven't fully uncovered the mechanisms that guide target recognition by PRMT1, these experiments lay a foundation for an accessible strategy to improve our knowledge of cellular health. We have yet to investigate if the aforementioned chimeric and R-K modifications to protein targets impact binding or kinetic efficiency of PRMT1. Further modifications with different varieties of targets could also be made. While just a first step, this knowledge could provide valuable insight to the function and key regulatory components of PRMT1 catalysis that are necessary for contextualizing cellular health. It provides us tools with which to view the catalytic nature of PRMT1, enabling better design of potential therapeutics and modulators of PRMT1 activity down the road.

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REFLECTIVE WRITING

I did not start out my degree knowing I enjoyed research. I started volunteering in a biochemistry lab to gain some experience and learn about benchwork. Rapidly I became engrossed with the charismatic, question-asking aspect of research. I started out spending 10ish hours in the lab, becoming familiar with procedures, literature, and developing the question-asking skills necessary to understand the content. Within a couple months, I applied for grants to work on my own projects and found it thrilling to be investigating an aspect of something that no one had ever considered. Here, I built relationships with the graduate students, undergrads, and my incredible mentor, Joanie Hevel. Now a senior lab member, I have worked to train undergraduate and graduate students on protein purification techniques and enzymatic characterization assays and take an active role in the preparation of a manuscript recently submitted to *Protein Science*.

Doing research for this capstone was not without struggle for me, however. After many, many failed experiments (some of which took whole days to perform), phone calls to my mentor late at night (thank you!), and broken supplies (sorry!), I came to realize that, unlike my bedside manner which I pride myself on, I was not born with a natural ability to research. My multitasking abilities are sub-par and I frequently act and speak faster than I think, needing to slow my processes down to eliminate errors. However, I came to learn that I am naturally curious, enjoy asking questions, and can understand and investigate complex topics given sufficient focus ability. I can accessibly explain topics to those with little background, and I enjoy collaborating with multi-disciplinary teams to get tasks done. I am not afraid to ask for help when I need it, nor am afraid of embarrassing myself to ask for clarification.

In specific, having a project of my own was a remarkable chance to test my skills as a researcher. It involved investigating a series of pieces of literature, and formulating plans for experiments that would eventually tell a story that could help people. From here, managing time and communication to implement these experiments was the next challenge, as well as troubleshooting when experiments inevitably didn't turn out the way they were planned. Seeing some experiments come together and gain usable data was the most rewarding part of this process— it helped me feel like the efforts were truly worth all the hours spent. This process let me see what it what like to develop a manuscript from start to finish—from the birth of an idea from my mentor, noticing patterns in the literature and asking questions, to creating and publishing figures and anticipating reviewers' concerns.

Upon performing this capstone, biochemistry became real. It allowed me to use the concepts from my classes for my first two years of school in areas that were important and applicable. In my general chemistry and biochemistry classes, I never thought I would use acid-base chemistry, unit analysis, cellular communication, and biomolecular techniques as much as I have through working on my capstone and in the Hevel Lab. Due to this use, I feel more confident and have greater understanding of a wide variety of topics because I have seen how they contribute to science and cellular health. It helped make school fun and contributed to my professional development as I was fostering the skills that I will need in any field.

In presenting our research on Capitol Hill, my public speaking, and collaborative abilities increased dramatically. It was an exciting challenge to distill biochemistry in a fashion that is digestible for legislators and work with my partner and mentor to come up with analogies that explain the importance of our work. Too often I find that STEM fields are undervalued in policy because they can be inaccessible, but it felt empowering to show legislators what goes on in

biochemistry labs and why it is essential that funding and support continue. We did find a couple of legislators that had been in science fields, such as Representative Lesser, who was a physician before entering the House. How cool!

This capstone and associated lab experience was a defining factor that even changed my career plans drastically, drawing me to consider an MD/PhD instead of just a medical degree. Having worked with patients since I was 16, I was sure that this was the only path for me. I loved being able to help patients feel special, and valued the one-on-one connections I made in assisted living and at the hospital. However, as with many decisions in life, a combination of factors whittled at my stubbornness and headstrong belief that I only wanted to do medicine for the rest of my life. The multiplicative nature of research leads me to believe that in some areas, the potential to save lives could be exponentially greater as a researcher than a physician who helps (and can have significant meaning to) one patient at a time. I also learned that research is not the lonely, isolated work that I thought it was (I envisioned a lonely grouchy grad student sitting at a bench by himself for 80 hours a week counting bacterial colonies, or something of the sort), and instead involves significant collaborative efforts that I enjoy. An in-depth understanding of the biochemical mechanisms that lead to health can create a more competent physician, and greater contextualization of the practicality and process of disease can create a researcher that better sees the big picture.

The significant growth I experienced during this process transformed me from an overeager box-checker to an undergraduate researcher with genuine curiosity, initiative, a growth mindset, and a considerable work ethic. It gave me the confidence to believe that I could be a quality scientist that could help many if given the time, focus, and proper guidance. I am incredibly thankful to my mentor, Dr. Hevel for her innumerable patience, wisdom, and guidance

throughout the process, as well as the stellar team of undergraduate that she selects and with whom I have become very close.

WC: 1010

AUTHOR BIO

Bella Lonardo studied Biochemistry and Spanish at her time at USU. Throughout her time in the Hevel Lab, she has presented at numerous departmental, schoolwide, and statewide conferences, received a College of Science Minigrant and an Undergraduate Research and Creative Opportunities grant, and was awarded the Legacy of Utah State Award for the College of Science. She has loved science since she was very young, and cites joining the Hevel Lab as the best decision she has made in all of college. Looking forward to doing science for the rest of her life, she is in the process of applying to MD/PhD programs for 2025 and is planning to spend six months living in Latin America before attending graduate school.