# Communicating Research to the General Public

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# Metabolism-Driven Epigenetic Reprogramming:

# Dissecting the Role of Methionine and S-adenosylmethionine in Histone Methylation and Heterochromatin Stability

By

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# Chapter 4

# COMMUNICATING RESEARCH TO THE GENERAL PUBLIC

## Wisconsin Initiative for Science Literacy

### 4.1 Prelude

Science communication is the heart of all innovation. “You could be the most brilliant scientist in the world,” an instructor once explained to our class of incoming PhD students, ”but if you can’t explain your research well to those around you, who’s going to believe you? If no one can understand your logic or the importance of your results, your research will end with you.” That, she explained, is how innovation dies.

Here’s the thing: scientists love talking to other scientists. To us, our research is the equivalent of an all-consuming hobby that we can’t shut up about (much to the dismay of our non-STEM family members and friends). Have you ever watched someone’s eyes search for a social savior after you launch into your fascinating description of seeing a rare vermillion flycatcher while birding last year, or how you FINALLY perfected your sourdough recipe after many, many, *many* alterations? Similarly, when it comes to our research, scientists often find themselves pigeon-holed within niche communities where they discuss the finer details of minute processes they have been observing for decades. While this *can* foster brilliant think-tanks where ideas are rapidly exchanged between experts, it can also impede progress when the *same* ideas and ways of thinking become the standard. After all, complex solutions to difficult problems often require us to think “outside of the box”.

We as humans are stronger and more brilliant when we share our knowledge and communicate to find innovative solutions. As you’ll read below, my thesis lies at the intersection of some major scientific fields: metabolism and epigenetics. Both fields have their own sets of general beliefs that have been upheld for decades within their own echo chambers. The interdisciplinary approaches I’ve applied through my research have allowed me to connect the hidden dots between critical cellular processes to untangle and challenge untested assumptions in the fields. Altogether, my hope is that my research is able to build bridges between communities to better foster collaboration and step outside our comfortable niches. Beyond this,

I strongly value the importance of sharing scientific discovery with the general public, to promote transparency for the scientific methods we apply and the broader implications for our results. This belief has transitioned into a passion of mine, and I hope to continue expanding the scope of scientific communication. In line with this, I am thrilled to have the opportunity to share my research with you all through the efforts of the Wisconsin Initiative for Science Literacy (WISL), with a huge thanks to my talented WISL editor in this process, Elizabeth Reynolds.

### 4.2 “Nature versus Nurture”: Your Genes Are Only Half the Story

What does “nature versus nurture” mean to you? This phrase pops up a lot in both biology and psychology studies, and refers to the way that scientists believe some of our traits are fixed by our biology (i.e. nature), whereas other traits are more flexible and can be altered by our environment (i.e. nurture). Now think of a professional athlete. Their height and frame will be dictated by their biology and are largely unchangeable, but things like muscle mass, endurance, and skill can all be learned and trained. While many scientists still argue over whether nature or nurture has the greater effect, the general consensus is that BOTH drastically impact the outcome. Our DNA, or “nature”, we are born with gives a broad stroke framework of who we will be, while our actual life experiences, environments, and stressors will fine-tune the process as we grow and adapt. Now, we can zoom in on the biology aspect a little bit.

DNA is our genetic blueprint which decides everything from what color hair we have, to how tall we will be when we grow up, and even whether we develop chronic illnesses later in life. This is our “nature”, or what we were inherently born with. Cells then access and copy different portions of DNA sequences to build proteins to carry out various cellular functions. Sleep, diet, and exercise are some of the top factors that affect the health of your cells. When you change these factors in your life, there are chemical consequences on a cellular level. What I mean by this is, while your DNA sequence itself does <u>not</u> change over your lifetime, how it is *used* and *regulated* does change! But first, let’s get into some DNA basics…

*4.2.1 What Do You Mean My DNA is Regulated?*

We're going to start from the beginning here. Each and every cell in your body has the same exact copy of genetic make-up, or DNA, which ultimately makes you, you! If we were to take the DNA from inside one of your cells and stretch it out, it would reach 6 feet in length. In order to fit all this DNA inside the nucleus of a cell smaller than the head of a pin, DNA must be wrapped and folded *very* carefully. Kind of like organizing a storage closet, there are some items that are rarely used and can be hidden deep within the mess, while regularly used items need to be stored for easy access. This makes the way that cells organize and wrap their DNA a super critical and tightly regulated process, otherwise we wouldn't have life!

So how do they do it? First, DNA is wrapped around special proteins called **histones**. This DNA-histone interaction is commonly referred to as a "beads on a string" model, where the histone "beads" can slide along the DNA "string" (Figure 4-1). Together, they make up **chromatin**. Like we touched on before, the structure of chromatin is very important for life. After all, you have specialized cells in your body that do all kinds of different things. Muscle cells that contract for movement, pancreatic cells that secrete insulin to help digest meals, neurons that transmit signals to the rest of the body, and so much more! So, if all our cells have the same blueprint, why aren't they all the same? Even though all your cells have the exact same DNA sequence, the ways they USE those genetic instructions is different for each cell type. Ultimately, this boils down to how chromatin is wrapped and which genes within DNA sequence are turned ON or OFF.

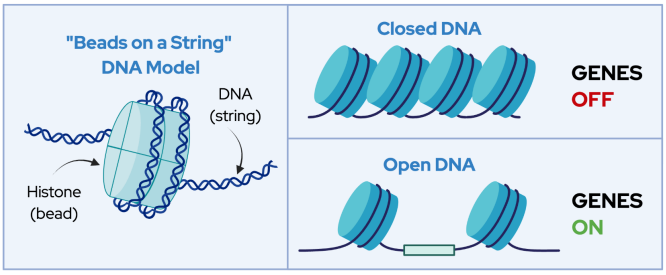


**Figure 4-1**. The structure of DNA in our cells is referred to as a "beads on a string" model, where DNA "string" is wrapped around histone protein "beads". These beads can either be tightly compacted to keep genes off, or loosened to turn gene expression on. Teal box represents an accessible gene region

There are two main structures that chromatin exists in. First, let's bring back our "beads on a string" model. When we only have a few beads on the string, there are plenty of open regions of DNA, or string, that the cell can physically interact with and utilize. This open and relaxed state of chromatin is called **euchromatin**, and is associated with gene expression being turned ON (Figure 4-2). Now, imagine that you fill the string with beads completely so that you can no longer see any gaps of string between the beads. This is called **heterochromatin** and is associated with turning genes OFF. Because heterochromatin is wrapped so tightly, the genes within DNA sequences are no longer accessible as histone proteins act as a physical barrier preventing the blueprint from being read. Therefore, the open or closed structure of chromatin can directly affect which genes are turned on or off.

The active expression of genes results first in a single-stranded RNA copy of the gene (called transcription) from double-stranded DNA. This new messenger RNA sequence is then translated by cellular machinery to create proteins (Figure 4-2). Proteins are involved in nearly every cellular process and ultimately dictate the function of different cell types. This unidirectional process of DNA → RNA → protein synthesis is the **Central Dogma of Biology**, and helps to cement the importance of chromatin structure on the fate of our cells.

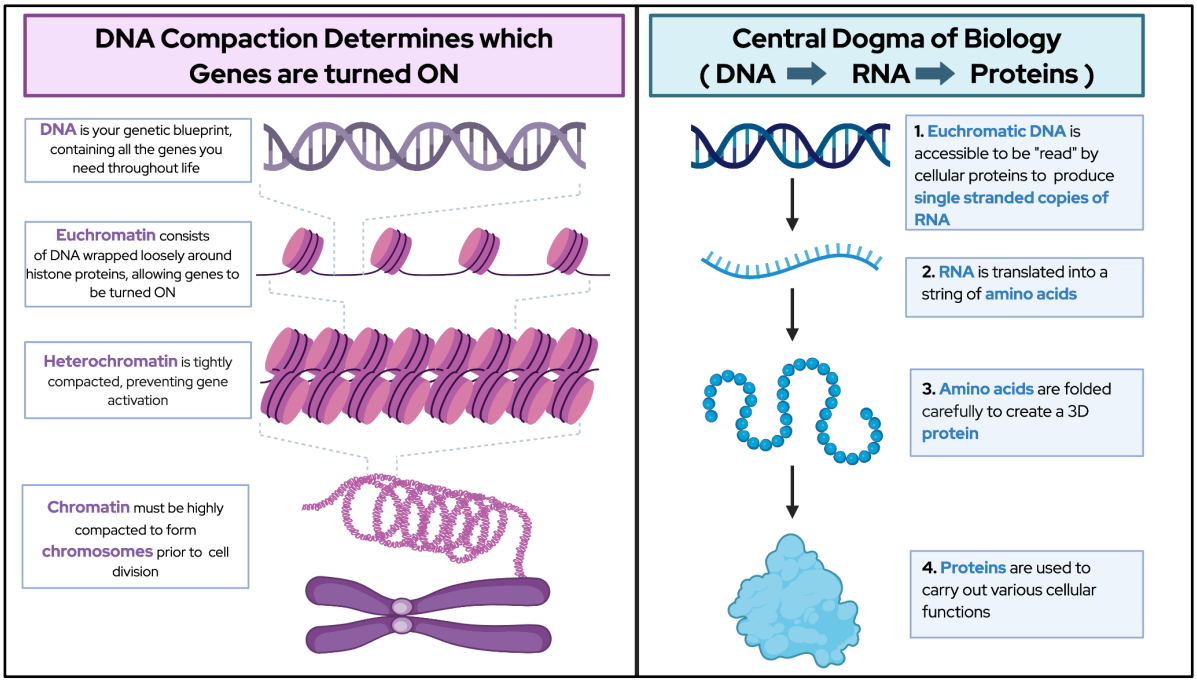


**Figure 4-2.** (Left) DNA requires careful compaction to fit within the nuclei of cells. This includes wrapping around histone proteins, which gives DNA structure and alters gene expression by opening or closing regions containing genes. (Right) The Central Dogma of Biology describes the process in which accessible DNA is transcribed into RNA, which is then translated into a string of amino acids that can be folded into functional proteins.

*4.2.2 What the Heck is Epigenetics?*

Okay, so now we know that DNA in our cells is regulated by its open/closed structure, AND changes to the structure of DNA determines which genes are turned on and which proteins are subsequently made. This is when our "beads on a string" model gets a little more complicated however, because histone proteins don't function simply as inert "beads". First of all, each histone has a long and flexible "tail" which can be chemically modified by cellular proteins. Secondly, each "bead" is actually made up of eight histone proteins bound together. This means each bead has eight histone tails, each of which can be modified in various ways leading to a near limitless combination of chemical signals on chromatin. These chemical signals are reversible and are called **Post Translational Modifications**, or **PTMs** for short.

The two main types of chemical modifications on histone tails are acetylation and methylation (Figure 4-3). **Histone acetylation** directly opens up chromatin structure by weakening the electrostatic interaction between histones and DNA, allowing for a more loose and accessible structure. **Histone methylation** is a little more complex. Rather than *directly* affecting chromatin structure, methylation can be thought of as a signal that must be "read" by different proteins to *either* loosen or tighten chromatin, therefore turning genes on *or* off. The consequences to histone methylation (e.g. are genes turned on or off?) depends on the specific location within a histone tail that is methylated and how heavily methylated that location is. For example, acetylation can only occur once at each location and functions like a light switch in the ON or OFF position. Histone methylation, in contrast, can occur up to three times at each location (called **mono-, di-,** or **tri-methylation**) and functions more like a dimmer switch, to fine-tune gene expression depending on the level of methylation. These PTMs are therefore critical drivers of chromatin structure and gene expression.

There are a host of proteins that are involved in "writing", "reading", and "erasing" these chemical modifications. This allows for a wide diversity of reversible signals which can be placed

on chromatin to quickly change its structure and gene expression. The study of genes is termed **genetics**, and when we study the chemical marks placed "on top of" genetic material (i.e. chromatin) we call this "**epigenetics**". Epigenetics is the foundation of what allows our cells to have different functions in our bodies, like muscle, heart, pancreatic, and brain cells, despite them all having the same DNA sequence. It's as if each cell type reads just a portion of the DNA blueprint to build one piece of the whole to make up you!

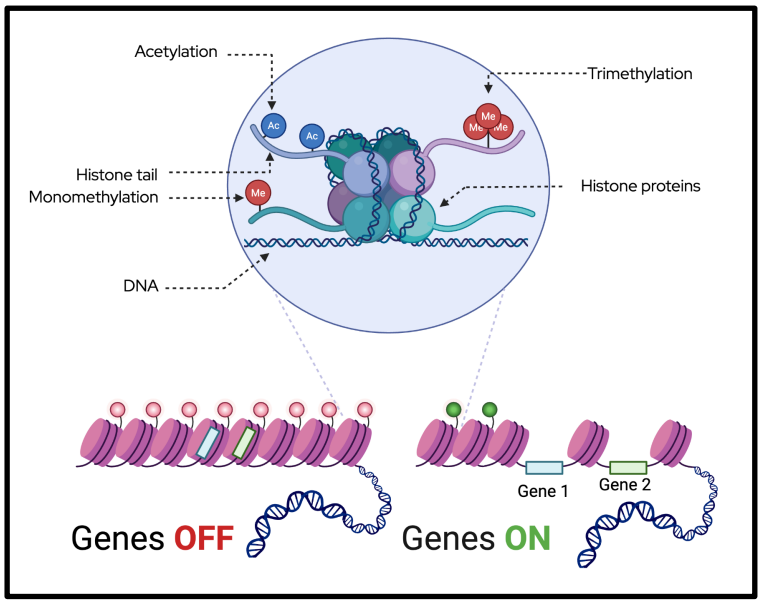


**Figure 4-3:** Epigenetic marks alter the structure of chromatin, resulting in changes in genes being turned on or off. DNA is wrapped around 8 histones which are bound together. The flexible tails of histones can be highly modified by the addition of reversible chemical additions, called PTMs, like acetylation (turns genes on) and methylation (turns genes on or off)

### *4.2.3 Your Lifestyle Affects Your Epigenome*

Let's take a quick recap over what we've covered and put it all together. 1) We know that all of the cells in our body have the exact same DNA sequence which encodes all of the information needed to make you into a functioning human. This information is hard-wired and cannot easily be changed. 2) DNA is wrapped around histone proteins to give it organized structure, which we call chromatin. Open chromatin (i.e. euchromatin) allows genes to be turned on, while very tightly compacted chromatin (i.e. heterochromatin) makes DNA inaccessible and turns genes off. 3) Epigenetics refers to the chemical signals placed on chromatin, which help open or close regions of DNA to control gene expression. They function like control switches for our genes.

I like to think of epigenetics as the "nurture" portion of cellular biology, because it is continuously changing through life as we grow and adapt to all kinds of stresses. If you get sick, your immune cells will incorporate signals to turn on the genes your body needs to fight infection. But even under normal, healthy conditions, your epigenome is changing as you age too! Think about all the developmental stages you go through as you grow from an embryo to an infant to a full adult. Each of those stages requires changes to your cells' epigenome to switch on new genes needed to grow, and at the same time switch off the genes which are no longer needed to preserve cellular energy. In fact, as we age these epigenetic processes tend to get just a little more messed up each time our cells divide. When these tiny errors start to add-up again and again, this is one of the main reasons why our bodily functions start to decline as we age. That's why it's super important that we try to protect our genome and epigenome as best we can! Here's where you might be asking, how in the world are we supposed to know what our DNA needs to be "healthy"? This is where the scientists come in!

### *4.2.4 Cellular Stress and Homeostasis: A Balancing Act*

Let's talk about stress. And no, I'm not talking about the kind of stress that comes from cramming for finals or hosting your in-laws for Thanksgiving. I'm talking about *cellular* stress, because believe it or not, your cells get stressed out too! Here's the thing, living organisms require balance, or **homeostasis**, in order to survive. Think about a tight rope walker balanced carefully on a rope strung between two buildings, his long balancing stick extending out on either side of his body which he uses to counterbalance against the push and pull of the wind. This is what homeostasis is like. It's a constant push and pull between forces, often as a counteracting response to one another, in order to remain in an overall state of balance. Little leans to the left and right won't throw off our tightrope walking friend, but if he can't counterbalance himself, he *will* fall. Cells are just like this. There are all kinds of stresses they have to deal with in order to survive, and if they are unable to adapt to stressors over a long enough period of time, they ultimately die. It's important to understand what the general stress limits are for our cells so that researchers can design appropriate therapies for humans.

My research, as we'll get into shortly, studies how one nutrient from your diet affects your cells' ability to signal through epigenetic mechanisms. Cutting out all the fancy language, I take away one nutrient from cells and measure how they adapt to nutritional stress. What I'm trying to highlight here, is that my years of research into understanding this process in cells is really only scratching the surface of what we can understand on a whole, organismal level. I can't tell you what you should eat, but I can tell you what happens to a cell when we starve them of an essential nutrient.

### *4.2.5 Fighting Cancer with Diet*

Next, we're going to take a look at how understanding stress pathways can lead to new cancer therapies. The word "cancer" can trigger a lot of feelings for a lot of people, but what does it actually mean? **Cancer** is clinically defined as uncontrolled cell growth. This uncontrolled cellular growth leads to the formation of tumors, or abnormal masses of cells. In order for cells and tumors to keep growing, just like humans, they need lots of food to fuel this process, but let's focus on one in particular: **methionine**. Methionine is found in nearly all the foods we eat, which is great, because all of your cells, including the healthy ones, need methionine to survive! The thing is, when cells become cancerous and begin to rapidly grow and divide, they actually start to rewire their metabolism to use even *more* methionine. (Fun fact: This is actually the basis of how PET scans work to detect whether cancers have spread in a patient. Doctors inject a special kind of methionine that can be seen on a scan if it accumulates in parts of your body. Because cancer cells consume and accumulate more methionine than non-cancerous cells, doctors are able to see if cancers have spread to other places of the body by looking for highly concentrated areas of methionine.)

**Metabolism** refers to the interconnected network of chemical processes that are needed to sustain life, like breaking down food into energy or building blocks for growth. When cancer cells rewire these metabolic networks, they develop a **methionine addiction**. In other words, the uncontrollable growth has changed their internal metabolism to be so dependent on methionine that if you take methionine away they no longer survive. When homeostasis, or balance, is shifted in some aspect, healthy cells usually retain the ability to respond and recenter against whatever unbalanced them. This is true in the case of methionine, where healthy cells are able to adapt to short periods of time without methionine, or very low levels of methionine, despite it being an essential nutrient. Methionine-addicted cancer cells, however, have already pushed the system past homeostasis to a state of unbalanced and uncontrolled growth. So now, what do you think

happens when you're unbalanced and add *another* stress? If you're the tightrope-walker, you might fall. If you're a cancer cell, that added stress may be just enough to push you over the edge as well. So, how do we add stress to unbalanced cancer cells? We take away what they've become addicted to: methionine.

Since its discovery, scientists have spent decades trying to understand how to target this methionine vulnerability of cancer. The problem is, methionine is in nearly everything we eat! Nutritionists can make human foods that don't have any methionine in them, but most patients find them unpalatable and struggle to stay on these diets long-term, largely making them an ineffective therapy. So, if we can't eliminate methionine completely from our diets, how else can scientists manipulate methionine levels in cancerous cells to push them towards cell death without negatively affecting healthy cells?

### 4.3 Entering Research

Though I consider myself a cellular biologist at this point, chemistry was my first passion. I was fascinated by the idea that the complexities of the universe could be broken down into atoms, which when simply rearranged, became the building blocks of life. When I first joined the Denu Lab at the University of Wisconsin-Madison for my Ph.D. studies, I loved the fundamental biochemical approaches they took to understanding basic cellular processes (Figure 4-4). It was here that I was able to pursue my fascination with the emerging field of epigenetics to understand the core processes driving diseases, like cancer. So finally, it's time to combine everything we've covered so far.

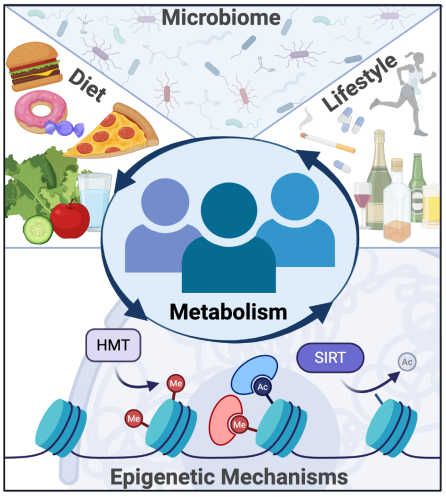


**Figure 4-4:** The Denu Lab at the University of Wisconsin studies how lifestyle changes (usually through diet) affects your epigenome!

During my training period, I was mentored by another graduate student, now Dr. Spencer Haws, who was asking "what happens to the epigenome of cancer cells when you completely remove methionine completely from the diet?" As we covered before, methionine is a critical dietary component of all cells, although cancer cells in particular rely on extra methionine to support their uncontrolled growth. Beyond metabolic pathways, methionine can also be converted into the signaling molecule, S-adenosylmethionine, which we'll call **SAM** for short. SAM is the molecule that actually gets used during all of these histone methylation reactions. Essentially, methionine can be either utilized by metabolic pathways OR be converted into SAM for methylation reactions, thereby connecting metabolism and epigenetic mechanisms through the shared utility of methionine and SAM as essential metabolites in both processes. What's even cooler, is that scientists have demonstrated that the levels of SAM within cells can actually influence their overall levels of histone methylation! This demonstrates that metabolism and

epigenetics have a reciprocal and dynamic relationship with one another, especially within the context of methionine.

To take these concepts from the micro to the macro, let's think about this dynamic relationship like a house where every room is controlled by a separate dimmer switch. In this analogy, methionine is like the power grid that supplies the house with all its electricity, while SAM is like the dimmer switch which controls the amount of light each room gets. When there is plenty of electricity (methionine), the dimmer switches (SAM) are fully operational and can maximally control the light in each room. However, if the power grid decreases in the amount of energy supplied to the house itself (low methionine), the overall light of each room will dim, even while the dimmer switches can still fine-tune the level. In this manner SAM is able to still control gene expression, though the extent of this control is limited by its supply, aka cellular methionine. To take this analogy one step further, you can picture each room as a specific metabolic pathway. Some of these rooms are central and lead into many other rooms, like central metabolism, while some secondary rooms/pathways are only accessible from specific rooms. If the power grid (methionine) is severely lessened (methionine-depletion), it might make sense to dim or even turn off the lights in some less-connected rooms in order to conserve energy for the most essential rooms in the home. Some rooms, after all, are more important to maintain a constant supply of electricity within, like the kitchen and its refrigerator to avoid spoiling stored foods. This is similar to central metabolism providing the building blocks for cells to grow. However, if you work from home, you might need to prioritize keeping the lights in your home office on so that you can work, make money, and purchase more power (methionine) for your house. As you can probably tell, it might take you a while to figure out how to best delegate a limited power source. This is what happens in cells, where they distribute essential methionine to multiple metabolic pathways to keep the cell running, with one main output being its conversion to SAM to be used as a signaling molecule to fine-tune gene expression.

### *4.3.1 Methionine Deprivation Triggers Heterochromatin Dysregulation*

Spencer tested the epigenetic consequences to a methionine-depleted (Met-D) diet in cancer cells. When he looked at histone PTMs following Met-D, he identified a general loss of tri- and di-methylation of histone proteins. These were not surprising results; if you take away methionine, cells can't use it for high levels of methylation (like dimmer switches operating under limited power). What was surprising though, was Spencer identifying that despite the loss of methylation potential (via decreased dietary methionine and SAM), cells were somehow able to maintain low levels of methylation at a specific histone tail location. This suggests that cells are using an unknown mechanism to scrounge up nearly non-existent levels of methionine and SAM, which is then prioritized to maintain specific histone PTMs rather than other cellular uses for methionine. A further interpretation is that there must be something important about these specific PTMs for cells to prioritize so much energy into their maintenance, so what do they do? All of these changing PTMs occurred within heterochromatin, those normally tightly wrapped and repressive regions of DNA. Another key feature of heterochromatin is the presence of ancient viral DNA, often called **transposable elements (TEs),** that have fused with our human DNA many generations ago. Before you worry, no, these viruses can't infect you anymore because they've acquired plenty of inactivating mutations over evolution. They can stress your cells though! If we look at the bigger picture, this means that changes in metabolism or diet can have immediate and drastic effects on the regulation of DNA. When DNA becomes less regulated (losing repressive tri-methylation at heterochromatin), this has negative consequences for our cells (ancient viral genes get turned on).

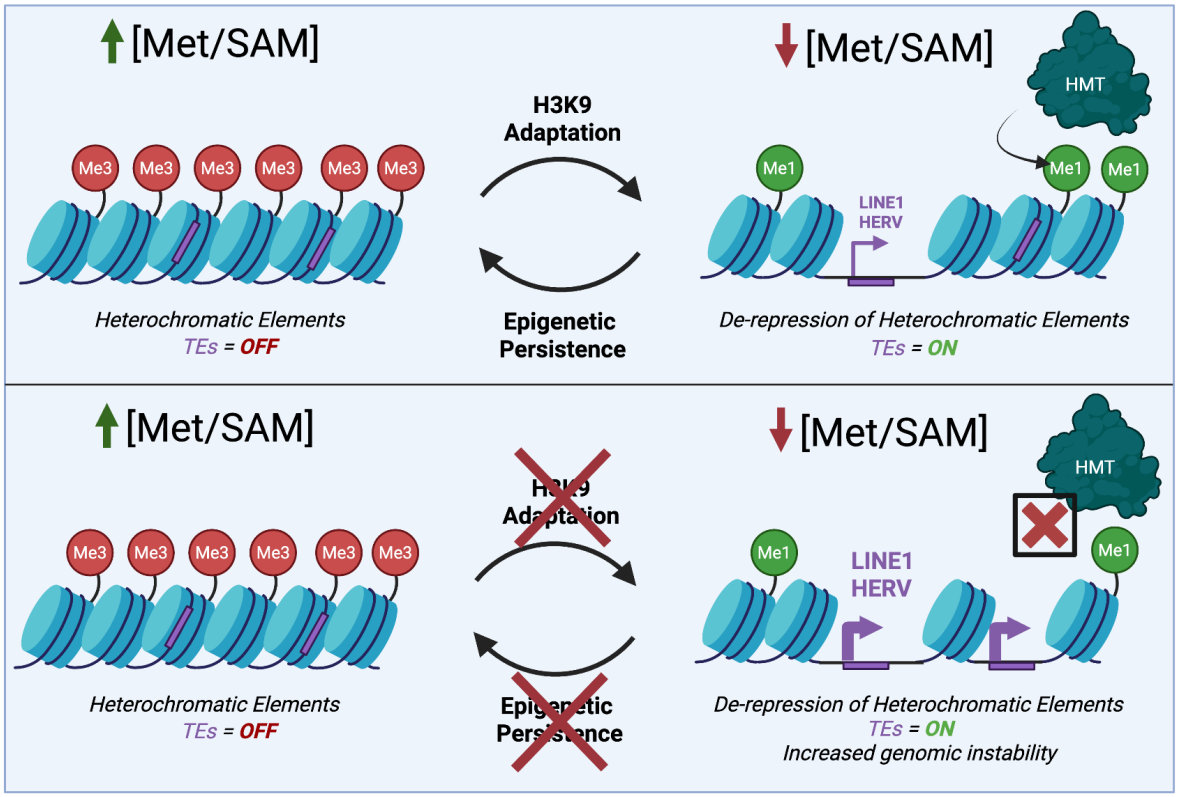


**Figure 4-5. Top**: Spencer found that when cells don't have enough methionine and SAM, heterochromatin cannot maintain proper regulation of H3K9me3 (red circles) to keep chromatin closed, and instead histone methyltransferases (HMT) uses active H3K9me1 (green circles) to minimally repress TEs (purple bars). If we give cells back methionine after depletion, they can recover their epigenomic homeostasis and properly repress heterochromatin again. **Bottom**: If we block active H3K9me1, there is a further increase in TE expression, which is a form of cellular stress. Importantly, without H3K9me1, cells cannot regain epigenetic homeostasis.

### 4.4 My Research Story

Wow. That was a lot of background to get to my part in this story, huh? Sometimes it can feel like verbally describing a mesmerizing piece of art and expecting your listener to fully grasp its beauty. Scientific writing isn't usually as colorful as a priceless work or art, but discussing our findings and continuing to build off the research of the past is how we make progress. Here, I will share my research story of untangling more of this Met-D stress pathway by following up on Spencer's discoveries. I will share my successes, my many failures, and how the results I've produced can be used by future researchers to move our understanding of these complex processes forward towards potential therapies.

### *4.4.1 Early Failures: Three Strikes and Still Swinging*

If you asked me for my advice before starting a biology PhD, I would tell you, “Get used to failure.” This might sound harsh, but one of the most important mental switches I’ve made is to understand that failing is an essential component to learning. Because guess what? I failed. A lot. But I rarely repeated a mistake, and with each test I performed with my own hands I cemented more knowledge into my brain than any Googled answer would have. What I hadn’t realized was that experiencing my own failures taught me so much more than reading about any other scientist’s successes. Throughout my description of my thesis, you’ll notice I use the term “failure” a lot, but I don’t say this with any shame or regret. Each failure taught me a lesson, some harder than others, but now I see it as a truly critical component for any type of growth.

My thesis plans were to follow up on Spencer’s research and see if I could identify some of the proteins that were involved in maintaining methylation at heterochromatin under Met-D, despite these cells having non-measurable levels of methionine (Figure 4-5). Understanding the inner workings of this process could increase our chances of identifying potential therapies to disrupt cancer’s methionine addiction. Primarily, I was interested in the protein named *Methionine AdenosylTransferase II*, or as we call it, Mat2A. Mat2A is classified as an essential protein, meaning that it is required for normal cell development and survival. It is the only protein that can use methionine to produce SAM, the signaling molecule used for every methylation reaction. To recap: methionine is an *essential* amino acid that comes from our diet and is used in many metabolic pathways to produce additional amino acids, metabolites, or proteins required for survival. Because SAM production requires methionine, Met-D cells dramatically lose both methionine and the signaling molecule, SAM, very rapidly. Therefore, if all histone methylation reactions require SAM production, and methionine/SAM levels are practically nonexistent, then I hypothesized that the protein Mat2A is likely a critical factor in providing the SAM needed for H3K9me1 and survival under Met-D.

I first investigated whether Mat2A can bind to DNA directly. If it did, this could indicate a mechanism for which Mat2A itself binds to chromatin and produces SAM at the specific DNA locations where active H3K9me1 is occurring. Mat2A has a protein binding partner called Mat2B. Mat2B cannot produce SAM like Mat2A, but when the two bind to each other, this interaction alters the structure of Mat2A slightly so that it is more stable (i.e. prevents Mat2A from being degraded naturally by the cell) and can better find methionine. This is like a flashlight (Mat2A) and a rubberized grip (Mat2B). Only the flashlight can actually produce light, but the rubber grip allows you to hold the flashlight steady to find what you're looking for (like methionine) more easily. Mat2B, therefore, could also be critical to this process by supporting Mat2A's activity to produce SAM more efficiently under Met-D.

To test whether Mat2A, or Mat2B, could bind to DNA directly, this first requires purifying large quantities of the proteins from E. coli cells. Next, I set up chemical reactions combining Mat2A, or Mat2B, with DNA fragments that were fused with a small chemical structure that expresses light (i.e. fluorescence). Essentially, we measure the light produced by DNA fragments on their own first, then see if this light changes when we add proteins to the mix. If there is a difference in fluorescence signal, this indicates your protein has bound to the DNA. When I tested Mat2A and Mat2B's ability to bind DNA, I was able to see that only Mat2B could interact directly. This indicated that though Mat2A did not bind directly to DNA, it could potentially be brought to regions of DNA through interacting with its binding partner, Mat2B.

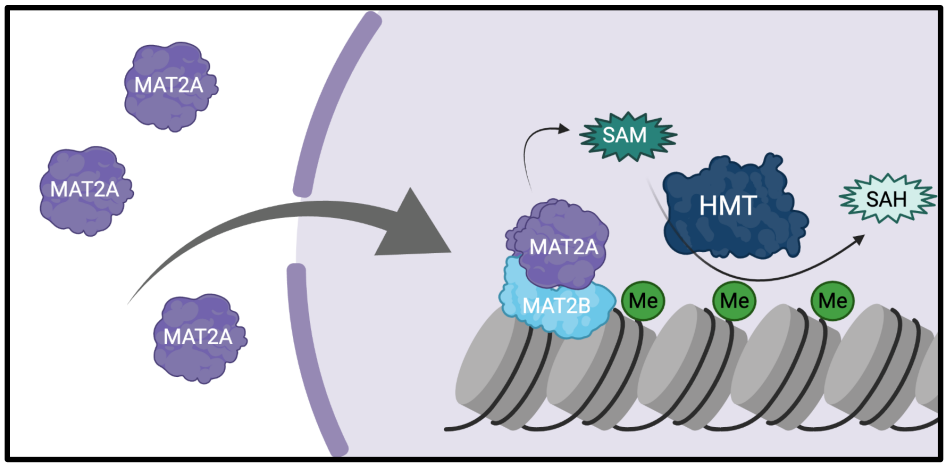


**Figure 4-6**: My first full hypothesis I based my preliminary exam on, which was ultimately, incorrect. I hypothesized that Mat2A (which produces SAM needed for the methylation of histones) binds to DNA through the help of its binding partner protein, Mat2B. This could be a way for the cell to produce SAM directly where it's needed for active H3K9 monomethylation for heterochromatin protection, rather than SAM being used in alternative cellular reactions. The figure depicts the nuclear translocation (i.e. migration into the nucleus) of Mat2A, where it binds with Mat2B at heterochromatin (grey). Mat2A creates SAM which is immediately used by Histone Methyltransferases (abbreviated HMT) to monomethylate H3K9 and maintain heterochromatin regulation. The byproduct of a methylation reaction is S-adenosyl homocysteine (SAH), which then re-enter metabolic pathways and can eventually be converted back into methionine.

This hypothesis surrounding Mat2B's potential role in bringing Mat2A to regions of heterochromatin DNA which desperately require SAM for active methylation was what I based my preliminary exam and future thesis project on (Figure 4-6). Because Mat2B cannot actually make SAM, it was not a largely studied protein and I was excited that my hypothesis could identify a totally new role for Mat2B under the context of methionine deprivation. The next step to test this hypothesis, was to eliminate Mat2B from cells, and test if heterochromatin regulation still occurs. Here, I wanted to use the expression of ancient viral genes as a proxy, or representation, for lost heterochromatin structure. Spencer's experiments had previously confirmed that expression of these genes is anti-correlated with the heterochromatin methylation (e.g. more methylation = less RNA). Therefore, I used the RNA levels of TE's such as HERV-R to identify how dysregulated (i.e. un-methylated) heterochromatin is (Figure 4-7, left). If my hypothesis is correct, and Mat2B

is essential for mediating H3K9me1 under Met-D, I would expect to see even higher expression (e.g. RNA) levels of viral genes upon the loss of Mat2B (Figure 4-7). Spoiler alert: I was wrong. The loss of Mat2B during Met-D did not increase viral RNA, indicating that heterochromatin regulation was still happening without Mat2B. My first central hypothesis, and the entire basis of my projected studies, was incorrect.

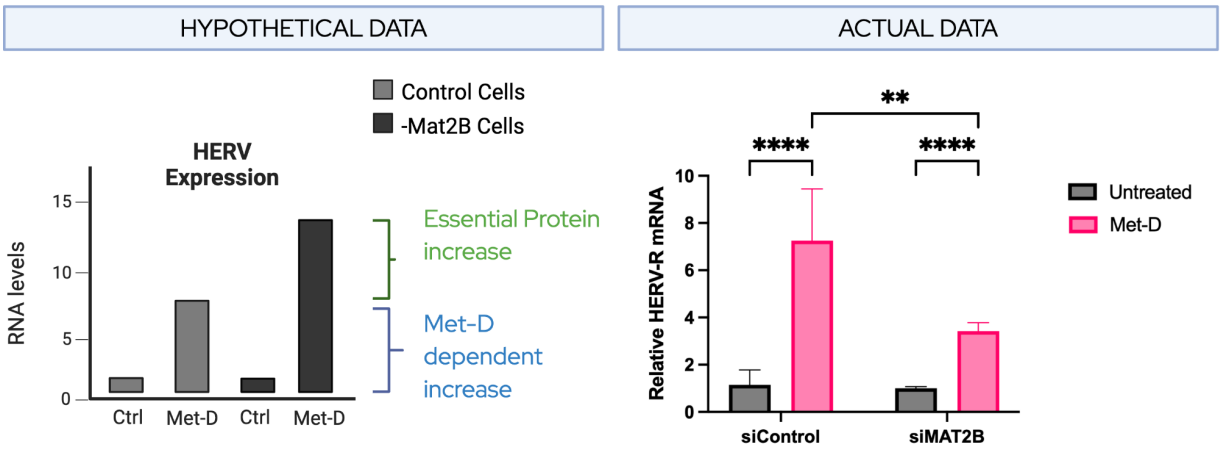


**Figure 4-7**. **Left**: Hypothetical results using RNA levels of TEs such as HERVs as a proxy for dysregulation (higher RNA = less heterochromatin). In blue, are the expected increases in expression levels due to Met-D alone. In green, is the hypothesized further increase in RNA if Mat2B, or SAMTOR, is essential for mediating H3K9me1 at heterochromatin. **Right**: Actual experimental results. Eliminating Mat2B during Met-D did not result in increased heterochromatin dysregulation/HERV expression.

After the failed Mat2B experiments, I switched gears to investigate a newly discovered protein named SAMTOR. SAMTOR normally binds to available SAM within the cell, however, when SAM concentrations drop significantly, unbound SAMTOR then associates with a major cell regulator (i.e. mTORC1) to inhibit growth signals. This allows SAMTOR to sense the availability of cellular SAM and coordinate cell growth when there are sufficient nutrients, like SAM. So, I eliminated SAMTOR in cells prior to Met-D and measured the RNA levels of viral genes as a proxy for heterochromatin dysregulation. Just like in the case of Mat2B, the loss of SAMTOR

under Met-D conditions did not increase viral RNA. This meant SAMTOR was dispensable for H3K9me1 heterochromatin regulation of viral genes under Met-D. Project “failure” number two had arrived.

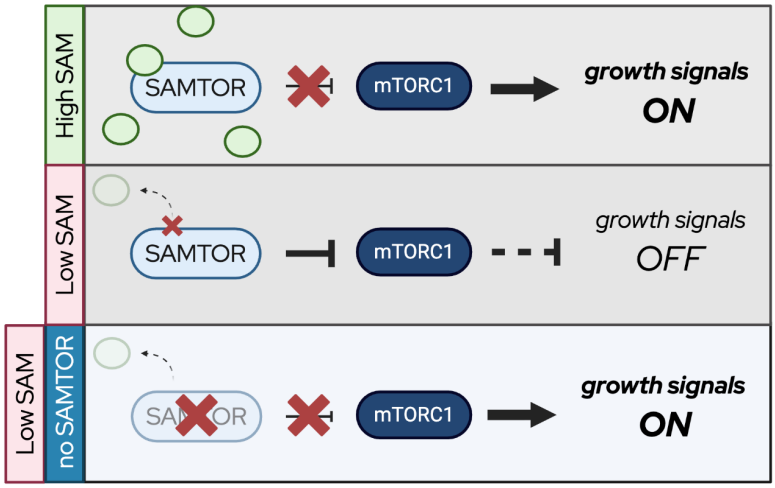


**Figure 4-8.** When cellular SAM concentrations are high, SAMTOR is bound to SAM and does not interact with mTORC1, leaving growth signals on. When SAM levels drop, unbound SAMTOR interacts with and inhibits mTORC1, thereby turning off growth signals. Eliminating SAMTOR from cells prior to Met-D removes the cellular “brake”, and causes mTORC1 to remain active and growth signals to stay on. I hypothesized that SAMTOR was therefore critical to sensing low SAM and shutting off growth for cells to adapt to dietary stress

By this point in my graduate career, I was pretty darn discouraged. Despite my inner pity party, I still had to fulfill my yearly program requirements to orally present my research to a wider audience of scientists. Remembering the advice of my mentor, I tried to highlight all the things I had learned from these project “failures”. Directly following my presentation, I was approached by a graduate student who had a lot of questions about a familiar protein: Mat2A. What started as a discussion over reagents, slowly turned into a full-blown collaboration between us. I’m not going to discuss the details of this project here, as not all of the results nor ideas are mine to claim, but driving this project with a fellow graduate student was a major boost to my self-confidence as a scientist. We came up with new ideas to test, taught each other new techniques, and worked closely for over a year and a half to turn our experiments into a joint publication. The data we were generating was promising and we had the support of our PhD advisors to complete this joint project and graduate from our respective programs. My collaborator and I, however, forgot one key concept in all our experimentation; we forgot to fail fast. Unfortunately, we had made

assumptions about a common scientific technique, which after careful testing, proved incorrect. This essentially negated all of our previous work, turning our results into rubbish. If we had prioritized failing fast, that control experiment would have been one of the first things we tested. Instead, we both learned a hard lesson.

### *4.4.2 Unexpected discoveries lead to unexpected results*

When this third hypothesis failed, I felt like a baseball batter with three strikes. Five years of dedicated research and I still had very little data to explain what was actually happening in the cell under Met-D. Looking back at my data, there was one experiment that had given me intriguing results. Instead of depleting methionine levels in cells, which consequently reduces SAM levels as well, I wanted to deplete SAM levels *without* affecting methionine content (i.e. remove the signaling molecule only). My hypothesis was that the heterochromatin regulation we see under Met-D is really due to the loss of the functional molecule, SAM, rather than methionine itself. If my hypothesis is correct, I would expect to see the same dysregulated heterochromatin response (i.e. increased viral RNA) when I limit SAM, but not methionine, via Mat2A inhibition. In a very surprising twist of events, the loss of SAM did not trigger heterochromatin dysregulation, and viral RNA levels remained low.

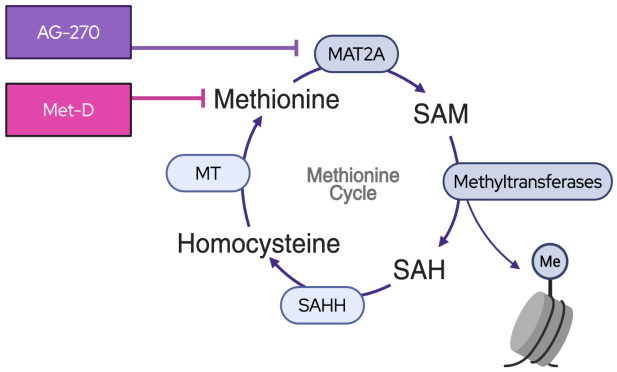


**Figure 4-9.** Simplified methionine cycle with key enzymes shown in ovals. Met-D eliminates methionine and SAM, while AG-270 treatment only prevents SAM from being made, without affecting methionine levels

These results really were surprising! The general consensus in published literature was that SAM was the key molecule here, especially with the discovery of SAMTOR being a cellular SAM-sensor when abundance is low. At the time I generated these results, I was confused at how there could be such drastically different effects on heterochromatin, despite the levels of SAM being similarly depleted under Met-D and AG-270 treatment. Now that my other research

directions had vaporized, I thought back to this experiment and decided to test it further. This time though, I was going to try my hardest to fail fast.

The first thing I investigated was the timing of SAM depletion during both Met-D and AG-270 treatment. We knew from previous experiments that when you give cells media lacking methionine, the methionine already within cells is used up in minutes and SAM levels drop rapidly to reach depletion within an hour. Importantly, we collect cells after 24-hours of Met-D because this allows sufficient time for cells to adapt their epigenome. Cells need time to sense stress and adapt appropriately, while epigenetic remodeling requires the coordinated action of many proteins. So, to apply my newly reinforced "fail fast" mentality, I sought to find ways I could prove my results wrong: "If Met-D treatment rapidly depletes SAM within an hour, allowing epigenetic changes to occur by hour 24, maybe the inhibitor works much slower and that's why I'm not seeing any heterochromatin dysregulation." In other words, even if SAM levels are comparatively low at the 24-hour snapshot I look at, there is the possibility that AG-270 treatment depletes SAM at a slower rate than Met-D, therefore not allowing sufficient time to see epigenetic changes by 24-hours. To test this, I treated cells with Met-D or AG-270 and collected cellular metabolites at multiple time points. It didn't fail. Results showed that AG-270 treatment significantly lowers SAM levels within 1 hour, and is fully depleted by the next time point taken at 3 hours. This data suggests that if the loss of SAM alone was triggering heterochromatin dysregulation, there is sufficient time for epigenetic changes to occur (similar to Met-D) by 24-hours.

Not a timing issue, great! My next attempt at failing fast was to see if I could generate the same results in a different cell type, which would suggest this was a general feature of all cells. Incredibly, my results were confirmed: SAM-depletion by inhibiting Mat2A with AG-270 does <u>not</u> stimulate the same heterochromatin response as seen in Met-D. This means that there is something specific about the loss of methionine, not SAM, that triggers an epigenetic response at heterochromatin.

The drastic difference in responses led me to test the hypothesis that Met-D specifically activates additional stress pathways, which are then responsible for modifying heterochromatin. I tested the RNA levels of genes involved in various stress pathways and found that the RNA of Activating Transcription Factor 4, or ATF4, was greatly increased. ATF4 is a key member of the Integrated Stress Response, a network of pathways that detect various cellular stresses and respond appropriately. These pathways generally converge to activate ATF4, which then turns on the expression of genes required to resolve the stress signal. One of ATF4's main targets was also observed to be highly expressed in Met-D, but not AG-270 treated cells. This indicates that the loss of methionine, but not SAM, triggers ATF4 activation, likely through some upstream signal to the integrated stress response.

Digging back through published literature to better understand how ATF4 ties into Met-D responses, I came across some interesting clues. ATF4's RNA is turned on by proteins who remove the repressive epigenetic signals at heterochromatin, under certain nutrient stresses. (Figure 4-10). These special proteins that remove methylation are called demethylases, and the family of specific demethylases which remove tri-methylation at heterochromatin are called KDM4s.. Intriguingly, the RNA levels of all KDM4s were significantly increased under Met-D only, indicating that depletion of methionine, but not SAM, triggers the activation of histone demethylases involved in removing repressive heterochromatin signals.

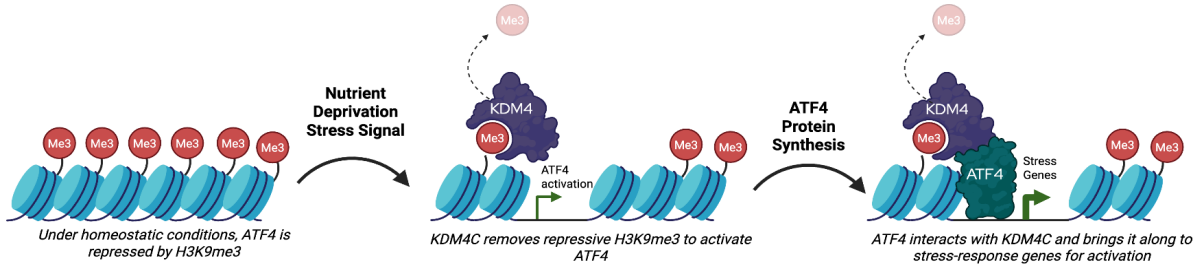


**Figure 4-10.** Histone demethylase, KDM4C, removes repressive PTMs to activate ATF4. Upon activation, ATF4 binds with KDM4C and together localize to stress-response genes for activation.

Time to look at the big picture of what we know so far. The loss of methionine activates a general stress response, including ATF4 and KDM4 histone demethylases, which then likely remove the repressive PTMs found at heterochromatin to free up metabolites which could theoretically re-enter the methionine cycle. Then, newly synthesized methionine can then be converted to SAM, which is used to maintain the new PTMs at heterochromatin. These new PTMs cannot repress ancient viral genes (LINE1 and HERVs) as well, resulting in their activation and an increase in their RNA content. Untangling the cascade of activated proteins involved in the upstream integrated stress response to systematically determine which specific proteins are critical for Met-D mediated heterochromatin dysregulation, is a tall task. That alone could be its own PhD thesis! So for now, we'll leave it at the integrated stress pathway, including ATF4 and KDM4s, are involved in mediating Met-D induced stress.

To round out the remainder of this project, there was one last question that always stuck in my mind. What is the purpose of the ancient viral gene expression? In other words, is their expression simply a *consequence* of lost heterochromatin regulation, or is their expression actually a *part of* the stress response mechanism? And if they are a stress signal on their own, what are the consequences to increasing their RNA levels?

Investigating these questions led me to some fascinating insights. Remember that integrated stress response (including ATF4) and how it is activated by various cellular stressors? As it turns out, viral RNA is one of these stresses. When the cell senses viral RNA in the cell, usually from viral infection, this activates a separate stress pathway so that your body can mount an immune response to clear the virus. Recent evidence describes similar activation of this response as a consequence of *endogenous viral genes.* Endogenous is synonymous with "internal" or "self", so "endogenous viral genes" refers to those ancient viruses that incorporated themselves into your ancestor's DNA thousands of years ago. When *endogenous* viruses activate an immune response, this is called **Viral Mimicry** because the products of your very own DNA

are mimicking an infection. There is still a lot scientists don't understand about this viral mimicry response, but there is plenty of evidence linking dysregulated heterochromatin, and the subsequent expression of LINE1 and HERVs, to immune activation. To test this, I measured the RNA levels of various immune genes and found almost all to be highly increased in Met-D conditions, but largely unchanged following AG-270 dependent SAM-D treatment. This data importantly suggests that depleting methionine triggers a viral mimicry response, likely through the increased RNA levels of endogenous viral genes due to lost regulation of heterochromatin.

### 4.5 Concluding Remarks: Finding connections one PhD at a time

All in all, my research identified additional stress pathways that are activated by dietary methionine stress. This includes a general stress response, ATF4 activation, histone demethylase activation, and viral mimicry response. These responses, as well as epigenetic adaptation, are consequences of lost methionine, but are importantly NOT triggered by the depletion of the signaling molecule, SAM. While it's not necessarily surprising that the loss of an essential and widely used dietary component like methionine triggers stress responses through multiple avenues, the fact that eliminating SAM (used for all methylation reactions) did not stimulate ANY stress response, was quite surprising. Importantly, this data contradicts some untested assumptions in the field. Protein and histone methylation is a quick way for cells to signal stress across pathways, and it was generally accepted that since SAM is the critical determinant for whether protein methylation occurs or not, it must be SAM itself that is “sensed” and adapted to. Lucky for us, we’ve learned the art of failing and not trusting in assumptions.

So where do we go from here? What benefits can my research provide in the long run? For one, the Mat2A inhibitor, AG-270, was recently used in a Phase I clinical trial to treat certain advanced solid tumors. These patients all had cancers in which a key enzyme involved in methionine recycling (called MTAP) is no longer functional. These specific tumors have pushed their equilibriums even farther than a traditional methionine-addicted cancer cell. Not only are they relying on excess methionine supplementation to support their growth, but they can’t recycle ANY intracellular methionine due to the inactivation of MTAP (which is commonly inactivated in ~15% of cancers). AG-270 treatment on its own, as my research indicated, is relatively well-tolerated by cells and results in little to no adaptation of heterochromatin or gene activation for growth to continue. However, combining this generally well-tolerated stress with a cancer type that is unable to recycle any methionine, was enough to slow down the metastasis and disease progression. This is why testing and understanding the complicated mechanisms cells use to adapt or escape stress, is critical for designing new human therapies

In conclusion, there are just a few points I really want to nail down. 1) Failure IS learning. The more you fail, the more you learn and the more you grow. 2) Your DNA is highly regulated by its structure, which ultimately decides which genes are turned on and which proteins are made. This is the basis which allows you to have specialized cells in your body! 3) Cancer cells are addicted to methionine, which is a key nutrient used for growth and regulation of chromatin. 4) By studying the ability of cells to respond to different stressors, we can find ways to push cells away from homeostasis and into cell death. For example, scientists have been working for decades to determine how to best target the methionine-addiction vulnerability of cancer cells. 5) Scientific discovery is a lengthy and expensive endeavor, which requires constant communication and collaboration. Here, I was able to build off of Spencer's discovery of Met-D heterochromatin adaptation, and show that this response is completely dependent on methionine levels rather than SAM concentration. These are all small steps towards understanding the larger picture, but progress, nonetheless.