Communicating Research to the General Public

The WISL Award for Communicating PhD Research to the Public launched in 2010, and since then over 100 Ph.D. degree recipients have successfully included a chapter in their Ph.D. thesis communicating their research to non-specialists. The goal is to explain the candidate's scholarly research and its significance—as well as their excitement for and journey through their area of study—to a wider audience that includes family members, friends, civic groups, newspaper reporters, program officers at appropriate funding agencies, state legislators, and members of the U.S. Congress.

WISL encourages the inclusion of such chapters in all Ph.D. theses everywhere, through the cooperation of PhD candidates, their mentors, and departments. WISL offers awards of $250 for UW-Madison Ph.D. candidates in science and engineering. Candidates from other institutions may participate, but are not eligible for the cash award. WISL strongly encourages other institutions to launch similar programs.

Wisconsin Initiative for Science Literacy

The dual mission of the Wisconsin Initiative for Science Literacy is to promote literacy in science, mathematics and technology among the general public and to attract future generations to careers in research, teaching and public service.

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Pyrrolemania:

Metabolite-dependent regulation of brown adipose tissue thermogenesis via heme biosynthesis

By

Dylan Jake Duerre

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Chapter 7: Wisconsin Initiative for Scientific Literacy (WISL) Chapter

Title:

Metabolic Manipulation: Altering the Cellular Supply Chain to Discover Novel Approaches for Metabolic Disorders

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Introduction

Prelude

Throughout my pursuit of a PhD over the last five years, I have been forced to think about many perplexing questions. However, the one question that has been most difficult for me to answer has not come from experiments, my mentors, or scientists in my field. Rather, it has been a question coming from friends and family, who frequently ask “what are you researching?” Although it seems basic in principle, offering a sufficient and accurate answer to that question has been remarkably challenging. Initially, the response I provided was the same with no regard for who I was talking to, whether it was my thesis committee or my parents. Filled with scientific jargon and lacking context, I quickly recognized that my explanations were met with confusion and disorientation, and I had completely failed the exercise.

Often, I would leave those interactions with a sense of imposter syndrome, feeling like I was not a good scientist. To protect my ego from that sense of shame and defeat, I had to figure out a way of dealing with those feelings. After discussing this topic with a colleague early in my training, I came to the opinion that the best strategy would be avoidance. Rather than engaging deeply with

a family member or friend who had genuine curiosity about the work I was doing, I would instead provide short, single word answers like “metabolism” or “fat”. This approach gave me a path where I could simultaneously answer truthfully and avoid feeling like a phony. While this strategy temporarily helped, it also created a sense of cognitive dissonance.

My adoption of an avoidance strategy coincided with broad shifts in public perception of science and medicine during the pandemic, starting in 2020. The ensuing mistrust of institutional scientific organizations and the concomitant rise of alternative sources can be attributed in part to at least two things. First, strong incentives motivated both well-intentioned individuals and opportunistic charlatans to make engaging content related to science and medicine. Unfortunately, much of the novel information was ill-informed at best, and at worst, outright false and potentially dangerous to the public’s health and wellbeing. Second, the rapid expansion of social media-sharing outlets had created new platforms for misinformation to spread. Regardless, the outcome was greater public mistrust, reflecting a broader failure of the scientific community to adequately meet the moment with effective science and medical communication at scale.

Although just a graduate student, I felt I had a duty to be an active part of the solution that would help re-establish trust in “Science”. But when I began reflecting, I was forced to confront my own hypocrisy. My unwillingness to work on public science communication skills was in direct conflict with my desire to help address the problem. The nature of my approach towards communicating my thesis research was not only extremely superficial, but also deeply patronizing towards those who wanted to learn. I was doing a disservice to myself and others.

As evidenced by my own friends and family, the general public is hungry for scientific knowledge. Taxpayers and private donors who support research should feel entitled to explanations that help

them better understand how we as scientists contribute knowledge that advances the human experience.

Since that inflection point, I have decided to make it a priority to develop my skills in communicating scientific data and ideas. During the last two years of my PhD training, I presented my research project to other scientists and experts dozens of times, including in lab meetings, departmental research symposiums, and international conferences. These experiences, many of which were supported by the goodwill of public and private funding, gave me the opportunities needed to test different approaches in scientific storytelling and data delivery.

Although presenting in these environments is quite different from answering the question of “what are you researching?”, they have been instrumental in teaching me that while it can be challenging to do well, effectively communicating science is worthwhile and rewarding. My decision to include a Wisconsin Initiative for Scientific Literacy (WISL) chapter in my PhD thesis is meant to reflect that belief, and now, passion. The WISL team operates an excellent program for communicating scientific research to the public, and I am grateful for their support throughout the writing and editing process, especially to Elizabeth Reynolds.

<u>So, what is the answer to “what are you researching?”</u>

From a thirty-thousand-foot view, it is indeed true that I study metabolism. One of the main reasons I chose to pursue a PhD was because my undergraduate studies and consumption of science content like videos and podcasts sparked a fascination regarding the role that metabolism plays in how our bodies develop, perform, and change over time. Since then, I have sought to contribute knowledge that helps us better understand how we live, grow, get sick, and recover. I was lucky enough to end up in the lab of Dr. Andrea Galmozzi, whose mission as a faculty member

at UW-Madison is to study how changes in metabolism affects how our genes are expressed, and how they can drive or prevent diseases such as obesity, type II diabetes, and cancer.

However, stating that I study metabolism doesn't provide much insight into what I was actually doing on a day-to-day basis over the last five years. My intention for this chapter is to properly tell a large portion of this story. In doing so, I want to explain it using a framework for thinking about metabolism as a cellular "supply chain" system. Thinking about it through this lens helped us make a couple exciting discoveries that may be important for human health. In this chapter, I wish to demonstrate the following principles about metabolism:

1. Metabolism is the underlying process in our cells and bodies that allows us to harvest and utilize things in our environment to grow, adapt, and survive.

2. Despite its complexity and thousands of moving components, metabolism exhibits a striking level of interconnectedness and coordination.

3. Understanding and mapping the paths of metabolism can teach us how our bodies work, how disease manifests when things go wrong, and how manipulating metabolism can be an approach to treat diseases and improve health.

Before diving into the research that I performed, it is important to adequately cover the major question this research aimed to answer, and why that question was worthy of pursuit.

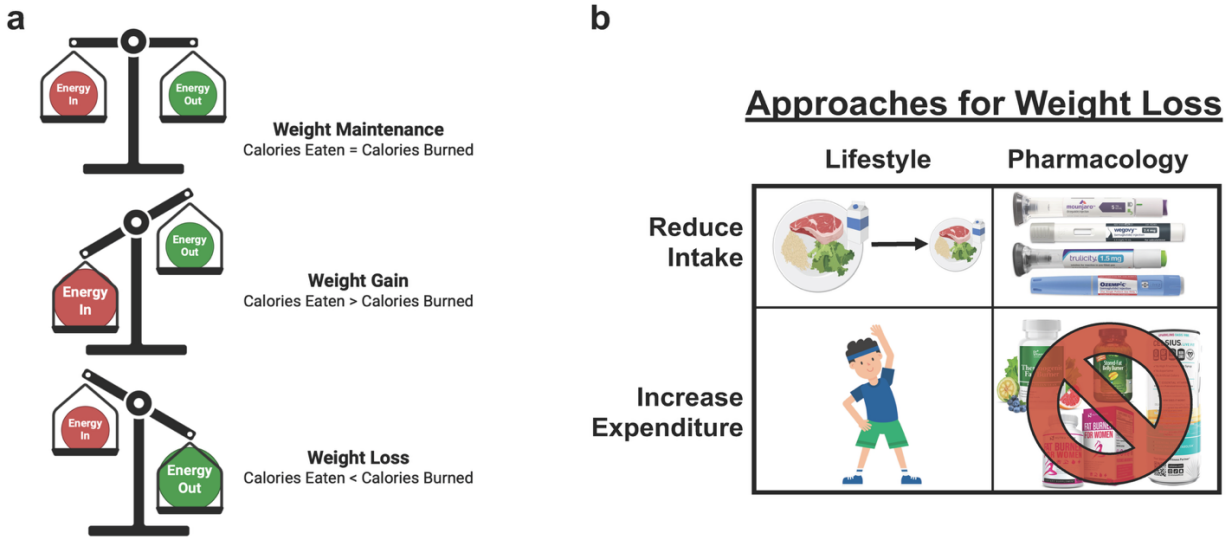
<u>Food, energy balance, and the obesity epidemic</u>

We will begin with something we are all familiar with, which is food. There are two fundamental truths underlying how we interact with food, and how food interacts with us.

First, what we put into our body matters. Even if the total calories are equal, it is easy to appreciate that the nutritional value of arugula salad and meat lover's pizza are not equivalent. We instinctively know that the foods we eat can affect many different aspects of how we feel, think, or sleep.

Second, when it comes to our body's composition and weight, the energy content of our food does matter. Given enough time, the energy balance equation (energy in vs. energy out, **Figure 1a**) reigns supreme. If energy consumed as food (calories in) and energy expended (calories out) are equal, weight will be maintained. However, imbalances between energy intake and expenditure will shift body weight. If someone maintains higher energy expenditure than intake (calories in < calories out), they will lose weight. In contrast, if someone consumes more energy than they use (calories in > calories out), they will gain weight.

Figure 1: Tipping the scales of energy balance.



Unfortunately, our modern environment makes it easier to consume more energy than we expend. In just the last 20 years, the prevalence of obesity and diabetes has exploded across both the

United States and much of the developed world. This is not only a healthcare problem, but an economic problem as well. The CDC estimates that the direct and indirect costs of obesity and diabetes to the US economy is over half a trillion dollars per year[1,2], and modeling indicates that it will continue to get worse as an aging population becomes more susceptible to these states of metabolic dysfunction[3]. To solve this epidemic of energy imbalance, we need systems and tools that help people restore metabolic health and lose unhealthy body weight.

When it comes to the energetics of weight loss, we can favorably tip the scale through two distinct, but not mutually exclusive methods (**Figure 1b**). The first is by lowering calorie intake. Historically, this has been achieved directly through dieting and reducing the total amount of energy consumed. In recent years, new FDA-approved diabetes and weight loss drugs such as semaglutide (Ozempic/Wegovy) and tirzepatide (Mounjaro/Zepbound) have emerged as powerful tools for weight loss, as their multifaceted effects, in part, reduce appetite and lower energy intake.

We can also drive weight loss by increasing energy expenditure through approaches like increased exercise. Historically, doctors and nutritionists believed that calories burned through exercise were additive. That is, if a person normally burns 2,000 calories per day, and then burns 500 additional calories through exercise, then the total energy expenditure for the day will be ~2,500. However, emerging research has suggested that it might not be so straight forward. Rather, calories burned through voluntary activity like exercise might not necessarily be additive[4]. Instead, the body senses an increase in calories burned and compensates by reducing the basal metabolic rate. In this example, despite burning 500 calories through exercise, the normal amount of energy burned per day is reduced below 2,000 calories, resulting in a total energy expenditure that is lower than 2,500 calories. This non-additive nature of voluntary energy expenditure may therefore limit maximal returns of calorie burning through exercise.

Furthermore, despite the marketing gimmicks and misleading advertisements for “fat burning” consumer supplements or products, there remains a lack of safe and effective compounds that can meaningfully burn extra calories. However, the current absence of such compounds doesn’t mean that we can’t develop new approaches to help burn calories. In fact, historical examples which failed due to toxicity concerns have taught us important lessons about safety and efficacy, and provided valuable insights about new approaches to drive weight loss.

Dinitrophenol as a weight loss compound: hard lessons learned

A great historical example of this is a chemical compound called 2,4-dinitrophenol (DNP) that was used in French factories to produce armor piercing ammunition during World War 1[5]. Women working in these facilities were frequently exposed to DNP and began reporting adverse side effects including nausea, dizziness, severe hyperthermia and sweating, coupled with profound weight loss. In multiple instances this exposure was particularly tragic, leading to severe injury or death. Why DNP was so toxic was not understood at the time, but its observed capacity to drive weight loss suggested that, if the dose could be right, it might be a useful weight loss compound.

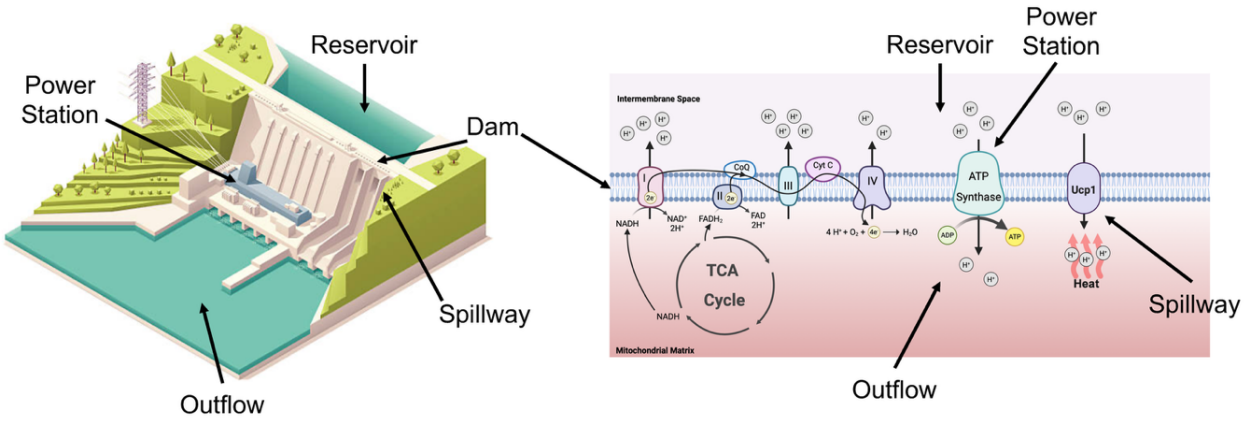
As a result, sale of DNP as a “cosmetic” slimming agent skyrocketed. However, it’s extremely small therapeutic window, that is, the range where DNP works and is safe to consume, made it difficult for consumers to get the dosing right. This unfortunately caused additional toxicity and deaths associated with its use. As a result, human consumption of DNP was made illegal following the passing of the Food, Drug, and Cosmetic Act in 1938. A decade after its removal, researchers at Stanford would identify the mechanism of action driving DNP-induced weight loss[6].

Mitochondria: a dam good analogy

To explain how and why DNP worked for weight loss, and why it was so toxic, we need to first discuss mitochondria. Known canonically as the “powerhouses” of our cells, mitochondria have

many parallels to hydroelectric dams that we engineer into our environments (see **Figure 2**). In principle, a dam captures potential energy in the form of stored water, where rivers and tributaries feed a reservoir that collects behind the wall of the dam. Rather than letting all of the water flow downstream, a dam controls the flow rate and directs water to spin turbines, effectively capturing its kinetic (i.e. movement) energy to produce electricity.

Figure 2. Mitochondrial energy production: a dam good analogy



Likewise, the energy in our food is captured through metabolism, that is, the chemical reactions that take place in our cells that enables us to utilize the energy within carbohydrates, proteins, and fats. To generate energy for our cells, metabolism produces molecules that contain high energy electrons (e^-), which are used to power a system called the electron transport chain (ETC). Much like the rivers and tributaries that feed the water reservoir behind a dam, the activity of the ETC feeds a reservoir of positively charged hydrogen ions (H^+; referred to as protons) on one side of a biological membrane within the mitochondria. This mitochondrial membrane acts like the wall of a dam to hold back the potential energy of the protons, which like the water within a reservoir, seek to flow “downstream” to restore a state of equilibrium on both sides.

While a dam feeds this flow of water through engineered channels to spin turbines, the mitochondria in your cells do this with protons. In the mitochondrial membrane, a protein complex called ATP synthase harvests the energy of the proton “flow” to make ATP, the energy currency of the cell.

The destructive uncoupling power of DNP in the mitochondria

Thinking about mitochondrial energy production through this analogy, DNP acts like an explosive. Disrupting the membrane integrity (analogous to the wall of the dam), DNP allows the potential energy in the stored reservoir to be unleashed in an uncontrolled manner. In our mitochondria, the unimpeded movement of protons across the mitochondrial membrane increases the activity of the ETC, accelerating metabolic rate and increasing energy expenditure.

In the case of a dam, the “uncoupling” of the reservoir’s potential energy from electricity production by uncontrolled flow would result in catastrophic consequences downstream. Likewise, uncontrolled mitochondrial uncoupling in the cells of our bodies is similarly consequential. The uncontrolled increase in metabolic rate by mitochondrial uncouplers like DNP produces extreme amounts of heat in every cell of the body. The observed toxicity of DNP was perfectly explained by this uncontrolled heat production, damaging the structural and functional integrity of the trillions of cells in our bodies.

We can learn two lessons from DNP. The first is that it should not be used for weight loss because it is not safe. Second, we learned that altering mitochondrial metabolism through uncoupling can increase energy expenditure and drive weight loss. This observation presented an opportunity: what if we could come up with an alternative approach to safely uncouple mitochondria? And if we could, could it be done in a more controlled way that is both safe and effective for weight loss? It turns out the answer to that question is yes, but before discussing how, let’s revisit the dam analogy.

Dams also serve additional purposes beyond energy production, including water storage for agricultural, industrial, and consumption purposes. Dams allow for control of water levels downstream, helping to prevent flooding in times of high precipitation, or maintain flow in times of drought. And just like dams, our mitochondria can use their reservoir for more than just energy production.

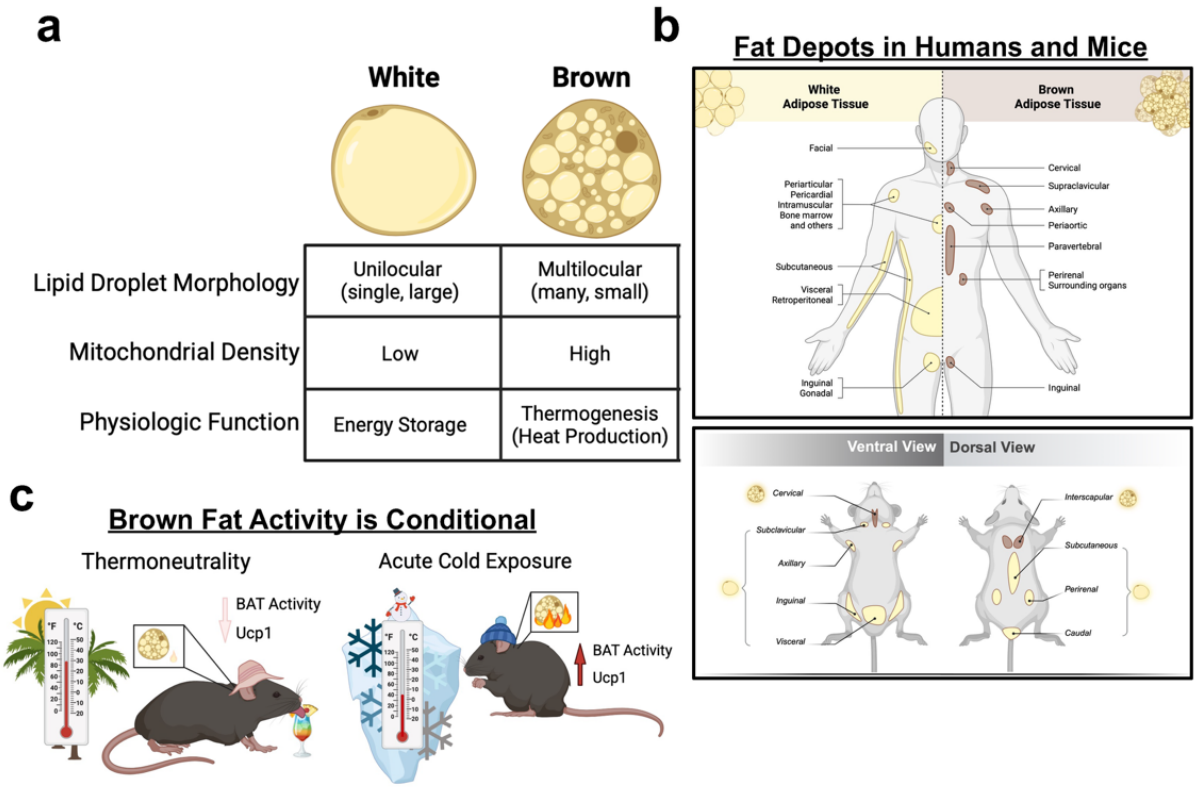
Similar to how a spillway in a dam allows water to safely flow through and not be utilized for electricity production (see **Figure 2**), specialized mitochondria present in a select few tissues of our bodies also have machinery paralleling a spillway. Rather than allowing protons to move safely across the membrane without being used for ATP synthesis, these specialized cells contain mitochondria with a "spillway-like" protein called uncoupling protein 1 (Ucp1). As its name suggests, Ucp1 "uncouples" the movement of protons from the production of energy through ATP synthesis.

Instead, the movement of protons back across the membrane by Ucp1 allows their energy to be converted to heat in a process appropriately called uncoupled thermogenesis (thermo = heat; genesis = production). Unlike DNP which induces chemical uncoupling within all cells of the body, Ucp1 safely mediates heat production only in cells where it is present. The presence of Ucp1 enabled specialized tissues to produce vast amounts of heat, which was a critical evolutionary trait that supported territory expansion and survival in environments where acute and chronic cold exposure was common[7]. Furthermore, the activity of Ucp1 is tightly controlled, allowing it to be turned on for heat production only under appropriate circumstances.

<u>Fighting fat with fat: brown adipose tissue thermogenesis</u>

So you might be thinking the following question: where does this take place? What tissues and cell types have Ucp1 present in the body? Well, it turns out that the answer is fat! This might be surprising considering that fat is the tissue we want to reduce in patients with obesity and diabetes. However, not all fat is equivalent (**Figure 3a-b**).

Figure 3. Differences between white and brown fat



When most of us think of fat, we think of white fat, which sits under the skin, on the limbs, around the tummy and hips, whose function is primarily for energy storage and physical padding. However, we also have a unique type of fat called brown fat. As the name suggests, brown fat is different than white fat, in both appearance and function. Rather than storing energy in the periphery, brown fat has evolved to form in specialized areas of the body for heat production and helps endotherms (i.e. animals that must produce their own heat) to maintain body temperature.

Accordingly, the primary function of brown fat is heat generation by uncoupled thermogenesis, which is primarily achieved through the presence and activity of Ucp1.

Brown fat function is especially important in smaller animals which are more vulnerable to disproportionate loss of body heat in cold environment*s*. As it is primarily used for heat production, the activity of brown fat thermogenesis is dynamic (**Figure 3c**). In rodent species like mice, brown fat activity is negligible in a warm state of thermoneutrality, where no additional energy burning is needed to maintain appropriate body temperature. However, when mice are exposed to cold at night or during cooler months, the activity and heat production of brown fat increases substantially.

Despite our limited exposure to cold stress due to the privileges of modernity, our brown fat is still responsive. Following identification of active thermogenic fat depots in humans, much interest has been garnered in developing strategies to promote its activity. Basic research aimed at understanding how brown fat works in mice revealed the signal and receiver that turns on brown fat. In response to cold exposure, a hormone called norepinephrine is released by peripheral neurons in the adipose (i.e. fat) tissue*,* which binds to a receptor on the membrane of brown adipocytes (i.e. fat cells). The binding of norepinephrine to this specialized receptor initiates a signal that results in activation of thermogenesis in the brown adipocyte, including mitochondrial uncoupling by Ucp1.

While norepinephrine is the molecule produced in our bodies that can bind to this receptor, we also have multiple drugs that can mimic it by activating its receptor on brown adipocytes. One of these compounds is a drug called mirabegron, which was FDA approved for treating overactive bladder in 2012. Mirabegron is capable of triggering expression of the *Ucp1* gene and activating its activity to induce uncoupled thermogenesis. Furthermore, unlike DNP, Ucp1 induction by Mirabegron activates heat production almost exclusively in fat cells.

This has made mirabegron administration an attractive strategy to test to increase energy expenditure. Excitingly, it is beginning to show promise in clinical studies. For example, a study from the National Institutes of Health (NIH) published in 2015 demonstrated that administration of mirabegron to young, healthy human subjects activated brown fat and increased energy expenditure[8]. While additional study is needed, these results suggest that activation and expansion of brown fat might be a feasible approach for development of new treatments for obesity and diabetes.

So now, let's revisit the differences in white and brown fat. Why are they different, and what makes the brown adipocyte uniquely suited to perform mitochondrial uncoupling and thermogenesis? The mantra of "form is function" is commonly used in biology and refers to the idea that the visual appearance of something is indicative of its importance and purpose. Accordingly, we might get a hint by starting with the color differences between white and brown fat. Where does the rust brown color of brown fat come from? The cartoon in **Figure 3a** provides the answer, although it might not be your first guess.

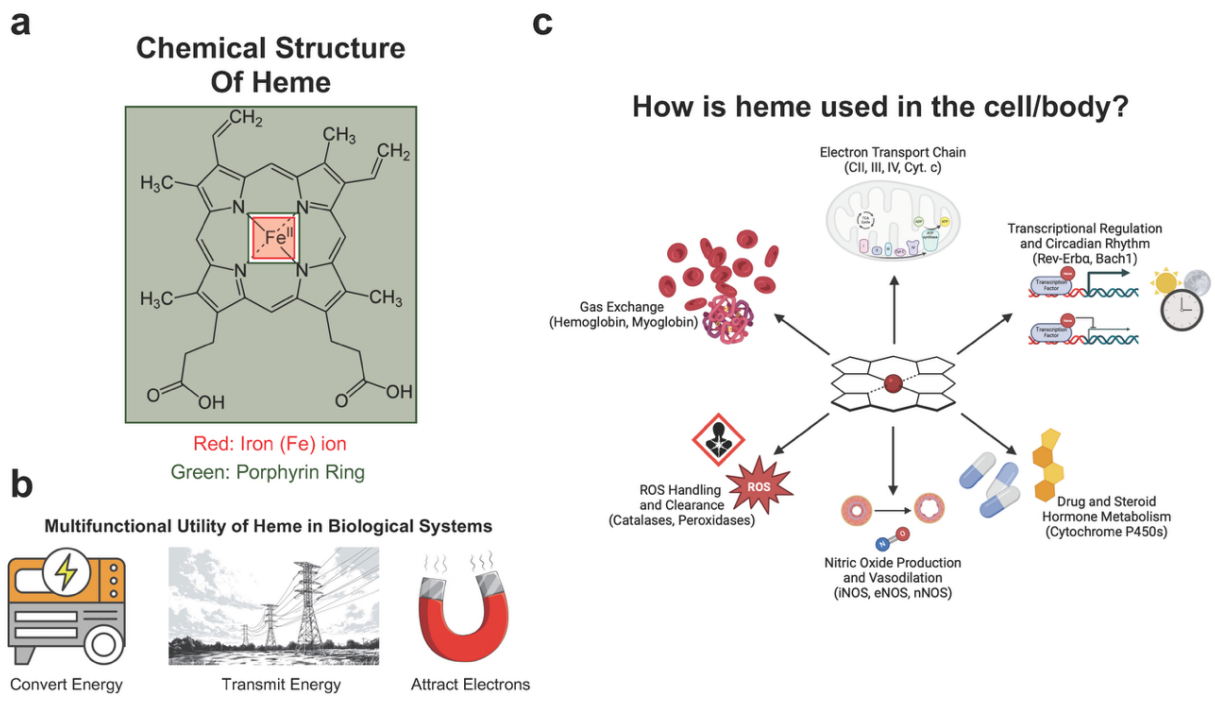
While it is true that brown adipocytes contain a smaller volume of stored fat than white adipocytes, the brown color is not a default. Rather, it comes from substantially greater density and content of mitochondria, those little organelles that produce energy and, in the case of brown fat, heat. But then the next question you might ask is why do mitochondria appear rust brown? Perhaps surprisingly, the answer is rust! Indeed, mitochondria get their distinctive color from their high concentration of iron-containing molecules, and in particular, a molecule called heme.

We are now going to turn our attention to heme, because it was the focus of my thesis research project. Let's begin by answering a couple questions first: what is heme, and what is its purpose in the mitochondria?

Heme: a critical component of the thermogenic furnace

Heme is a large molecule composed generally of two parts: a positively charged iron (Fe^{2+}) ion, and a porphyrin, a flat chemical ring that stabilizes and centrally coordinates the iron ion within the structure of the molecule (**Figure 4a**). When we think of iron in our daily lives, we commonly associate it with rust. The reason why iron-containing structures like vehicles and bridges rust is due to the tendency of iron to oxidize. At the molecular level, this means that iron likes to give its negatively charged electrons to oxygen. This process forms iron oxide compounds, one of which is rust. The same principle happens with the iron in heme.

Figure 4. Heme in biological systems



The preference of iron is to give away negatively charged electrons, making its associated ion become positively charged. This positive charge, similar to a positive pole of a magnet, attracts negatively charged electrons and molecules with an abundance of them.

The porphyrin ring of heme helps to stabilize the iron ion when its positive charge (or oxidation state) is altered through accepting electrons or interacting with negatively charged molecules. The ability of heme's iron to readily and stably change between different oxidation (that is, charged) states is leveraged in metabolism to attract or facilitate the transfer of electrons. These types of electron-exchanging reactions are called reduction-oxidation (redox) reactions and are extremely common in cellular metabolism. In some ways, these features enable heme to function as a multifaceted tool in the cell, acting as an energy converter, an energy distributor, and a magnet for electron-rich molecules within the cell (**Figure 4b**).

You are most likely to be familiar with heme due to its role in a protein called hemoglobin, the component of our blood that gives it its red color (**Figure 4c**). In this case, the heme in hemoglobin acts like a magnet to bind and transport the molecular oxygen (O_2) we breathe to cells and tissues across the body. However, this is not the only role of heme in the body, or even cells. There are numerous, and perhaps dozens of things heme can do in the cell. For the purposes of this project however, we will mainly focus on two of these functions.

The first is that heme is an essential component for the mitochondrial electron transport chain (ETC), which as we discussed earlier, allows us to generate energy and heat from the food we eat (see **Figure 2** and **4c**). Second, heme can also alter how our genes are expressed by modulating the activity of proteins called transcription factors, which control what, where and how much the genes in our DNA are turned on or off (**Figure 4c**).

We already know that heme dynamics are critical for brown fat function. Work from our lab has previously shown that a protein called Pgrmc2, which shuttles heme between the mitochondria and the nucleus where DNA is stored in the cell, regulates thermogenesis and the expression of the *Ucp1* gene. In fact, deletion of *Pgrmc2* in fat cells impairs heat production, in part through reducing the expression of the *Ucp1* gene[9].

It is clear that heme's multifaceted role in the cell is important for brown fat function. In a sense, it acts as critical infrastructure that is intertwined into almost every aspect of cell biology. Despite this knowledge, we as researchers did not have a clear understanding of how brown fat cells acquire the heme that they need to function properly.

Results

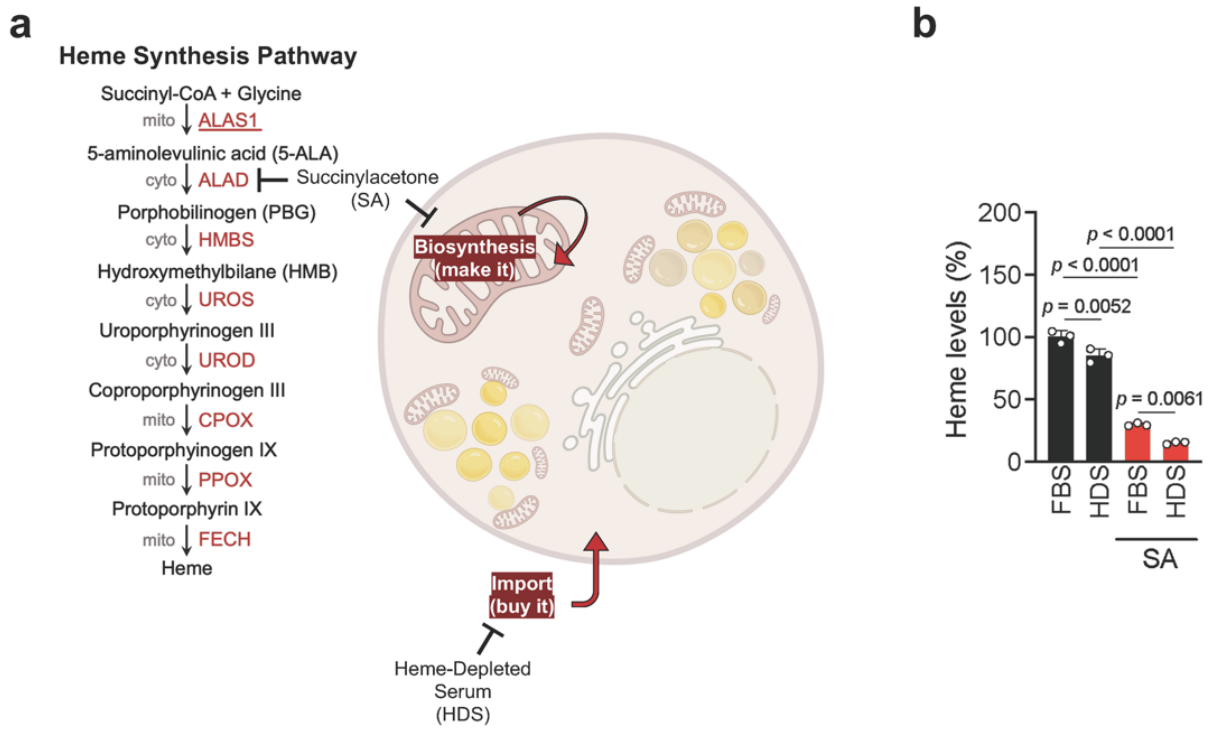
A gap in knowledge: how is heme sourced?

This basic question was really the foundation of this project. How do brown adipocytes acquire the heme they need? Is it produced locally, is it imported, or do both happen simultaneously? Put another way, how does the cellular heme supply chain work? As we dive into the data, keep this framework of a supply chain in mind, as we will return to its underlying principles throughout the story for guidance.

Looking at **Figure 5a**, you can see a visualization of the two routes of heme acquisition for a brown adipocyte. It can be made (that is, built in the cell through the heme biosynthesis pathway), or it can be imported (brought into the cell as a finished product). To determine the contribution of these two sources to the total pool of intracellular heme, we can simply interfere with the pathways and ask a simple question: how much do heme levels change? By doing so, we would be able to infer their relative contributions. To do this, we can block heme synthesis by treating cells with a chemical called succinylacetone (SA), which inhibits the second step of the production pathway.

Alternatively, we can remove heme from the environment that cells are grown in (i.e. heme-depleted serum; HDS) to prevent them from importing it.

Figure 5. Heme acquisition in brown adipocytes



This experiment was one of the first I performed as a PhD student, which I did alongside my lab mate, Juli. When we did this experiment and looked at the results (**Figure 5b**), we found that heme levels barely changed when we prevented import with HDS, suggesting that the contribution of import to the total heme pool is quite low. On the other hand, blocking heme synthesis caused a large reduction in heme levels, telling us that brown adipocytes primarily source heme by building it rather than importing it.

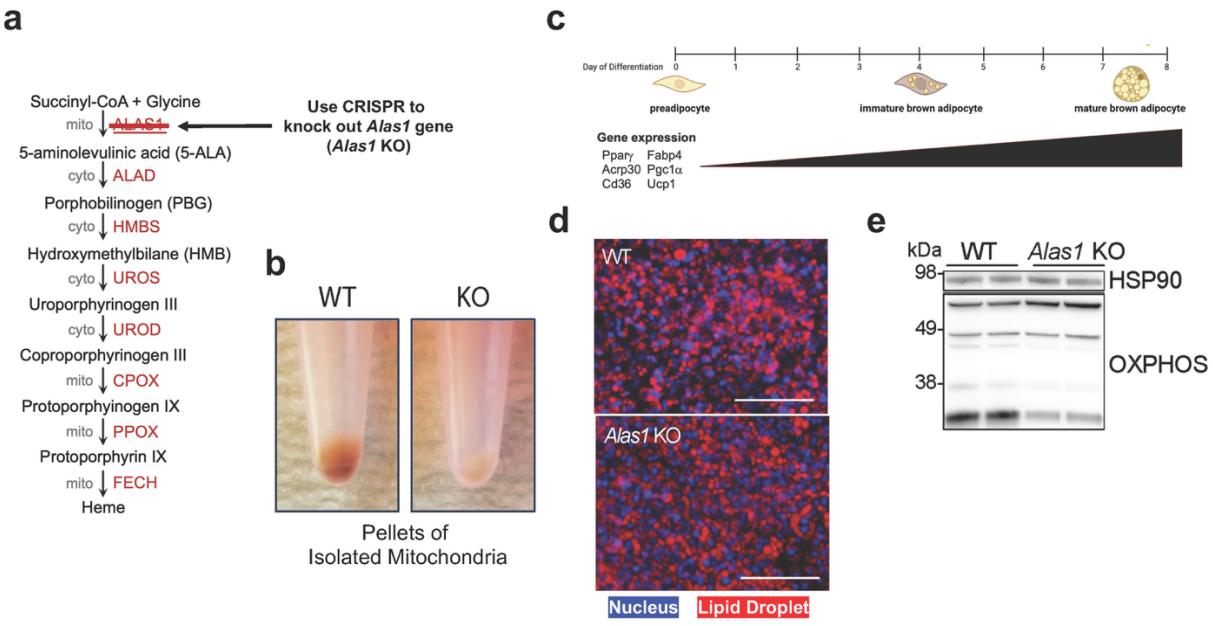
Assessing the contribution of heme synthesis to brown adipocyte function

Armed with the knowledge that the main source of heme in a brown fat cell is production through the heme synthesis pathway, I sought to change our approach which would allow us to answer

new questions. I wanted to avoid constantly treating cells with SA, which was inconvenient, and limit potential unintended side effects linked to using a drug. To solve this, I would instead block heme synthesis by removing the cellular machinery required for making it.

To do this, I utilized CRISPR, a tool that, much like a word processor, allows scientists to precisely edit and modify the DNA code. In my case, I mutated the gene *Alas1*, which produces a protein that performs the first reaction of the heme synthesis pathway. (**Figure 6a**). This CRISPR-induced mutation specifically disrupted only the genetic code for *Alas1*, blocking the ability to produce the associated Alas1 protein in the cell. Just like chemical inhibition of heme synthesis, *Alas1*-deficient (i.e. knockout) brown adipocytes (referred to as *Alas1* KO) had significantly less heme than normal cells (referred to as wild type, or WT). Further emphasizing this reduction in heme, I isolated the mitochondria from WT and *Alas1* KO brown adipocytes, and you can see the difference with your own eyes in **Figure 6b**. The characteristic rust brown color of the mitochondria pellet was completely lost when cells can no longer make heme.

Figure 6. Genetic disruption of heme synthesis gene *Alas1*



Now that I had confirmed that heme synthesis was necessary to maintain adequate heme levels, I next wondered how losing the ability to make it might affect adipogenesis, the process in which a class of committed stem cells called preadipocytes becomes mature fat cells. This process normally occurs in our bodies when the demand for more energy storage is needed (**Figure 6c**). Adipogenesis drives a change in appearance of the cell, including the formation of structures of fat-storing lipid droplets, which are visible under the microscope.

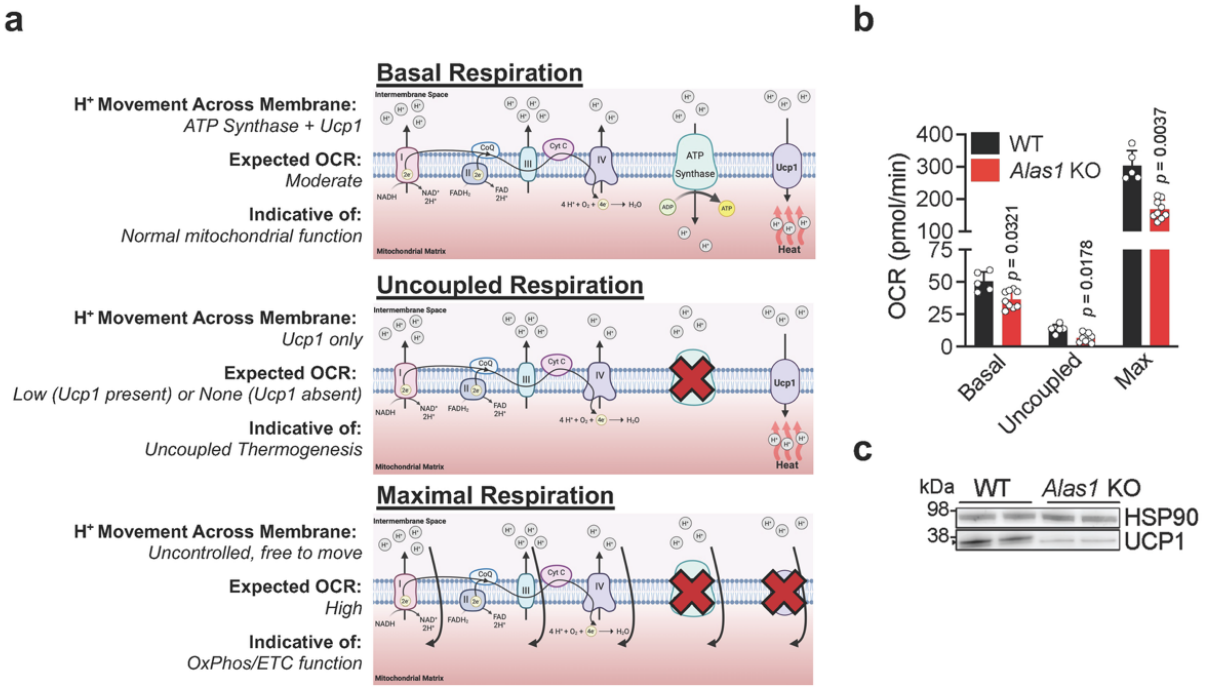
To see if this process was affected in *Alas1* KO cells, I stained the cells with a chemical which makes lipid droplets appear red when exposed to a specific wavelength of light. When I looked at lipid droplet formation under the microscope, I saw that they looked quite similar in both *Alas1* KO and normal WT cells, suggesting that blocking heme synthesis does not compromise adipogenesis (**Figure 6d**).

As mentioned earlier, heme is an essential component of the mitochondrial electron transport chain (ETC; see **Figure 4c**), not only for function, but also its construction by aiding in the proper assembly of its components called OxPhos proteins. As such, I wondered whether the abundance of OxPhos proteins would be affected in *Alas1* KO cells.

My hypothesis was that OxPhos proteins would be reduced in *Alas1* KO cells compared to WT. To assess this hypothesis, I measured their abundance using a technique called a Western blot, where the intensity of bands corresponds to the amount of protein present in the sample. Indeed, I found that *Alas1* KO brown fat cells had lower levels of OxPhos proteins compared to WT (**Figure 6e**). I expected this reduction in OxPhos proteins to lower the activity of the ETC. However, quantification of protein levels is not a direct assessment of their function. To assess that, I needed to use a different approach that directly measured activity.

The OxPhos proteins in the ETC are responsible for performing the final steps of aerobic respiration, the process by which we convert energy from our food into ATP. The term “aerobic respiration” gets its name because it is an aerobic, or oxygen (O_2)-requiring, process. The electrons used to power the ETC cannot exist freely and therefore their “disposal” is completed by passing them to a final acceptor. The final part of the ETC performs this step, donating the electrons to O_2, resulting in a chemical reaction that produces water (H_2O) (see **Figure 2**). As O_2 is converted in this process, its consumption rate is directly proportional to the rate of respiration, which is analogous to the activity of the ETC. Therefore, similar to how we measure the rate of fuel consumption in a car to understand its efficiency in the city or on the highway, we can measure the O_2 consumption rate (or OCR), under different conditions to learn about various states of mitochondrial respiratory activity in cells.

Figure 7. States of aerobic respiration in *Alas1* KO cells



Using this approach, I measured three different aspects of aerobic respiration in WT and *Alas1* KO brown adipocytes (**Figure 7a**).

1. Basal respiration: where O_2 consumption (OCR) is driven by the activity of ATP synthase and Ucp1 (if it is present)
2. Uncoupled respiration: where ATP synthase is blocked, allowing us to assess the contribution of Ucp1 to OCR
3. Maximal respiration: where the presence of a compound similar to DNP "disrupts the dam", pushing the mitochondria and OxPhos proteins into overdrive to achieve their peak OCR.

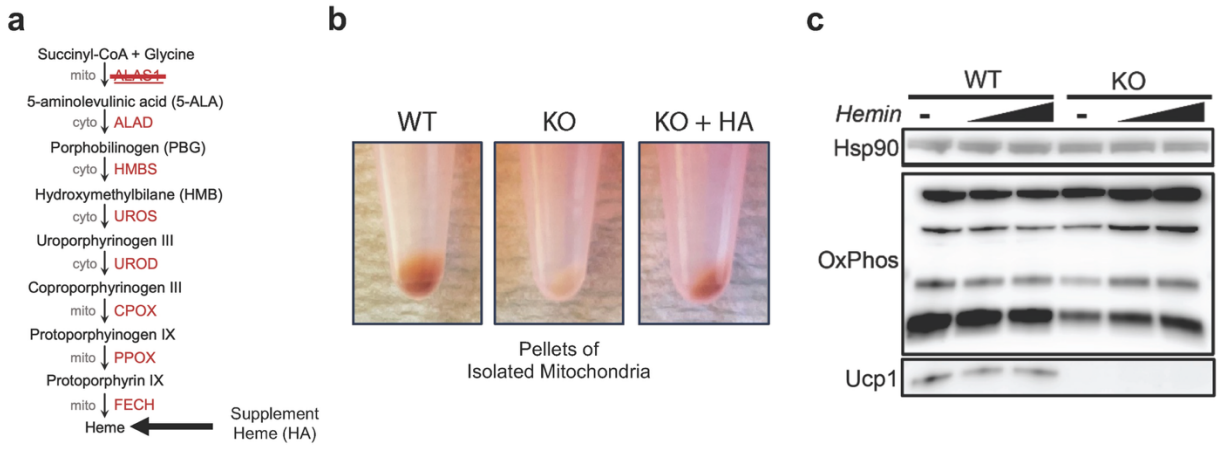
As seen in **Figure 7b**, I found that disruption of heme production significantly lowers respiration in all three of these states. While the reduction in OCR told us that *Alas1* KO cells had impaired ETC function, we were surprised by the result of uncoupled respiration. Recall that brown adipocytes should contain Ucp1, which if present and active, should contribute to O_2 consumption. Under this condition, it appeared there was almost no consumption of oxygen in *Alas1* KO cells, suggesting that Ucp1 was either not active, or not present. When I performed a Western blot to to see if the Ucp1 protein was present, I was shocked to find that it was completely absent in *Alas1* KO cells (**Figure 7c**).

<u>Supplementing heme to circumvent the cellular heme supply chain</u>

After making these observations, my initial hypothesis was that impaired mitochondrial function (indicated by lower OCR) and absence of Ucp1 in *Alas1* KO cells was straightforward. Deletion of *Alas1* would prevent heme production, and this insufficien*cy* impaired mitochondrial respiration and somehow prevented expression of *Ucp1*. In other words, the lack of sufficient heme was the problem. Therefore, I figured that if I could circumvent the need for heme synthesis by providing enough heme in the environment for import (**Figure 8a**), I would both fix mitochondrial respiration and restore the abundance of Ucp1 in *Alas1* KO brown adipocytes.

So, I did just that. I found a condition of heme supplementation (abbreviated as HA in **Figure 8a**) that was not only suitable to boost and restore intracellular heme levels, but it worked so well that it completely restored the characteristic rust brown color of the mitochondria pellet collected from *Alas1* KO adipocytes (**Figure 8b**). This restoration of mitochondrial color prompted me to check whether we had fixed the abundance of OxPhos proteins. When I checked their levels, I found that heme supplementation did increase OxPhos levels back to baseline (**Figure 8c**). But again, levels don't necessarily mean function.

Figure 8. Overcoming heme deficiency through supplementation



I returned to directly assessing respiration by measuring OCR, and what I found was puzzling. In this case, we can draw parallels between the mitochondria and a car engine, where mitochondrial OCR is similar to an engine's ability to consume fuel and generate power. When I measured maximal respiration, *Alas1* KO cells supplemented with heme had OCR equivalent to WT cells. Effectively, *Alas1* KO cells with sufficient heme had their engine's fuel consumption and power generating capacity fixed. However, despite "fixing the engine", heme supplementation only partially restored OCR in the states of basal and uncoupled respiration in *Alas1* KO cells. These results at first seemed contradictory. Why would basal and uncoupled respiration not be rescued

when we have completely restored OxPhos function? *In other words*, why would two engines of the same size and power have differences in various features of performance?

However, when I thought more deeply about the different components of respiration, I realized that the two states where I didn't get a complete rescue were the ones where Ucp1 was involved (see **Figure 7a**). So I went back to check the protein levels of Ucp1 and found that, in line with the respiration data, heme supplementation completely failed to restore any *Ucp1* expression in *Alas1* KO cells (**Figure 8c**). It was as if *Alas1* KO mitochondria were missing a turbocharger necessary to improve performance at submaximal states.

Despite normal mitochondrial respiratory capacity and adequate heme levels, Alas1 KO cells were somehow failing to induce the traditional Ucp1-linked thermogenic gene program. This result revealed two important insights:

1. Mitochondrial function is regulated by heme itself (heme-dependent)
2. Expression of Ucp1 is regulated by the manufacturing of heme (heme-independent)

<u>Revisiting the supply chain framework for insight</u>

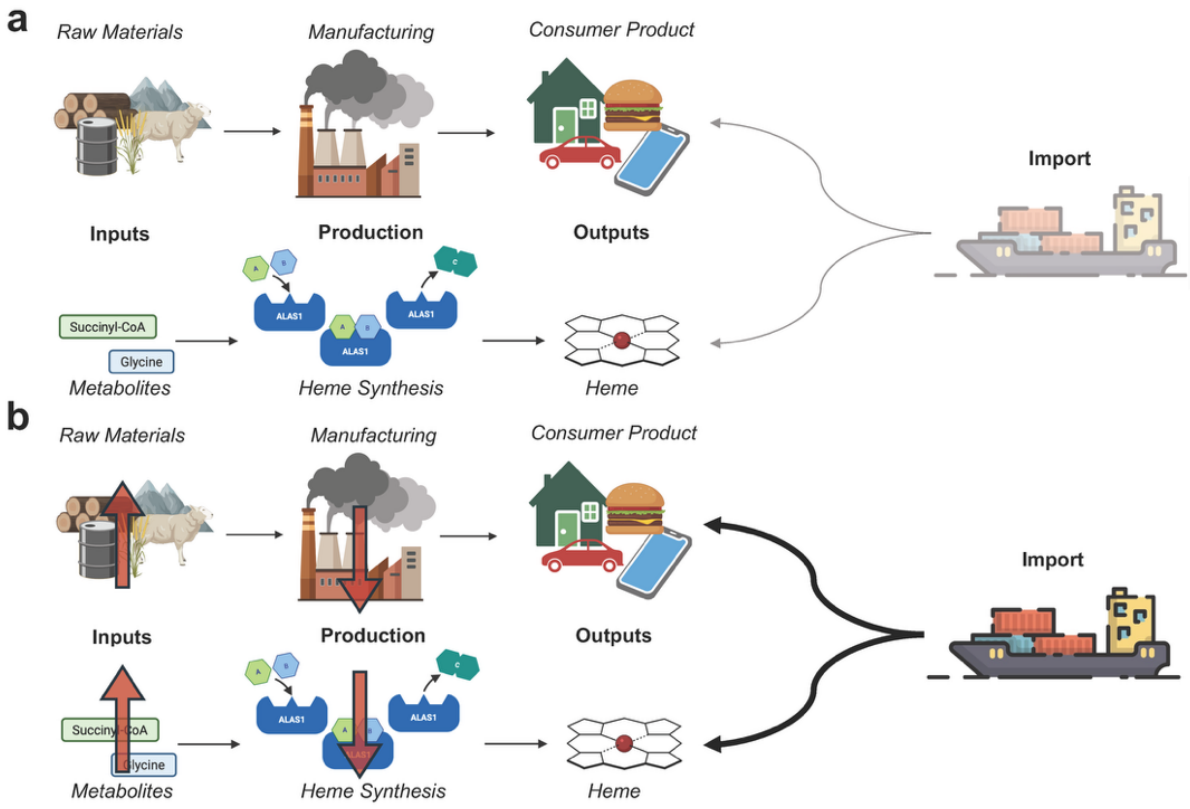
This divergence in regulation of these two characteristics forced my mentor and I to think about a new perplexing question. Why does *Ucp1* expression require heme production per se (i.e. the process of making it), but not necessarily heme (i.e. the end product) itself? What is the unique aspect of the heme synthesis pathway that mediates this effect? Let's return to the supply chain analogy that I brought up at the beginning now, because thinking about our question using this framework proved critical in helping us solve this puzzle.

In any supply chain (**Figure 9a**), raw materials are collected, processed into usable components, then combined to make a new end product with unique functions. We can think of heme synthesis

in the same way, where the metabolic substrates (that is, raw materials) for heme synthesis generate the processed components for cellular manufacturing of a heme end product.

However, any supply chain can be subject to disruption (**Figure 9b**). For example, if we suddenly come across a new source of an end product that can be directly imported to meet demand, then the need for manufacturing it goes away. As a result, our raw materials, which continue to be collected and delivered, are no longer in demand for use and will begin to accumulate, unless they are used for other purposes.

Figure 9. Conceptualizing heme synthesis as a cellular supply chain



Thinking about our question through this lens, I wondered: if the lack of heme wasn't responsible, could it instead be that accumulation of the raw materials for heme synthesis, specifically, the inputs that Alas1 utilizes to initiate heme synthesis, are responsible for blocking Ucp1 expression?

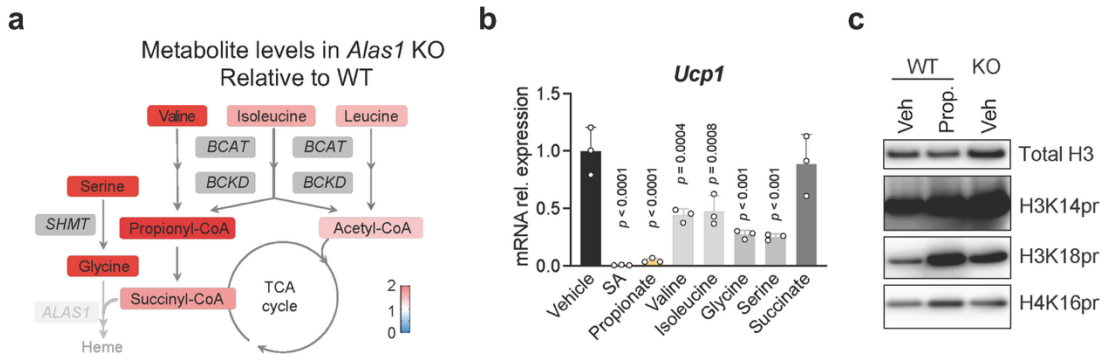
If this hypothesis was correct, we should make two observations. First, we should observe that metabolites that are inputs for heme synthesis are elevated in *Alas1* KO cells. Second, artificially elevating one or more of these metabolites should be capable of lowering *Ucp1* expression.

To test the first part of this hypothesis, I reached out to another group on campus for help. Working together with the lab of Jing Fan, we used a technique called metabolomics, which combines two powerful analytical methods (chromatography and mass spectrometry) to both identify and quantify individual metabolites within our cells. Using this method, we compared the abundance of dozens of metabolites in WT and *Alas1* KO cells. As I hypothesized, we found that the metabolites Alas1 uses to initiate heme synthesis, succinyl-CoA and glycine, were significantly elevated in *Alas1* KO cells compared to WT (**Figure 10a**). Furthermore, we also observed that multiple metabolites that can produce succinyl-CoA were also elevated. These included two metabolites called valine and isoleucine, and another metabolite made during their conversion to succinyl-CoA, called propionyl-CoA.

Secondary effects of disrupting the heme supply chain

Now that I knew heme synthesis substrates (that is, the raw material inputs) did indeed accumulate in *Alas1* KO cells, I sought to test whether having too much of them would be sufficient to inhibit expression of *Ucp1*. To do this, my lab mate Juli and I individually supplemented WT adipocytes with these metabolites and then measured gene expression and protein levels of Ucp1 (**Figure 10b**). When we looked at the results, one metabolite in particular stood out from the rest. Just like the effects following chemical disruption of heme synthesis with succinylacetone (SA), propionyl-CoA (i.e. propionate) completely blocked *Ucp1* expression.

Figure 10. Heme synthesis inputs accumulate to block *Ucp1* expression



With these results, it looked like we had a culprit. We had the who/what (propionyl-CoA), but we didn't yet have the why. What is special about propionyl-CoA that gives it the power to completely block the expression of *Ucp1*, the critically important thermogenic gene in brown fat? Over the next year, I tested many different ideas to try and answer this question. While I was able to rule out numerous explanations, the details regarding what propionyl-CoA was doing remained elusive. Although these results weren't failed experiments as we were learning new things through the process of elimination, I struggled to make progress on identifying how it worked. However, my mentor encouraged me to be persistent, think both deeply and creatively, and continue to read papers to try and find new ideas to test.

Eventually, my persistence paid off. I came across work from another group that eventually helped us down the right path. Their paper, published in 2022, described how metabolism of isoleucine, one of the metabolites that accumulated in *Alas1* KO cells, produces a pool of propionyl-CoA in the nucleus of the cell where DNA is found, which modified gene expression by acting as an epigenetic regulator[10].

The epigenome is a term that refers to compounds that can alter the structure of our DNA and how it interacts with certain proteins, without altering the DNA sequence itself. These changes,

which are often dynamic and reversible, can influence when, where, and how genes are expressed throughout the body. One class of proteins that commonly interact with DNA are histones, which facilitate the wrapping and coiling of DNA to control its accessibility. In short, these histone proteins can be chemically modified to affect how tightly DNA is packaged. One such modification is propionylation, the chemical attachment of propionate to histones.

Now that I had a plausible mechanism by which our metabolite of interest might affect expression of *Ucp1*, I checked to see if histones in *Alas1* KO cells showed differences in propionylation. As expected, normal (WT) adipocytes treated with propionate showed higher levels of three histone propionylation marks (**Figure 10c**). Interestingly, *Alas1* KO cells also showed similarly high levels even without added propionate, suggesting that epigenomic changes driven by propionyl-CoA might help explain the inhibition of *Ucp1* expression (**Figure 10c**).

So, now I felt pretty good that I had the motive-the answer to how and why. As of now, our lab is working to piece together the exact details of how it works. If we can figure out all the details of how it works, we might be able to interfere or manipulate this process to increase *Ucp1* expression in adipose tissue, and in doing so, test to see if it might be a suitable way to affect energy expenditure.

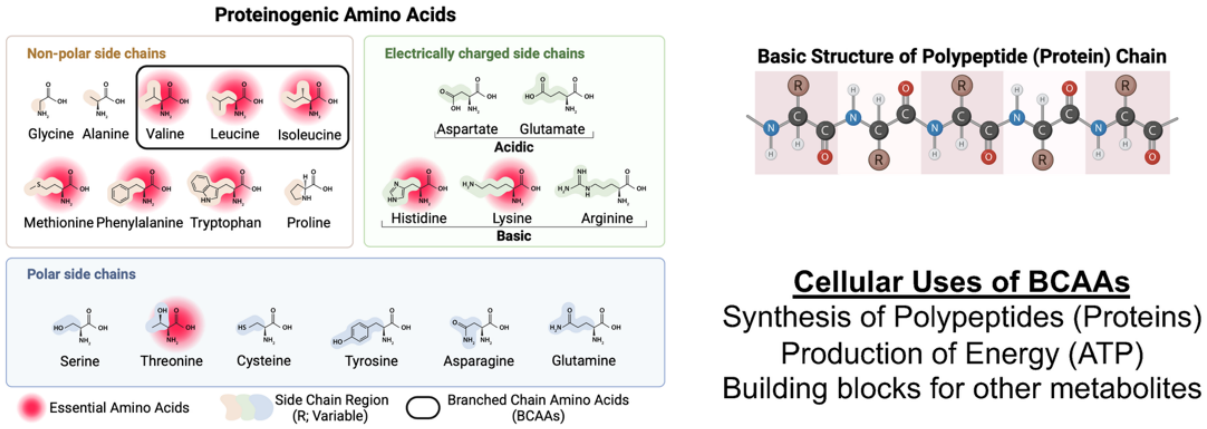
So let's do a quick review. When brown adipocytes cannot produce their own heme:

- The utilization of substrates for heme synthesis is reduced
- These substrates, including propionyl-CoA, are not sufficiently cleared through their typical pathways, leading to accumulation
- High cellular propionyl-CoA levels is sufficient by itself to block the expression of *Ucp1* in brown adipocytes

A new metabolic link: BCAAs and Heme

Now, we are going to switch gears, transitioning our focus from *Ucp1* expression towards a little more hardcore metabolism. The reason for this transition is the data forced us to ask a new question: Why does heme synthesis lead to an accumulation of propionyl-CoA, along with valine and isoleucine? Why do they, in particular, accumulate when we disrupt heme synthesis? At first glance, these two metabolic supply chains were thought to be independent of one another. Was it possible that they were instead connected?

Figure 11. Structure and function of branched chain amino acids (BCAAs)



Before discussing the experiments and data, it is important to note that valine and isoleucine are amino acids, one of the three main macromolecule classes that we get from our food. Specifically, they are classified as branched chain amino acids (BCAAs), whose name comes from their “branched chain” structure found in their variable region (**Figure 11**). BCAAs are three of the nine essential amino acids that we must get from our diets and can be found in animal products and protein-rich plant products. In our bodies, BCAAs have many uses (**Figure 11**), including:

1. Protein synthesis, where they account for nearly a quarter of the total amino acids found in proteins,

2. As raw materials which can be modified and converted into other useful metabolites that serve as building blocks for things like neurotransmitters, and
3. Production of cellular energy molecules like ATP in the mitochondria by breaking them down into two metabolites, succinyl-CoA and acetyl-CoA

Regarding this last function, one of the steps in the metabolic conversion of valine and isoleucine into succinyl-CoA results in the production of propionyl-CoA. Altogether, the accumulation of these three metabolites in *Alas1* KO cells suggested that the BCAA metabolic pathway might be somehow connected to heme synthesis.

BCAA utilization in brown fat: an ongoing mystery

In the last decade or so, work from multiple groups has demonstrated that, in addition to skeletal muscle which builds tons of proteins, brown fat is one of the primary sites in the body where BCAAs are utilized[11–14]. This hunger of brown fat for BCAAs has also been shown to be necessary for thermogenesis and maintenance of body temperature[11]. However, how BCAAs are utilized in brown fat has remained less clear.

Like our lab, dozens of groups across the world have been interested in trying to understand what BCAAs are doing in brown fat. A paper published in 2024 from one of these groups showed that the small nitrogen-containing component of BCAAs is used for production of an antioxidant called glutathione[12]. However, that nitrogen component is only a small piece of BCAA molecules. The general view in the field has been that the remaining nitrogen-free backbone was used for energy production. This assumption was based on the knowledge that the breakdown products of BCAAs in the mitochondria generate succinyl-CoA and acetyl-CoA, which can be used as fuel to support ATP *synthesis* or heat production. However, recent studies that have tracked the metabolic route

of BCAAs revealed their use as fuels for energy and heat production is very low[13,14]. These findings meant that BCAAs must be getting used for alternative purposes.

To summarize, BCAAs are taken up by brown fat, and their metabolism is necessary for thermogenesis. However, they aren't being used as fuel. So, what are they doing? Well, we know that the BCAAs valine and isoleucine generate the heme synthesis metabolite succinyl-CoA, and that BCAAs accumulate when we disrupt heme synthesis. Therefore, I hypothesized that heme synthesis and BCAA metabolism might be connected, which could help explain what BCAAs are being utilized in brown fat.

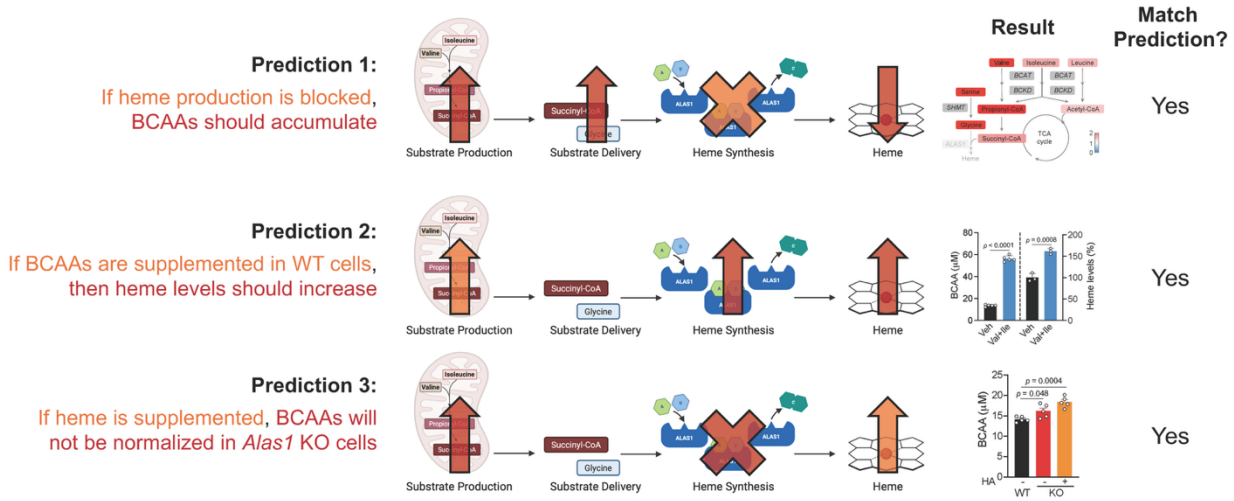
<u>An additional step in the heme supply chain</u>

Since valine and isoleucine metabolism produces succinyl-CoA as an end product, I suspected that this connection between the two pathways might be direct. Put in other words, I hypothesized that BCAA metabolism might be considered an "early extension" of heme synthesis. First, I sought to screen the behavior of this BCAA-heme relationship, once again utilizing a framework built around the idea of a supply chain. If my hypothesis was correct, I would see the metabolic behavior of these pathways act as expected under three separate circumstances (**Figure 12**):

1. If I block the production of heme, the levels of BCAAs should increase
2. If I give brown adipocytes extra valine and isoleucine, heme levels should increase
3. The perturbed mitochondrial function in *Alas1* KO cells should not account for the rise in BCAAs, so supplementation of heme should have no effect on BCAA levels

So, I went to test each of these three principles and see what happens (**Figure 12**). I already knew the answer to the first question, because we previously showed that BCAAs increase when heme synthesis is blocked by deleting the *Alas1* gene (see **Figure 10a**).

Figure 12: Predicted behaviors of a BCAA-heme supply chain connection



Next, I gave normal brown adipocytes extra valine and isoleucine, then measured heme levels to see how they change. As expected, heme levels went up with BCAA supplementation (**Figure 12**), consistent with my hypothesis predictions.

Finally, to make sure that the accumulation of BCAAs was not due to impaired mitochondrial function, I measured the concentration of BCAAs in *Alas1* KO cells supplemented with heme. Indeed, BCAAs remained high (**Figure 12**), suggesting that their accumulation was instead attributed to a lack of heme synthesis.

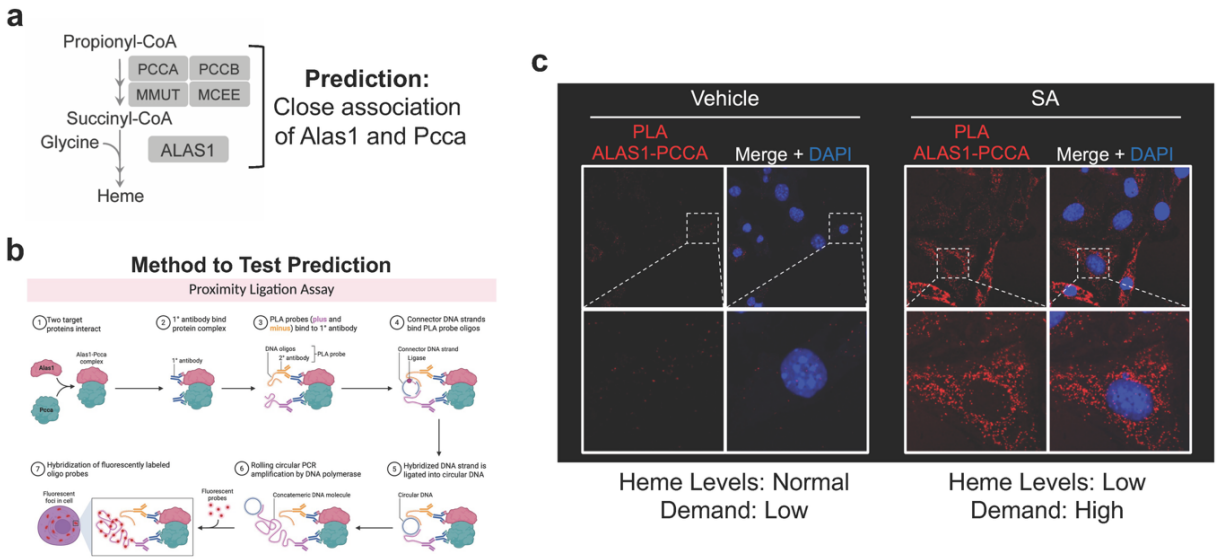
Altogether, the data suggested that the BCAAs are directly supporting heme synthesis. However, this brought forward a new question. The metabolism of valine, isoleucine, and propionyl-CoA produces succinyl-CoA, which can be used for energy/heat production, or heme synthesis. Yet, we knew that brown fat does not readily use BCAAs for energy production. So, how does the brown fat cell do this? In other words, how is the cell managing to exclusively utilize succinyl-CoA from BCAAs for one purpose, but not the other?

Supply chain logistics: feeding BCAAs to heme synthesis

Once again, we can return to our supply chain analogy for insight. One way to improve efficiency in a supply chain is to place the site of manufacturing that uses the raw materials close to the source of their production. This feature of a supply chain also enables the raw materials produced at the site to readily be used for that manufacturing process.

Likewise, my mentor and I envisioned that brown adipocytes might do something similar, where the site of heme synthesis is localized close to the site of BCAA conversion into succinyl-CoA (see **Figure 13a**). If so, then Alas1 and an enzyme that aids in the conversion of BCAAs into succinyl-CoA called propionyl-CoA carboxylase A (Pcca), should be close to one another in the cell.

Figure 13. A physical connection of BCAA metabolism and heme synthesis



To assess the proximity of proteins in live cells, we can use a technique called proximity ligation assay, or PLA. The methodology of PLA is shown in **Figure 13b**, but what you need to know about

this method is that when two proteins closely associate with each other (that is, 40 nanometers, or around 1/100th of the thickness of a human hair), we can detect it by looking under the microscope for the presence of fluorescent dots within the cell (called puncta).

Accordingly, I hypothesized that Alas1 and Pcca would be found near each other in normal brown adipocytes. Through a collaboration with our lab neighbors who regularly perform PLA experiments, we did just that. Under normal conditions, Alas1 and Pcca were in proximity, but at low levels (**Figure 13c**).

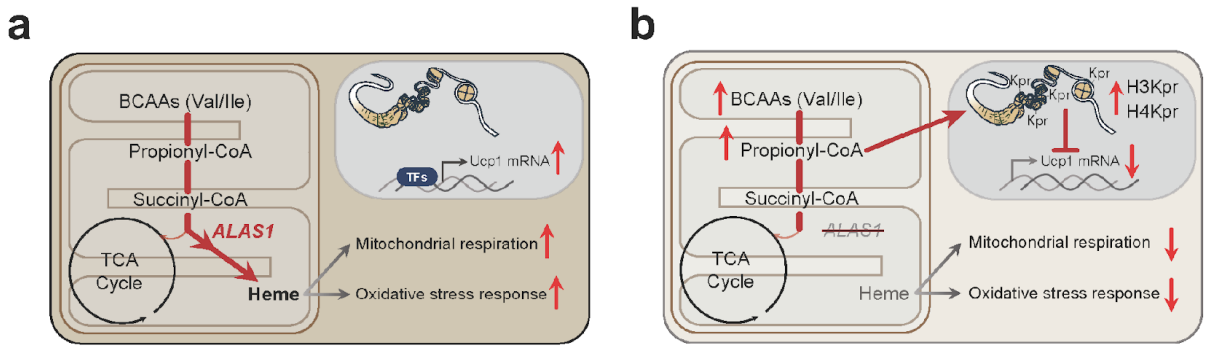
But what about conditions where demand for heme is greater? I suspected that increased heme demand should induce this association. When we reduced heme levels and increased heme demand using SA, the cells responded by forming many more Alas1-Pcca puncta (**Figure 13c**). This result demonstrated that the connection of these two metabolic pathways is not only direct, but also dynamic and varies in response to stress, helping the cell to adapt to environmental changes.

Discussion

Building a new model: The BCAA-Heme-Ucp1 connection

In sum, we have put together a model based on the data of how we think this works in brown adipocytes. This work was published earlier this year in Nature Metabolism, of which I was the first author[15,16]. Under normal conditions, BCAA metabolism supports mitochondrial respiration and function in part through supplying heme synthesis with the raw materials it needs (**Figure 14a**). However, disruptions to heme synthesis, such as deletion of *Alas1*, lowers heme levels and disrupts mitochondrial function. At the same time, the brown adipocyte undergoes a massive supply chain shock, as demand for BCAAs is reduced (**Figure 14b**).

Figure 14. A working model of Ucp1 regulation by the BCAA-heme metabolic axis



Much like how the demand for gas and oil evaporated at the beginning of the pandemic, the disruption of BCAA utilization for heme synthesis in brown fat leads to the accumulation of valine, isoleucine, and their intermediate propionyl-CoA. This elevation is linked to epigenome remodeling, which we suspect may be responsible for the suppression of *Ucp1* expression.

What comes next?

As we discussed at the beginning, the induction of brown fat thermogenesis and Ucp1 activity is emerging as an attractive approach to increase energy expenditure and help patients with obesity and diabetes lose unhealthy body weight. Understanding the mechanisms that the cell utilizes to control the levels of Ucp1 in fat can inform our efforts to develop new tools and approaches to manipulate it.

Accordingly, our work has revealed a novel mechanism controlling the adoption of *Ucp1* expression in adipose tissue. While we think it is linked to changes in the epigenome, we don't yet have a clear picture of the entire series of events that lead to its inhibition. However, if we can identify the mechanism of action, that is, the steps by which this takes place, we might be able to identify compounds or conditions that relieve the inhibitory break that prevents *Ucp1* from being expressed and instead promote its expression in fat cells to increase energy expenditure.

Conclusion

Let's revisit the key messages from the beginning that I hope this work helps to emphasize about what metabolism is and how studying it can help us better understand ourselves and improve the human condition.

First, metabolism is the underlying process in our cells and bodies that allows us to harvest and utilize things in our environment to grow, adapt, and survive. This project demonstrates the importance of sourcing those raw materials for cells by highlighting how heme synthesis is necessary for brown adipocytes to perform their critical functions.

Second, despite its complexity and thousands of moving components, metabolism exhibits a striking level of interconnectedness and coordination. Through the efforts to understand why heme synthesis is required for *Ucp1* expression in brown adipocytes, we identified a novel connection between two distinct metabolic pathways that uniquely supports brown adipocyte function.

Third, understanding and mapping the paths of metabolism can teach us how our bodies work, how disease can manifest when things go wrong, and how manipulating metabolism can be a tool to treat diseases and improve health. The discovery of a novel regulator that governs the adoption of *Ucp1* expression in fat cells has armed the scientific community with new information about methods to control its presence. Figuring out how this works at the molecular level can give us new information needed to develop strategies to increase *Ucp1* expression in fat and test its effectiveness for treating metabolic disorders such as obesity and diabetes.

A final (personal) note

To put a bow on this chapter, I want to share that the last five years have been exceptionally transformative in both my personal and professional life. When I entered graduate school, I came in with a confidence that I had science figured out, and that I could achieve anything I set my mind to. I quickly learned that this presumptuous overconfidence in my own knowledge and ability to achieve great things was misplaced. This cognitive bias is known as the Dunning-Kruger effect. The PhD experience forced me to confront this bias and inflated ego head on. During my training, I frequently failed, made mistakes, and had hypotheses proven wrong many more times than I was right. The lab gave me a safe space to learn the importance of recognizing the frequency at which I am wrong. It forced me to adapt, to be resilient, and to overcome challenges and circumstances that are out of my control. Importantly, I learned that being wrong is not a reflection of my identity or self-worth, but rather a chance to take in new information and learn from it.

While I recognize my development and growth as a scientist, I am (and always will be) a work in progress. I still regularly experience the Dunning-Kruger effect in my life inside and outside of the lab. However, my PhD training has better equipped me to recognize when I am wrong or do not know what I am talking about. Accordingly, I am doing a better job of saying “I don’t know” and am growing more comfortable with recognizing my own ignorance in all aspects of my life.

Executing the scientific method on a daily basis has instilled in me a framework to catch myself from being too confident or committed to ideas. It has given me a way to question, observe, and learn new things. In a way, identifying these gaps in knowledge continues to spark new curiosities about things I see, read, and encounter. The scientific training I have received will help me to use my innate curiosity as fuel towards better understanding myself, others, and the world around me.

Thank you for taking the time to read this chapter. I hope you enjoyed learning about my scientific journey, and I hope that it serves as a reminder of the value of scientific thinking, the importance of curious exploration, and the purpose of seeking new knowledge.

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