PUTTING THE PIECES TOGETHER: AN EXPLORATION OF STUDENT THINKING ABOUT METABOLISM AND THE ROLE OF CCRK IN CILIARY MAINTENANCE

by

KUSH SONI BHATIA

(Under the Direction of Paula Lemons and Jonathan Eggenschwiler)

ABSTRACT

While the two parts of this dissertation might seem disparate at first glance, both projects ultimately seek to understand the basic ideas underlying the larger phenotypes we observe. The first part of this dissertation investigates student thinking about metabolism, a core concept of biochemistry courses. This study aims to characterize the ideas students activate and utilize when solving a metabolism problem they have not been exposed to before, utilizing the knowledge- in-pieces framework. Once the ideas students were using were identified, we also set out to understand how students assembled the independent pieces of knowledge into an explanation. By categorizing and attempting to understand how students assemble those categories of ideas, insight into instruction and future research projects were gained. The second part of this project investigates the role of Cell Cycle-Related Kinase (Ccrk) on downstream effector proteins and on the phenotypes of intraflagellar transport (IFT) machinery movement. Two downstream

effectors known to impact ciliogenesis were confirmed to be differentially phosphorylated when Ccrk is knocked out in a cell. When Ccrk is knocked out, we also see lower frequencies of IFT particle movement within cilia and lower frequencies of the import of new IFT machinery into the cilia. These phenotypes seem to be linked. In order to further explore the link, stable cell lines expressing fluorescent cargo alongside fluorescent IFT were generated to assay cargo phenotypes. Together, this work serves as an interdisciplinary effort to add to the bodies of literature in both developmental biology and discipline-based education research in biochemistry.

INDEX WORDS: STEM education, biochemistry undergraduate education, problem solving, knowledge-in-pieces, negative feedback inhibition, metabolism, resource graphs, Ccrk, Cell cycle related kinase, Map4, MyosinIIa, IFT, cilia, FRAP,

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Dedication

In loving memory of Anita Nadkarni Odak

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Chapter 1

Introduction – Part 1

Discipline-based education research (DBER) investigates student thinking and learning within specific disciplines (Singer et al., 2012). Research on student thinking and learning yields insights about the development of expertise within the specific field in order to identify, design, and implement specific instructional approaches, as well as translate research findings directly into classroom practices to aid student instruction (National Research Council, 2012). As science and technology permeate more and more aspects of our world, building a fundamental understanding of scientific principles, or scientific literacy, is becoming more critical. In Vision and Change, a report published by the AAAS, many scientists across the country agreed that to be scientifically literate, students need to refine a set of overarching core concepts of biology and develop core competencies. These biology core concepts were evolution; pathways and transformation of energy and matter; information flow, exchange, and storage; structure and function; and biological systems (AAAS, 2011).

Research groups expanded on the broad biology core concepts by collecting instructor feedback and input nationwide. For example, they identified three overarching principles that a biology major must master before graduation pertaining to the core concept of "pathways and transformation of energy and matter." Those principles are: energy and matter cannot be created or destroyed but can be changed from one form to

another; energy captured by primary procedures is necessary to support the maintenance, growth, and reproduction of all organisms; and natural selection leads to the evolution of efficient use of resources within constraints (Brownell et al., 2014). These principles echo many of the same ideas underlying topics in biochemistry education. Biochemistry has a fundamental focus on metabolic pathway dynamics and regulation, and their link to how these pathways interact and allow an organism to function.

Metabolic pathways and transformations of energy and matter have been noted as core concepts of biochemistry by many educators for years (AAAS, 2011; Loertscher et al., 2014). Loertscher and a group of biology and biochemistry colleagues' defined this concept as one of the threshold concepts of biochemistry (Loertscher et al., 2014). A threshold concept represents an idea within a discipline that a student must possess understanding of in order to develop an expertise in the discipline. In order for a student to exhibit an understanding of metabolism, there are major classes of knowledge they must possess: reactions within a biological system are dynamic and reversible; the directionality of processes depend on the free energy and relative concentrations of reactants and products available and flux through pathways are the net result of both forward and reverse reactions taking place; and enzyme activity is highly regulated and controls the rate of forward and reverse reactions, allowing pathways to be responsive to the needs of the organism (Loertscher et al., 2014). Metabolism draws upon many elements taught to students outside of a broader context. For example, the ideas of reversible chemical reactions are taught independently in introductory chemistry courses. In contrast, in introductory biology courses, students learn about cellular functions and equilibrium, and the role feedback inhibition plays. However, students might not

recognize or receive instruction that, in a biochemical context, they should recognize, integrate, and apply knowledge from both biology and chemistry.

Student thinking and learning always occur within contexts, or the conditions surrounding learning. These conditions vary and are unique to students due to the physical, social, cultural, or historical factors involved in the effectiveness of instructional activity. Different contexts elicit different patterns of thinking (diSessa et al., 2004; Louca et al., 2004; Wagner, 2006; Nehm & Ha, 2011; Sherin et al., 2012; Watkins & Elby, 2013; Chao et al., 2018; Gouvea & Simon, 2018; Goodhew et al., 2021; diSessa & Sherin, 1998; National Research Council, 2001; Clark & Linn, 2013). Investigating student thinking requires researchers to adopt a perspective on student knowledge, as this impacts research methods, analysis, and applications for instruction. The DBER field has coalesced around two contrasting views of student thinking based on the extent to which students' knowledge is coherent versus fragmented (Elby, 2000; Sherin et al., 2012; Clark & Linn, 2013; Maskiewicz & Lineback, 2013; Leonard et al., 2014; Lira & Gardner, 2020). Evidence supporting students displaying coherent, theory-like views of student knowledge supports the coherence or misconceptions framework (Scherr, 2007; Clark & Linn, 2013; Leonard et al., 2014; Gouvea & Simon, 2018). Conversely, there is also evidence supporting students displaying independent ideas, and they assemble these ideas based on the context in which they are presented (diSessa, 1988, 2017, 2018; diSessa & Sherin, 1998; Hammer, 1996; Hammer & Elby, 2003; Wagner, 2006, 2010; Nehm et al., 2012; Sherin et al., 2012; Gouvea & Simon, 2018). In the following sections, we present the two contrasting perspectives.

Misconceptions Framework

Misconceptions are understandings or explanations that differ from what is known to be scientifically correct (NRC, 2012). Many other frameworks have adopted this term to represent noncanonical ideas that students express (Pugh et al., 2010; Hickey & Zuiker, 2012; Opfer et al., 2012; Heddy & Sinatra, 2013). The misconceptions framework uses the perspective that knowledge is stored in layers or tiers (Gilbert & Watts, 1983). As learners acquire new knowledge, knowledge in earlier tiers must be built upon correctly (Gagne, 1970). This perspective also implies that ideas emerge, fully formed, from cognitive structures leading to stable, theory-like ways of thinking, regardless of whether they are scientifically accurate or inaccurate (Hammer, 1996; Taber, 2008). Therefore, learning new information is based upon the existing knowledge. Thus, if a student holds scientifically incorrect ideas organized into stable theories, it will impede the student's ability to construct or integrate higher tiers of concepts. In this perspective, students' scientifically incorrect ideas are considered flaws in their knowledge that need to be eliminated or replaced with scientifically correct ideas for new knowledge acquisition to happen (Hogan & Maglienti, 2001).

Knowledge-in-Pieces Framework

A core assumption of the knowledge-in-pieces framework is that students have a collection of pieces of knowledge, or knowledge elements, that they construct into an understanding of visual representations, scientific ideas, or phenomena (Elby, 2000; Hammer & Elby, 2002). These knowledge elements are dynamic patterns of activity utilized in various contexts by grouping them into a system, which is activated and linked with other systems dependent upon the context (i.e., the dashed arrow and circled bar in a

metabolic pathway indicates negative feedback). This system is an assembly of interrelated elements that together can be used by a student to represent a more complex function (diSessa & Sherin, 1998; diSessa, 2002).

Student thinking is activating or utilizing these pieces of knowledge in particular contexts, and learning can be thought of as the changes in the patterns and consistency of these activations over time (Gouvea, 2023). The knowledge in pieces framework argues that knowledge from novices and experts is structured as a system of related elements. A novice student's knowledge is a more extensive collection of disconnected elements that is organized into useful systems with the development of expertise, as they realize when, where, and how to utilize and link these knowledge elements. There is continuity, or a spectrum, between novice and expert. An expert's understanding evolves from the foundational knowledge novices gain (Clark, 2006). As a novice's knowledge developed, their ideas are not necessarily replaced but modified, co-opted, and pulled in the appropriate context to perform new functions in experts' understanding (Smith, diSessa, and Roschelle, 1994; Clark, 2006; Sherin, 2006). The differences between novice and expert knowledge should not be assessed one element at a time, but how they are applied in relation to others withing specific contexts (Smith, diSessa, and Roschelle, 1994).

Utilizing the knowledge-in-pieces framework, one can identify commonly used resources or systems that students are likely to activate (Sabo et al., 2016; Robertson et al., 2021). A common goal in education is for students to arrive at specific ideas (Sikorski & Hammer, 2010). If an instructor labels knowledge elements as incorrect, wrong, or unproductive, this would discourage students from utilizing them ever again. This may inadvertently cut off students productively using these ideas in a different context where

those knowledge elements are correct or help lead to productive thinking (Ball et al., 1993; Hammer, 1997; Warren et al., 2001; Rosebery et al., 2010). Rather than view these knowledge elements as correct or incorrect, the knowledge-in-pieces framework argues that they should be assessed as productive or unproductive inside of the student's specific learning context. Instruction can bring unproductive knowledge elements to the learner's attention and aid in coordinating knowledge elements. Rather than focus on identifying fine-grained elements that alone have no explanatory power, the knowledge-in-pieces framework focuses on the processes students are using to organize these knowledge pieces with other knowledge pieces into these systems and analyzing how these systems change and are utilized in relation to context (Thelan & Smith, 1994; Rodgers & McClelland, 2003; Nicholson & Dupre, 2018). The knowledge-in-pieces perspective attempts to understand the roles of these knowledge elements in specific contexts and how these elements interact with each other.

Researchers and educators should aim to understand how and why students assemble individual knowledge elements in specific contexts to improve learning (Gouvea, 2023). The learning context provides insight and context for students' thinking the way they do. An idea that seems incorrect on the surface is still intuitive or valuable to the student in a particular context, and understanding how these ideas interact within and across contexts can inform the instruction within that context (Scherr, 2007). By exploring the diverse array of student thinking, we can become informed on how educators can productively engage with students during the learning process.

This dissertation presents a study that approached student thinking about biochemistry from the knowledge-in-pieces perspective of learning. We used the knowledge-in-pieces perspective because we hypothesize that students' ideas about science are naturally independent and context-reliant. As students build up their knowledge of metabolism, there is an expectation that students learn what knowledge elements to utilize to combine understandings of the dynamics of a pathway, how pathways change and respond to changes in the environmental context, ideas about free energy, and the directionality of chemical reactions and regulation. By understanding students' knowledge and how they utilize it, we can help students refine their intuitions when exposed to metabolism problems. The goal of instruction is to help students recognize these contexts and refine and adapt their intuitions of what knowledge elements to pull and how to use them. All the knowledge elements pulled can be productive pieces towards building a more sophisticated understanding network. The research presented aims to identify and characterize these knowledge elements that students assemble and how they are used to construct understandings of visual representations or scientific ideas when addressing questions related to metabolism.

Chapter 2

PUTTING THE PIECES TOGETHER: STUDENT THINKING ABOUT TRANSFORMATIONS OF ENERGY AND MATTER

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Abstract

Research on student thinking facilitates the design of instructional materials that build on student ideas. The pieces framework views student knowledge as consisting of independent pieces that students assemble in fluctuating ways based on the context at hand. This perspective affords important insights about the reasons students think the way they do. We used the pieces framework to investigate student thinking about the concept transformations of energy and matter with a specific focus on metabolism. We conducted think-aloud interviews with undergraduate introductory biology and biochemistry students as they solved a metabolism problem set. Through knowledge analysis, we identified two categories of knowledge elements cued during metabolism problem solving: 1) those about the visual representation of negative feedback inhibition; and 2) those pertaining to student focus on different metabolic compounds in a pathway. Through resource graph analysis, we found that participants tend to use knowledge elements independently and in a fluctuating way. Participants generally showed low representational competence. We recommend further research using the pieces perspective, including research on improving representational competence. We suggest that metabolism instructors teach metabolism as a concept, not a collection of example pathways, and explicitly instruct students about the meaning of visual representations associated with metabolism.

Introduction

Designing high-quality instructional materials and pedagogical approaches requires research on student thinking about life sciences concepts and how students interact with instruction to build their knowledge. We define student thinking as the knowledge students bring to the learning context, which includes a mixture of intuitions, experientially-grounded notions, and scientific ideas (diSessa & Sherin, 1998; National Research Council, 2001; Clark and Linn, 2013). Research characterizing student thinking reveals the challenging process of knowledge refinement students undertake when learning new concepts. For example, research on student thinking about natural selection revealed that students tend to use the word "adapt" in an everyday way rather than a scientific way and that students apply more scientific ideas when solving problems about familiar organisms than unfamiliar organisms (Nehm and Schonfield, 2008; Nehm and Ha, 2011). Research on student thinking about noncovalent interactions revealed that students approach the topic using definitions and heuristics and must be guided to use causal mechanistic reasoning (Cooper *et al.*, 2015; Halmo *et al.*, 2018). This type of research has improved instruction while also raising new research questions about learning (e.g., Williams, *et al.*, 2015; Halmo *et al.*, 2020). Biochemistry offers a particularly useful context for student-thinking research as it requires students to integrate knowledge from biology and chemistry. The work presented here addresses the critical need to investigate student thinking by examining the ideas students use to solve problems about a core concept in biochemistry.

We investigated student thinking in the context of biochemistry because of the crucial role biochemistry plays in life sciences education. Many undergraduate science students take biochemistry because of its importance to their career paths. Most pre-health professionals must take biochemistry as part of the admission requirements for professional school. Opportunities in engineering are abundant for students who are prepared to consider how the chemical principles of life can be leveraged to solve real-world problems. Biochemistry also sits at the junction of numerous areas of research that impact human health and the environment, making it a desirable course for students who plan to go to graduate school. Students take introductory biology and general chemistry as early as high school, yet they typically do not consider the chemical foundations of biology phenomena nor the biological applications of chemistry concepts until their first biochemistry course. Biochemistry courses challenge students to think about molecules and systems of molecules by applying basic chemistry knowledge.

Transformations of energy and matter is the core concept that explains how biological systems grow and change through pathways of chemical transformation (American Association for the Advancement of Science, 2011; Brownell *et al.*, 2014). This concept pertains to biochemistry due to the discipline's central focus on metabolic pathway dynamics and regulation (Loertscher *et al.*, 2014; American Society for Biochemistry and Molecular Biology, 2020), hereafter referred to as metabolism. Metabolism deals with the linkage of chemical reactions in pathways and how pathways interconnect and allow an organism to function (Loertscher *et al.*, 2014). As students build their knowledge of metabolism, they improve their ability to make predictions about the dynamics of a pathway, including how a pathway will respond to fluctuations

caused by changes in the cellular environment. Biochemistry students must integrate numerous ideas to solve problems about metabolism (Loertscher *et al.*, 2014), including ideas about free energy and directionality of chemical reactions, enzymes and catalysis, and enzyme regulation. Students develop these ideas in general chemistry and introductory biology courses, sometimes as early as high school and continuing into introductory college classes. This study examines student thinking about metabolism among undergraduate life science students.

Students who learn metabolism also must decode visual representations, and these visuals may or may not be properly designed (Loertscher *et al.*, 2014; Offerdahl 2017). When solving metabolism problems, students must decipher symbols such as letters, arrows, and compound names and make inferences to produce a dynamic mental construct of the phenomena being represented (Sherin *et al.,* 2012, Lira and Garnder, 2020). They must then integrate this visual information with ideas brought to working memory from long-term memory to generate an explanation of how the pathway works (Sherin *et al.,* 2012, Fiorella and Mayer, 2015). This process is made more difficult for learners because of shortcomings in the design of visual representations. Biochemists often design visuals based on their intuition and extensive knowledge base, and these visuals can prompt unintended student interpretations (Tversky *et al.*, 2000; Smallman & John, 2005). Moreover, biochemists lack agreed-upon conventions for visually representing metabolism. An analysis of arrows used in biology textbooks showed that the same arrows were used in many different contexts, and several different arrow types were used to present the same concept (Wright *et al.*, 2018). For these reasons, students' interpretations of visual representations of metabolism warrants investigation.

Theoretical Perspective: Student Thinking as Knowledge in Pieces

Investigating student thinking requires taking a perspective on the characteristics of student knowledge. There has been a long debate in the literature about the extent to which students' knowledge is coherent and theory-like versus fragmented and context-dependent (Sherin *et al.*, 2012; Clark and Linn, 2013; Maskiewicz and Lineback, 2013; Leonard *et al.*, 2014; Lira and Gardner, 2020). Indeed, evidence exists for both views (Scherr, 2007; Sherin *et al*., 2012). On the one hand, evidence shows that students display coherent, context-independent, stable ideas that are resistant to instruction (e.g., Vosniadou and Brewer, 1992, Vosniadou *et al.*, 2008; Sherin *et al.*, 2012; Coley and Tanner, 2015). This body of work is referred to by multiple names (e.g., framework theory, coherence perspective (Scherr, 2007; Clark and Linn, 2013; Leonard *et al.*, 2014; Gouvea and Simon, 2018)), but we will call it the misconceptions perspective (Scherr, 2007). On the other hand, evidence shows that students' ideas are more like independent elements assembled in the moment based on context (e.g., diSessa, 1988; Smith *et al.*, 1992; Hammer, 1996; diSessa & Sherin, 1998; Hammer & Elby, 2003; Wagner, 2006; Wagner, 2010; Nehm *et al.,* 2012; Sherin et al., 2012; diSessa, 2017, 2018; Gouvea and Simon, 2018). This body of work has been referred to by a variety of names, each with a different emphasis (e.g., phenomenological primitives, facets of knowledge, resources, conceptual dynamics (Minstrell, 1992; diSessa, 1993; Hammer, 2000; Southerland *et al.*, 2001)), but we will refer to it as the pieces perspective (Scherr, 2007).

The misconceptions and pieces perspectives share some things in common, yet they have different research agendas (Scherr, 2007; Clark and Linn, 2013). For example, both perspectives describe students' knowledge as constantly developing and evolving

(Clark and Linn, 2013). However, research from the misconceptions perspective aims to identify conceptual frameworks, while research from the pieces perspective aims to identify individual knowledge elements (Scherr, 2007; Clark and Linn, 2013; Lira and Gardner, 2020). Misconceptions researchers search for the key models students use coherently yet incorrectly, regardless of context. They imagine students' ideas as coordinated, unified, and rigid. Pieces researchers search for more fine-grained elements of student thinking and focus on how these elements interact within and across a particular context. Pieces researchers assume that students have good reasons for thinking the way they do and that an idea they express that may seem incorrect is likely useful in another situation. Thus, the research agenda is to identify the independent knowledge elements accessed during student reasoning and how the use of these ideas fluctuates based on context (Scherr, 2007).

The misconceptions and pieces perspectives also differ in their instructional agendas. The misconceptions model argues that students must dismantle and discard non-canonical ideas by becoming aware of the inadequacy of those ideas, discovering more robust ideas, and resolving the contradictions (Scherr, 2007,Nehm and Reilly, 2007; Kallinowski *et al.*, 2010). This process is difficult but leads to permanent change.The pieces model promotes the notion of instruction as helping students refine their intutions (Elby, 2001), which are not considered to be incorrect but inappropriately applied in a given context. Thus, instruction is meant to help students recognize the utility of their intutions in some circumstances and the need to refine them for a given situation. Adaptation of ideas can come relatively easily according to the pieces model, but it may

be unstable. That is, a student may refine their intuitions and then later forget the importance of the refinment (Scherr, 2007; Robertson *et al.,* 2015).

We approached this investigation from the pieces perspective because we hypothesized that students' ideas about metabolism are more independent, loosely connected, and context-dependent than they are theory like. We thought this because metabolism draws upon numerous ideas that are not typically linked together until late in the life sciences curriculum. Some ideas are taught more so in general chemistry (e.g., free energy and the conditions of reversibility for a chemical reaction) while others are taught more so in introductory biology (e.g., enzymes and catalysis). In fact, many students may never receive explicit instruction, even in a biochemistry course, that prompts them to recognize, integrate, and apply pieces from biology and chemistry to metabolism. Many biochemistry instructors possess tacit schemas about metabolism that they assume students also have (Meyer and Land, 2003; Loertscher *et al*., 2014). Only recently have biochemistry educators acknowledged this limitation and started to tease apart the numerous concepts students could and should coordinate when solving problems about metabolism (Loertscher *et al*., 2014; Villafañe *et al.*, 2021). Additionally, we hypothesized that visual representations of metabolism would activate a variety of ideas among students. Biochemists have not agreed upon conventions for representating metabolism (Offerdahl, 2017), and representations of metabolism rely heavily on arrows to convey meaning, even though arrows are used inconsistently in biology (Wright *et al.*, 2018). Finally, we were compelled by the evidence for an instructional stance that privileges student thinking as intuitive and rational, even if sometimes misplaced, and

that focuses on deciphering the origin of students' ideas and facilitating refinement (Robertson *et al.*, 2015).

Thus, we aimed to add to the body of literature on student thinking about core life sciences concepts (Nehm and Reilly, 2007, Hartley *et al.*, 2011; Andrews *et al*., 2012; Wright *et al.*, 2014; McFarland *et al.*, 2016; Prevost *et al.*, 2016; Halmo *et al.*, 2018; Scott *et al.,* 2019) by characterizing the knowledge elements students use during metabolism problem solving. Specifically, we investigated the following research questions: What knowledge elements do life sciences undergraduates use when thinking about metabolism? How do students assemble these elements during problem solving?

Methods

We conducted a qualitative interview study using a think-aloud protocol where participants answered a problem set that examined their understanding of metabolism. Participants answered each problem while thinking aloud, and we further probed their understanding for clarity. We analyzed participants' responses using qualitative content analysis, and this led to the identification of two categories that describe the use of knowledge elements across the entire sample. We then created resource graphs (Wittman, 2006; Rodriguez and Towns, 2019; Rodriguez *et al.*, 2020) for three purposefully-selected students to address how individual students assemble knowledge elements about metabolism to make predictions across three different problems.

Participants and Context

We recruited participants from an introductory biology course in fall 2016 and introductory biochemistry courses in fall 2018. All courses were taught at a large, public

research university in the Southeastern United States. One instructor in a single section taught all of the introductory biology participants. Multiple professors in multiple sections taught the introductory biochemistry participants. The introductory biology course serves as a foundational course for life sciences majors, and at the time of the interviews, general chemistry was a prerequisite. This course enrolls mostly first- and second-year students. We refer to participants from this course as biology students. The introductory biochemistry course focuses on the structure and function of biological molecules, enzyme kinetics, metabolism, and molecular biology. The introductory biochemistry course enrolls mostly second- and third-year students. We refer to participants from this course as biochemistry students.

Although this is a basic research study to discover the range and assembly of student knowledge elements for metabolism problem solving, we provide some information about the instructional approaches for the purpose of context. The introductory biology course from which participants were recruited focused on metabolism in terms of understanding the basics of reactants and products of each step in cellular respiration and photosynthesis. Students were taught to focus on the movements of ADP/ATP and the electron carriers. Biology students encountered many visual representations, in a wide variety of styles. Some visual representations gave an indication of how intermediates change in structure throughout the reaction, but even in those cases, students were directed in the related questions and discussion to focus on the incoming reactants and outgoing products instead. The course emphasized metabolism solely in the context of achieving specific ends to support life in organisms—most specifically energy processing.

The biochemistry participants received metabolism instruction focused on regulatory mechanisms of pathways, but they were generally covered in terms of: "This molecule inhibits/activates this enzyme/process, so what happens when there is a lot of it or a little of it?" The specific ways regulation can occur were touched on but not the primary focus. The primary focus articulated for these students was understanding free energy and reaction favorability. Students were typically asked to work in-depth with named and visually depicted pathways and enzymes in a more elaborate way than in the introductory biology course. Introductory biochemistry also used a diverse set of visual representations for metabolism, but the diversity was more narrow than that in introductory biology.

Recruited participants had previously completed a 26-item problem set as part of their enrollment in either introductory biology or introductory biochemistry. This assessment measures student learning about noncovalent interactions in biomolecular structure and metabolism. The investigation reported here focuses on the metabolism items, which we describe below. Our investigation of the noncovalent-interaction items is reported elsewhere (Halmo *et al.*, 2018, Halmo *et al.*, 2020, King *et al.*, in preparation). We recruited a total of 44 participants, including 22 participants from introductory biology and 22 participants from biochemistry. None of the biochemistry participants had previously completed the assessment during introductory biology. Of the 22 biology participants, 11 were male and 11 were female. Of the 22 biochemistry participants, 2 were male and 20 were female. Gender identities of the sample were obtained from the Office of Institutional Research. Students received a monetary incentive of $20 for their participation in this study.

This study was approved by the UGA IRB under exempt status (STUDY00000660 and PROJECT000000090).

Data Collection

Problem Set Development

We developed a metabolism problem set that was designed to uncover the knowledge elements students use when solving problems about metabolism. We aimed to address concepts that students learn in introductory biology and chemistry courses and build upon in biochemistry courses. After piloting three initial pathways with students and interviewing for construct validity purposes, we refined the problem set to the pathway used in this study that asks students about an unfamiliar pathway (Figure 1). Thus, students need to rely on their knowledge of metabolism and visual representations in order to make predictions about the pathway. In pilot interviews, when the pathways involved more familiar metabolic compounds, students used knowledge elements more related to the particular compounds (e.g., ATP or cholesterol) than to principles of metabolism. We piloted the problem set with students from both introductory biology and biochemistry and with Ph.D.-level biologists, chemists, and biochemists. We also conducted follow-up interviews with students and Ph.D.-level biologists, chemists, and biochemists to obtain detailed feedback and recommendations for improvement of the problem set.

The finalized problem set contains seven multiple-choice, three multiple-true/false, and three constructed-response items for a total of thirteen items. The complete problem set is presented in Supplemental Material Figure 1. All thirteen items refer to the

metabolic pathway presented in Figure 1. The objective items reference the image in Figure 1A, and the constructed-response items reference the image in Figure 1B. The thirteen items probe a variety of aspects of metabolism including the meaning of visual representations, enzyme properties, reaction reversibility, and flux.

This paper reports results from our analysis of participants' responses to a subset of five items, including one multiple-true/false, one multiple-choice and three constructed-response items. These items reveal knowledge elements about the concept of negative feedback inhibition, a mechanism that governs the dynamics of pathway regulation. Based on pilot studies with experts and students, we represented negative feedback inhibition using a dashed arrow and circled bar (Figure 1). There is no consensus on how to represent negative feedback inhibition. Negative feedback inhibition occurs when an excess of product accumulates and inhibits an enzyme earlier in the pathway. In the case of our problem, when IV-CoA accumulates, it binds to enzyme B at a site other than the active site (i.e., an allosteric site), inhibits enzyme B, and decreases the amount of IV-CoA produced. The levels of all metabolic compounds in the pathway are affected by negative feedback inhibition at a single step.

Interview Protocol

All participants had previously completed the problem set as part of their course enrollment. In the interview, we asked participants to complete the assessment again using a think-aloud interview protocol (Ericsson, 1980; Ericsson and Simon, 1998; Keys, 2000; Cooper *et al.*, 2013). This protocol reveals cognitive processes that are not normally available in written answers alone. Twenty-seven of the interviews focused on

problems related to both noncovalent interactions and metabolism. The remaining seventeen interviews focused entirely on problems related to metabolism.

We modeled our protocol after established protocols in other studies (Keys, 2000; Cooper *et al.,* 2013, Halmo *et al.,* 2018). At the start of the interview, we asked participants to think aloud while solving the problems presented to them, including anything they were reading and looking at in the figures. We limited interruptions to statements encouraging participants to "Please keep thinking aloud." At breaks in the interviewee's thinking, we occasionally prompted with "Can you explain what you mean by [scientific term]?" or "Can you further elaborate on [idea]." Students viewed the problem set via Qualtrics. For the multiple true/false and multiple-choice items, we asked students to click their response in Qualtrics, which provided us with a record of their selection in addition to their verbal response. For the constructed-response items students verbalized their answers. They did not write them.

We video recorded all interviews and applied pseudonyms. Pseudonyms that begin with B refer to biology participants, while pseudonyms that begin with C refer to biochemistry participants. Pseudonyms are gender-neutral because we did not examine gender as a factor in our analysis.

Data Analysis

Student performance

We determined student performance on the problem set as an indication of the extent to which students think about metabolism in a scientifically accurate way. We evaluated student performance on the multiple true/false and multiple-choice items by

comparing the answers students clicked in Qualtrics with the key we generated in advance. We evaluated student performance on the constructed-response items by categorizing the ideas within their verbal explanations as purely scientific (e.g., aligning with scientific explanations), purely non-scientific (e.g., different than scientific explanations), or a mixture of both.

Qualitative content analysis

We conducted qualitative content analysis (Strauss and Corbin, 1998) of participant interviews using a process that aimed to ensure the trustworthiness of our findings (e.g., Elo *et al.*. 2014). We aimed to identify the full range of knowledge elements students brought to the problem-solving space. First, we developed an *a priori* codebook consisting of ideas and problem-solving approaches we expected participants to express based on prior work (e.g., see Problem Development). Second, author K.B. refined the codebook by listening to all interviews to determine how well the *a priori* codebook aligned with the ideas presented by participants. Third, K.B. repeated the listening process and made additional refinements to the codebook as new insights emerged. Throughout steps two and three, K.B. consulted with author P.P.L. This process led to a coding scheme of 40 total codes that were derived from participant language rather than solely our preliminary ideas. Fourth, authors K.B. and A.S. applied the coding scheme to all interviews. They independently coded interviews and then compared their coding to resolve all discrepancies. Fifth, K.B. and A.S reviewed all coded interviews and narrowed the focus to 26 codes that deal with students' interpretations of the dashed arrow and circled bar and their attention to metabolic compounds in the pathway. At this stage, K.B., A.S., and P.P.L. also reorganized several codes to better capture the

variability within the original codes. This process led to the twelve codes presented in Tables 2 and 3. The process of refining our codes is illustrated for two of the final eleven codes in Supplemental Figure 2.

Resource Graph Creation

A resource graph is a representation of linked knowledge elements (Wittman, 2006; Rodriguez and Towns, 2019; Rodriguez *et al.*, 2020). Resource graphs reveal student reasoning because they display the coordination of individual knowledge elements that are activated when students solve a problem. We purposefully selected three students from our sample and created resource graphs of their solutions. Authors K.B. and P.P.L. selected three students (of 44 total) who utilized five of the seven distinct ideas about the dashed arrow and circled bar and focused to different extents on various metabolic compounds. Author K.B. returned to these students interviews and documented each distinct statement they made. Generally speaking, K.B. defined a knowledge statement as a sentence. For example, K.B. counted the following sentence from participant Charlie as a knowledge statement: “Without enzyme C, there would be barely any production of DMA-CoA.” While documenting knowledge statements, K.B. also documented the connections between knowledge statements, which he determined because statements were sequential or because the student used linking words that explicitly connect different knowledge statements. Authors K.B. and P.P.L. then organized knowledge statements into resource graphs (Figures 3-5).

Results

Here we present results that address our research questions: What knowledge elements do life sciences undergraduates use when thinking about metabolism? How do students assemble these elements during problem solving? We focus on research participants' interview responses to five items. We first report participants' scientific accuracy on these items. Accuracy is not the point of our research, yet we provide these data to orient readers to the way professional biologists, chemists, and biochemists think about the problem set and to present an overview of the extent to which students think in this way. The subsequent two sections focus on the use of knowledge elements during metabolism problem solving. Section two presents categories of knowledge elements derived from qualitative content analysis, and section three presents resource graphs from a subset of participants to address the assembly of knowledge elements. Taken together, these data illustrate that our problem set cued multiple independent knowledge elements that most participants assembled in a contextually-fluctuating way.

Performance on a metabolism assessment

Participants in our study answered one multiple true/false item, one multiple-choice item, and three constructed-response items. The multiple true/false item asked about the meaning of the dashed arrow and circled bar (Figure 1). Participants could select one to seven different responses, with "negative feedback or inhibition" as the correct response and all other responses as false (Table 1A). In multiple true/false items, each true/false item is independent, so it is possible to capture multiple ideas simultaneously held by students. Biology participants responded to this item with variability. The majority of biology participants (N=15) selected negative feedback or

inhibition, but even more biology participants selected IV-CoA being added as a reactant (N=16) or IV-CoA being recycled (N=18). Biology participants also selected other responses (e.g., alternate pathway, etc.). In contrast, all biochemistry participants recognized the dashed arrow and circled bar for its intended purpose (N=22), even though a small subset simultaneously thought of the arrow in other ways (Table 1A). These data provide initial evidence that the dashed arrow and circled bar activated multiple knowledge elements for biology participants in our study and most strongly activated the negative feedback element for biochemistry participants. We will more fully explore participants' knowledge elements about the dashed arrow and circled bar in the next section.

Second, we asked participants a multiple-choice question about the outcome of a perturbation in the visualized pathway. Specifically, we asked them what would happen to enzyme B activity if enzyme C were inhibited. The scientifically accurate view of this perturbation is that IV-CoA would build up due to decreased conversion to DMA-CoA by enzyme C and that the increased concentration of IV-CoA would in turn decrease the activity of enzyme B. This would occur due to negative feedback inhibition. Nine biology participants selected the scientifically accurate response, while eleven selected that the perturbation would have no effect on the activity of enzyme B. This result aligns somewhat with the diversity of biology participants' knowledge elements about the dashed arrow and circled bar. Participants who thought, for example, that the symbol represented IV-CoA being recycled, might have reasoned that inhibiting enzyme C would either increase the activity of enzyme B or have no effect. Twelve biochemistry participants selected the scientifically accurate response (i.e., the activity of enzyme B

would decrease). This number is much less than the number from Table 1A who said that the dashed arrow and circled bar represent negative feedback and inhibition. Apparently some biochemistry participants who recognized the dashed arrow and circled bar as negative feedback inhibition did not use this knowledge element for the multiple-choice question. These data point to the importance of uncovering the range of knowledge elements activated by the visual representations used for metabolism.

We also characterized participant performance on three constructed-response items. Each item presented participants with a different context, a particular perturbation to the pathway. For each perturbation, we asked participants to make and explain their prediction about the impact on flux through the pathway. For example, participants were asked about the impact on flux if IV-CoA could no longer bind to enzyme B. We focused our performance analysis both on participants' decisions that flux is or is not affected and whether they used purely scientific explanations, purely non-scientific explanations, or a mixture of both. We observed a similar pattern for biology and biochemistry participants (Figure 2, Figure S3). Most participants stated that flux would be affected by the perturbation, which is the intended response. A few participants used purely scientific explanations and a few used purely non-scientific one, but the typical explanations were a mixture of scientific and non-scientific ideas. The only noteworthy distinction between biology and biochemistry participants was that biochemistry participants' explanations were somewhat more scientifically accurate overall. These data suggest that while participants may have recognized that perturbations affect pathway flux, their reasoning included many knowledge elements, some of which needed refinement. The primary purpose of this paper is to report the range of knowledge elements participants use when

solving metabolism problems and to examine how participants assemble these ideas in a problem solution. We present these data in the following two sections.

Knowledge elements cued during metabolism problem solving

Analyzing participant performance only tells a partial story. Identifying the variety of knowledge elements cued during problem solving explains participant performance and reveals future avenues for research and instruction. Our qualitative content analysis showed that participants' solutions can be explained by two categories of knowledge elements: those pertaining to the dashed arrow and circled bar and those pertaining to the metabolic compounds students focus on. We describe these categories below by providing examples of knowledge elements using quotes from the data. Quotes have been lightly edited for clarity and readability. We use gender-neutral pseudonyms for participants. Pseudonyms starting with the letter B indicate biology participants, and pseudonyms starting with the letter C indicate biochemistry participants. We begin with a look at knowledge elements activated by the dashed arrow and circled bar.

Knowledge elements about the dashed arrow and circled bar

As reported in Table 1A, participants could select one to seven choices about the meaning of the dashed arrow and circled bar. Participants' interview explanations revealed which of these choices most strongly cued their thinking (Table 2). For a few biology participants and the majority of biochemistry participants, the dashed arrow and circled bar cued the knowledge element "negative feedback inhibition," but the representation prompted other knowledge elements as well, including some not provided in the problem set (Table 2).

The most prevalent set of knowledge elements cued by the dashed arrow and circled bar, particularly among biology participants, was that the dashed arrow and circled bar represent IV-CoA going back to be part of the previous chemical reaction (i.e., the conversion Fatty Acyl Thioester to IV-CoA). Some participants described this notion as IV-CoA being "recycled" (Table 2). These participants explained a process in which IV-CoA serves as a necessary component for the conversion of Fatty Acyl Thioester into IV-CoA. Consider Brooklyn's description of what the dashed arrow and circled bar represent:

> Brooklyn: *[IV-CoA is] being added back in right here to B, so that it can somehow bond with the fatty acyl thioester and make some more of itself.*

Similarly, participants described the dashed arrow and circled bar as IV-CoA being "reverted" back to a previous compound, usually fatty acyl thioester (Table 2). Some individuals thought that this was a necessary part of the process of creating IV-CoA. Still others saw the dashed arrow and circled bar as equivalent to a "reversible reaction," to be used when the pathway needed more fatty acyl thioester. An example of this idea is shown here by Casey:

> Casey: *Then the reaction is just gonna essentially go back to [enzyme] B and the thioester because of the buildup of IV-CoA that's going to push it back up the chain.*

It is rational for participants to have thought that the dashed arrow and circled bar symbolize IV-CoA going back to participate in the previous reaction. In fact, the other "back" arrows shown in the pathway are intended to signify participation of that

compound in a chemical conversion, and all participants understood this fact (data not shown). We suggest that participants who thought of the dashed arrow and circled bar as the intermediate "going back" simply need to refine this knowledge element. They need help distinguishing the solid and dotted arrows and associating the dotted arrow with negative feedback inhibition.

Another knowledge element activated by the dashed arrow and circled bar was "negative feedback because of the negative sign" (Table 2). This was true of both biology and biochemistry participants, including Bryce and Camron:

> Bryce: *I feel like the circled bar would have something to do with it being negative. So, in that thinking, I would say that this would be negative feedback or inhibition, and it would not be positive. And I'm only saying that because I see this as being a negative sign.*
>
> Camron: *So the dashed arrow implies regulation of some sort of the metabolic compound on the activity of the enzyme. And the circled bar, since it's a negative – a minus sign – it would be negative inhibition."*

Bryce and Camron, and participants like them, were using a rational thought process. We designed the visual representation with a bar precisely because it signals "negative." Yet these participants need to recruit knowledge elements beyond "bar equals negative," including ones that have to do with the molecular interactions that account for negative feedback inhibition.

Interestingly, some participants did recruit a resource for the molecular mechamisms of negative feedback inhibition. These participants noted that the dashed arrow and circled bar indicate IV-CoA binding to and inhibiting enzyme B. We found this resource among both biology and biochemistry participants (Table 2). Consider Cheyenne's description of the dashed arrow and circled bar:

> Cheyenne: *[IV-CoA] is attaching to this [enzyme B]. When a lot of [IV-CoA] is present, it's signaling to enzyme B to stop working basically, so this reaction stops because you already have a lot of this product. So that's allosteric inhibition.*

Finally, the dashed arrow and circled bar caused one biochemistry participant to think of electrons. This student associated the circled bar with the negative charge of electrons and imagined that an electron was being added back to step B. This student used a knowledge element that is appropriate for biochemistry (e.g., in the context of full and partial charges on atoms, Halmo *et al*., 2020), but needs to be refined. A negative sign indicates electrons in some circumstances and negative regulation in others.

In summary, these data show that the dashed arrow and circled bar cued a variety of productive knowledge elements for students. Some need refinement and others need supplementation with additional knowledge elements, particularly those pertaining to the molecular mechanisms of negative feedback inhibition.

Knowledge elements pertaining to student focus on different metabolic compounds

Metabolic pathways, like the one presented in our problem set, are interconnected systems. Metabolic compounds are not lined up in the cell to react in order. Instead, they move around in cellular space like vegetables in soup. Reactions occur simultaneously.

Perturbations in the concentration of one metabolic compound can impact all other aspects of the pathway as well as other pathways. With this reality in mind, the ultimate aim of our problem set was for participants to discuss how each metabolic compound would be affected by changes to a single pathway component. Yet participants mostly made predictions about pathway dynamics with a focus on single reactions and rarely dealt with impacts on the initial reactions of the pathway. Thus, a second category of knowledge elements we identified deals with the metabolic compounds to which students directed their attention during problem solving: thiamine phosphate, fatty acyl thioester, IV-CoA, DMA-CoA or IB-CoA (Table 3).

We found that the problem set most strongly cued students to focus on IV-CoA, IB-CoA, and DMA-CoA (Table 3). All biochemistry participants and almost all biology participants described effects on IV-CoA, IB-CoA, and DMA-CoA. Bobbi's prediction about the impact of Enzyme C not working anymore typifies participants' strong tendency to think about the problems from the vantage point of these three compounds:

> Bobbi: *"[Enzyme C not working anymore] would affect only this pathway," <they indicate the reaction from thiamine phosphate to DMA-CoA> "So it wouldn't affect the whole system. It would affect this way because if [enzyme C] stops working, then it's gonna stop working to produce the DMA-CoA, but it won't be affecting this one…" <they indicate the pathway from IV-CoA to IB-CoA> "…since it's using a completely different enzyme."*

In contrast, few participants paid attention to thiamine phosphate and fatty acyl thioester (Table 3). Even when the interviewer specifically asked about these compounds, participants were not prone to think that the impacts on thiamine phosphate and fatty acyl thioester were noteworthy. For example, the interviewer asked if introducing a branch point in the pathway would affect flux prior to the branch point. Camron and Cheyenne exemplify the responses in our sample:

> Camron: *"No, it would only affect the concentration of DMA-CoA and IB-CoA, because it's just working with how much IV-CoA is available at this predetermined place."*
>
> Cheyenne: *"... since the IV-CoA is now being distributed amongst two branches, there's less of that in general, so there's less of it to inhibit enzyme B. ... So it's not changing anything about the thiamine phosphate to the fatty acyl thioester, but it could be decreasing their concentrations, if anything.*

Even when participants expressed there might be some change in the amounts of thiamine phosphate and fatty acyl thioester, they minimized the importance of that change. Cheyenne suggested a decrease in concentrations as an afterthought—a side effect that may or may not occur, and that would have no groundbreaking significance either way.

It is not surprising that participants focused on IV-CoA, IB-CoA and DMA-CoA. We asked participants to consider perturbations directly connected to IV-CoA, so utilizing this resource is expected and appropriate. Likewise, participants may have focused on IB-CoA and DMA-CoA because their biology course and to some extent their biochemistry

course encouraged them to think of metabolic pathways from the perspective of the end products (see Methods). Also, drawing pathways from top to bottom like we did may bias students to focus on the compounds that come "last." Yet it is important for students to focus on all intermediates in a pathway because doing so opens them to the connectedness among different pathways and the impact of single changes on an entire system. Our data suggest the need for explicit instruction to support students to consider all of the metabolic compounds in a pathway.

Assembly of knowledge elements into solutions

The previous section reported the knowledge elements participants used when thinking about metabolism. We also wanted to learn how participants assemble these resources during problem solving, which we addressed using resource graphs. Here we present the resource graphs for three purposefully-selected participants who used different knowledge elements about the dashed arrow and circled bar and focused to different extents on the various metabolic compounds. Each resource graph reveals a particular participant's coordination of individual knowledge elements. We begin with Bailey and Carson to show the independent, fluctuating nature of participants' resource use, which was most characteristic of our sample. We end with Charlie to show how student thinking can advance to greater coherence and stability, a rare occurrence in our sample.

Bailey: Independent, fluctuating knowledge elements with a focus on IV-CoA, IB-CoA, and DMA-CoA.

We present biology student Bailey's resource graph in Figure 3. Bailey's resource graph shows utilization of a mixture of independent knowledge elements about the dashed arrow and circled bar and a view of the pathway as a step-by-step process meant to make DMA-CoA and IB-CoA, rather than a multi-component system controlled by feedback inhibition (Figure 3).

When Bailey was asked what the dashed arrow and circled bar mean, they drew upon four knowledge elements: negative feedback because of the negative sign, negative feedback inhibition, the intermediate being recycled, and the intermediate being added back as a reactant (i.e., reverted) (Figure 3, left side). Bailey likened the dashed arrow and circled bar to a similar representation from their biology class, where one has to input some ATP to make more ATP product and have a net gain. Bailey also pointed out that the dashed arrow looks "sort of like a recycling sign." Bailey appears to have used these knowledge elements independently because when they were later asked to make predictions about pathway perturbations, they abandoned the elements about negative feedback inhibition and only carried forward the elements about IV-CoA being added back to the previous reaction. For example, Bailey explained that when IV-CoA can't bind to Enzyme B, IV-CoA can no longer go back into enzyme B. They reasoned that because IV-CoA binding to B is essential for IV-CoA to be recycled, Enzyme B won't work properly and the reaction will not proceed. Thus, they concluded that less IV-CoA, DMA-CoA, and IB-CoA will be produced, and flux will be affected. Additionally, Bailey's use of knowledge elements about the dashed arrow and circled bar fluctuated as they progressed through the three contexts. In the first context (i.e., introduction of a branch point), Bailey never considered the dashed arrow and circled bar. In the second

context, when the problem explicitly cued them to think about binding between IV-CoA and enzyme B, Bailey did shift their attention strongly to the dashed arrow and circled bar. Yet in the third context (i.e., enzyme C no longer works), Bailey only utilized the recycling element at the very end of their solution.

In terms of their focus on the distinct metabolic compouds, Bailey used just three of these compounds for problem solving, IV-CoA, IB-CoA, DMA-CoA (Figure 3). Bailey seemed to think of IV-CoA as being able to go in three directions, IB-CoA, DMA-CoA, or back to the fatty acyl thioester to IV-CoA reaction. Bailey gave some indication that metabolic compounds exist in pools of molecules (i.e., "there will be less DMA-CoA now with the introduction of the branch point"). Yet this notion fluctuated, because other times they did not consider that less production of one compound would create the possibility of more production of a different compound (e.g., "Enzyme C not working will affect the DMA-CoA branch, but not the IB-CoA branch").

In summary, Bailey discussed several knowledge elements about the meaning of the dashed arrow and circled bar, but they only saw the relevance of a couple of these elements, and only for one of the three problem scenarios. Bailey's ideas about the dashed arrow and circled bar seem loosely held and specific to the context at hand. We would not expect Bailey to apply these ideas in a different context, for example, if negative feedback inhibition were visually represented in a different way. Bailey also did not see the relevance of thiamine phosphate and fatty acyl thioester. Instead, they thought of the pathway as primarily about the end products.

These results suggest that students like Bailey need explicit guidance to draw forth more knowledge elements about negative feedback inhibition, reconcile those elements with the notion of recycling and reversion, and practice using elements about negative feedback inhibition to explain how changes in any pathway component can affect every pathway component.

Carson: Independent and linked knowledge elements with a focus on all metabolic compounds.

Figure 4 shows the resource graph for biochemistry student Carson. Carson drew upon multiple knowledge elements about the dashed arrow and circled bar, some of which they linked and used variably during problem solving, and others which they kept independent and eventually abandoned. Carson also focused attention on every metabolic compound during problem solving (Figure 4).

Carson drew upon multiple knowledge elements when asked what the dashed arrow and circled bar mean: an electron donated to step B, IV-CoA being recycled, IV-CoA being added back as a reactant, and negative feedback inhibition. One of these ideas compelled Carson the most, and they linked it to two other knowledge elements. Carson kept the other knowledge element completely independent. Their solution unfolded in this way. As soon as Carson encountered the dashed arrow and circled bar, they stated that the representation looked like an electron being donated to reaction step B. Carson then considered the multiple true-false prompt “IV-CoA being recycled” and reasoned like this: because the charge (i.e., the electron) going back to reaction step B contributes to the entire pathway, the symbol potentially indicates recycling. Carson did something similar when considering the multiple true-false prompt “negative feedback inhibtion,”

stating that “an electron is negative; [therefore], this is negative feedback inhibition.” In contrast, when Carson read the prompt “IV-CoA being added as a reactant,” they generated an independent line of thought. Abandoning the electron, recycling, and negative feedback inhibition ideas, Carson stated that IV-CoA is the reactant for the IV-CoA to DMA-CoA reaction, so the dashed arrow and circled bar represent IV-CoA being added back as a reactant. Thus, with Carson we see expression of a variety of knowledge elements, some that they thought of independently and some that they linked.

Next Carson went on to consider the impact of perturbations to the pathway. Here they carried forward the ideas of an electron being added to the previous reaction, but they did so with variability. When asked about the effect of adding a branch point, Carson stated that the electron being added is powering reaction B, but they did not use this idea to make a prediction about the branch point. When asked what would happen if IV-CoA can no longer bind to enzyme B, Carson drew upon the electron addition idea once again. They stated that when the electron is added to step B, the reaction goes back to fatty acyl thioester and back down, which is important for the pathway to function. This part of the process would be unable to function anymore (i.e., if IV-CoA can no longer bind to enzyme B), so the entire reaction pathway won’t work. This would limit the reaction to IV-CoA, DMA-CoA and IB-CoA, and affect the flux through the pathway. However, when asked what would happen to the pathway if Enzyme C doesn’t work anymore, Carson set aside the electron donation idea and simply walked through the steps of the pathway.

In terms of Carson's focus on distinct metabolic compounds, they considered all five compounds during problem solving (Figure 4). Unlike Bailey, who seemed to think of the pathway as existing for the formation of DMA-CoA and IB-CoA, Carson considered every compound in their solutions. When thinking about the branch point problem, Carson pointed out that the conversion of fatty acyl thioester to IV-CoA is powered by an electron. When considering the impact of IV-CoA no longer binding to enzyme B, Carson predicted an affect on thiamine phosphate and fatty acyl thioester but not DMA-CoA. Finally, when considering what would happen if enzyme C doesn't work anymore, Carson noted that the steps catalyzed by enzymes A, B, and D would still be working. These results suggest that Carson viewed every metabolic compound as an important factor to consider.

In summary, Carson displayed a number of different knowledge elements about the dashed arrow and circled bar, some which they abandoned and others which they linked and used variably during problem solving. Carson also utilized all five metabolic compounds during problem solving, showing the intuition that a metabolic pathway is a system where a change in one component can affect every other component.

These results suggest that students like Carson need encouragement to continue attempting to link disparate knowledge elements while simultaneously receiving guidance to sort through the conditions under which to apply elements. For example, Carson would benefit from an instructor pointing out that, yes, negative signs often symbolize negative charge (i.e., electrons), but that they also symbolize negative regulation (i.e., decreasing activity). Like Bailey, Carson needs additional guidance about negative feedback inhibition, whether it be opportunities to build upon related ideas (e.g.,

the interaction between IV-CoA and enzyme B is like a ligand binding to a receptor) or opportunities to learn new terminology (e.g., the interaction between IV-CoA and enzyme B is an allosteric interaction). Unlike Bailey, who needs guidance to consider every pathway component, Carson only needs encouragement to continue this practice.

Charlie. Coherent, stable knowledge elements with attention to most metabolic compounds.

Figure 5 shows the resource graph for biochemistry student Charlie. Charlie drew upon a single resource when asked about the dashed arrow and circled bar, and they used this resource stably across all three problem contexts, while also considering most of the metabolic compounds in the pathway.

Charlie recognized the dashed arrow and circled bar as negative feedback inhibition. Unlike Bailey and Carson, Charlie did not state that the symbol represented any other thing (e.g., IV-CoA being recycled). Charlie then defined negative feedback inhibition using the context of the problem: "production of IV-CoA inhibits enzyme B." Charlie also went further than other students in our sample and stated that with negative feedback inhibition "there must be an allosteric enzyme," as this tends to be the mechanism by which negative feedback inhibition functions.

When asked to analyze perturbations in the pathway and their effect on flux, Charlie utilized their coherent understanding of negative feedback inhibition for reasoning and also focused attention on the pathway as a whole, not just the end products. With the introduction of the branch point, Charlie stated that the new branch would decrease the amount of free IV-CoA available. With less IV-CoA available, there would

be less negative inhibition of enzyme B, resulting in an increased conversion of fatty acyl thioester into IV-CoA. In the second scenario where IV-CoA can no longer bind to enzyme B, Charlie again used the idea of negative feedback inhibition, pointing out that it would be disrupted. Charlie then predicted the outcome on concentrations of fatty acyl thioester, IV-CoA, DMA-CoA, and IB-CoA. When asked about enzyme C not working, Charlie reasoned that there will be less production of DMA-CoA from IV-CoA, resulting in a buildup in the concentration of IV-CoA. He then pointed out that this would affect negative inhibition, as the buildup of IV-CoA would result in increased negative inhibition of enzyme B to slow the production of IV-CoA. Interestingly, Charlie never made a prediction about thiamine phosphate.

In summary, Charlie showed coherent understanding of the dashed arrow and circled bar and negative feedback inhibition. While Bailey and Carson displayed multiple, competing ideas about the dashed arrow and circled bar and utilized only one or two during problem solving, Charlie stuck with their singular understanding through every problem scenario. All of Charlie's statements converged on the underlying principle that IV-CoA negatively regulates enzyme B and, thus, controls pathway flux. Also, Charlie considered the impact on all metabolic compounds except thiamine phosphate.

Students like Charlie have refined their intuitions and learned to see the problem in a way that resembles that of experts. Thus, they respond in the scientifically expected way. However, students like Charlie certainly still have limits to their use of scientifically accurate knowledge elements. Charlie could benefit from more complex problems. For example, how would Charlie respond if asked to consider the utilization of IV-CoA by

other pathways (e.g., as a regulator of another enzyme with a different binding constant)? We predict that Charlie would draw upon additional knowledge elements and that they would need guidance to sort out their intuitions in this more complex situation.

Limitations

We were able to identify numerous knowledge elements cued by a metabolism problem set by interviewing 44 students at two undergraduate levels. The knowledge elements we discovered are unlikely to be the only ones about metabolism that exist among undergraduate life sciences students, because the problem set used in this study provides only one way of visually representing metabolism and only three problem-solving scenarios, all within the same pathway. The pieces framework predicts that the diversity of knowledge elements would increase with research using different problem sets and contexts. Additionally, students in other samples may bring additional knowledge elements to bear on metabolism problems. Finally, think-aloud interviews only reveal the ideas that participants say aloud. Participants may have used other knowledge elements that they did not expressed them verbally.

Discussion

In this study, we explored the knowledge elements introductory biology and biochemistry students used when solving problems about metabolism. We discovered a diverse array of knowledge elements, which most participants utilized independently and with fluctuations across contexts. Given our theoretical perspective, we view these results as the interaction among participants' intuitions, experientially-grounded notions, and scientific ideas, the assessment, and the interview setting. Here we present several

implications for research and instruction and conclude with a statement of the impact of research on student thinking.

Research Implications

Researchers who take the pieces perspective search for the independent, fine-grained elements of student thinking and how these elements interact within and across problem-solving contexts (Scherr, 2007; Gouvea and Simon, 2018; Rodriguez & Towns, 2019; Lira and Gardner, 2020; Rodriguez *et al.*, 2020). Indeed, we found evidence for the fragmented, piece-like nature of student thinking about metabolism. Students in our sample used a wide range of knowledge elements to describe the dashed arrow and circled bar and focused mostly on downstream pathway components rather than upstream components. Our sample consisted primarily of students like Bailey and Carson who drew upon these knowledge elements independently and in a way that fluctuated with the problem context. These data support the pieces perspective. With Charlie, we found one rare instance of coherent and stable use of knowledge elements. We argue that Charlie's problem-solving performance does not refute the pieces perspective. Instead, Charlie's performance illustrates how students' can refine their once varied, fragmented, and fluctuating knowledge elements to a more unified set of scientific ideas that cohere and are stably applied.

Pieces researchers assume that students have good reasons for thinking the way they do. They assume that students' seemingly incorrect ideas are instead misapplied or indicative of ambiguous science instruction. We see rational reasons for the variety of knowledge elements students expressed in our study, some of which we described alongside our results. Consider the dashed arrow and circled bar. We intended for this

symbol to represent negative feedback inhibition, and many participants gravitated toward this idea. Yet participants also thought that it represented the intermediate going back to be part of the previous chemical reaction. This is logical because "back" arrows in chemical reactions almost always symbolize reverse reactions. Also, perhaps participants thought of recycling because the visual resembles the circular loop that denotes recycling in student's everyday life. Participants may have made a subconscious connection to recycling when they did not immediately recognize the visual. For many participants in our sample, the dotted, curved arrow simply was not a strong enough cue to indicate an entirely different process than that indicated by the solid, straight arrows. Further, consider participants' focus on downstream vs. upstream components in the metabolic pathway. The presentation of pathways in a stepwise pattern (fatty acyl thioester gets turned into IV-CoA, which gets turned into DMA-CoA) may play upon students' natural biases (i.e., this pathway exists to make DMA-CoA). This could lead to underlying notions that every pathway has tangible goals and the end products are what matters. These findings warrant at least three lines of further research from the pieces perspective.

First, we need to discover the knowledge elements activated by various representations of metabolism and the reasons they are activated. Conventions do not exist for visually representing metabolism (Offerdahl, 2017, Wright *et al.*, 2018). We designed the visual representations in our study based on feedback from experts, but experts did not agree on the best way to represent negative feedback inhibition. For example, some experts liked the representation we used, but others wanted a curved line ending in a flat, perpendicular line instead of an arrow. Yet even if experts agreed on the

way to represent metabolism, their intuitions would be different from students (Tversky *et al.*, 2002; Smallman and John, 2005). What we really need is basic research that inquires into the knowledge elements activated by students in response to different visual representations, including ones commonly used in instruction and ones that are intentionally designed with attention to principles of human perception. Such work could follow a trajectory like the work of Novick and Catley on cladograms (e.g., Novick and Catley, 2007; Catley and Novick, 2008; Novick and Catley, 2013; Novick and Catley, 2014). Novick and Catley's work revealed that tree-formatted cladograms promote better student learning than ladder-formatted cladograms. One cause for this difference is that human perceptual tendencies cause students to interpret ladder cladograms in a way that conflicts with the scientifically accurate segregation of taxa into nested levels (Novick and Catley, 2007). This research led to shifts in the presentation of cladograms in textbooks and instructional materials and, thus, to improved student learning (Catley and Novick, 2007; Schramm *et al.*, 2021). A systematic inquiry into the knowledge elements cued by different visual representations of metabolism, and visual representations of all core biology concepts for that matter, will show us what we can expect from students during instruction. If we find one set of visuals is better than another at aligning student thinking with scientific thinking, we can move forward to design instructional materials in that way.

Second, research is needed that reveals students' knowledge elements related to the causal mechanisms of negative feedback inhibition. It was rare in our study for participants to discuss the molecular-level process by which a compound like IV-CoA binds to the allosteric site on an enzyme, changes the structure of that enzyme, and thus,

regulates the enzyme's catalytic activity. Another recent study on metabolism assessment showed the same finding (Villafañe *et al.,* 2021). We hypothesize that students do, indeed, posses relevant knowledge elements. For example, the process of negative feedback inhibition is similar to that of a ligand binding to a cell surface receptor and triggering events inside the cell, which is commonly taught in introductory biology and physiology courses. We need research to discover how best to help students make connections like these and move beyond recognizing negative feedback (e.g., because of the negative sign), which many of our participants did, toward causal mechanistic understanding.

Third, and more generally, the pieces perspective should be applied to additional investigations of student thinking in the life sciences. Only a handful of pieces' studies exist in biology education research (Nehm and Ha, 2011; Gouvea and Simon, 2018; Lira and Garnder, 2020; Slominski *et al.*, 2020), even though the perspective has been productively utilized in physics (e.g., Hammer, 2000; Sayre and Wittmann, 2008; Scherr and Hammer, 2009; Weliweriya *et al.*, 2019) and chemistry (e.g., Heisterkamp and Talanquer, 2015; Becker *et al.,* 2017; Becker and Towns, 2012; Rodriguez *et al.*, 2018; Rodriguez and Towns, 2019; Rodriguez *et al.*, 2020). The pieces perspective facilitated our discovery of numerous knowledge elements students used when solving metabolism problems, and the biology and biochemistry education communities are now better equipped to create instructional materials that support student learning. Biology education has much to gain from the pieces perspective precisely because this perspective privileges and prioritizes student thinking.

Implications for Instruction

The pieces model promotes the notion that instruction should help students refine their intuitions by recognizing the utility of their intuitions in some circumstances and the need to refine their intuitions for other situations (Elby, 2001). Our results provide important insights for instructors who want to guide students in this refinement process.

Overall our data suggest that metabolism should be taught as a concept in its own right, not as a collection of examples. Metabolism is typically taught by marching through a series of scientifically-understood pathways. In introductory biology, this may include glycolysis, cellular respiration and photosynthesis. In biochemistry, the number of examples often expands to include gluconeogenesis, lipid metabolism, amino acid metabolism, and others. Yet our data illustrate that conceptual understanding of metabolism and problem solving requires integration of knowledge elements about visual representations and processes like negative feedback inhibition. Some students, like Charlie, may eventually activate, refine, and integrate these knowledge elements as they examine numerous example pathways. However, we suggest that a more expedient and successful approach would be to explicitly instruct students to apply, and refine relevant knowledge elements (e.g., as discussed above regarding the causal mechanisms of negative feedback inhibition). Inspired by our findings, we created an activity that we used in a biochemistry course that prompts students to activate and integrate knowledge of negative feedback inhibtion, free energy, and flux in an example pathway (Supplemental Material Figure 4). The point of the lesson is not to learn the particular inputs and outputs of the pathway, but to examine the pathway as an example of ideas in

play across all metabolic pathways. We encourage other instructors to follow suit, and we suggest the inclusion of three key points.

First, metabolism activities should help students develop their intuitions about visual representations of metabolism. As with our participants, the visuals in instruction and assessment may not adequately align with students' knowledge elements. Students will likely build more coherent understanding of metabolism if they are explicitly instructed about the meaning of common visual representations. We recommend that instructors walk students through the visual representations used in a metabolic pathway, including the different types of arrows and other symbols, to bring to light common interpretations of the visuals and help students sort through which interpretations align with the intent of the scientific community.

Second, metabolism activities should teach students the causal mechanisms of negative feedback inhibition implied by symbols like our dashed arrow and circled bar. This symbol represents a process with numerous steps, including diffusion of compounds within a cell, binding through noncovalent interactions, changes to the structure and catalytic activity of enzymes, and changes in flux through individual reactions and the pathway as a whole. As discussed above, our data suggest that the everyday, multipurpose arrow and circled bar do not cue these complex molecular-level ideas for most students. When we examined the instructional materials used by our participants' instructors, we found that introductory biology did not spend any time on feedback inhibition. Biochemistry instruction did cover feedback inhibition, but the materials focused on the impact of negative feedback on flux, not how negative feedback occurs or how it alters the catalytic rate of enzymes. The intermolecular interactions that facilitate negative

feedback are typically taught in separate sections of a biochemistry course. In the majority of our participants, we saw a reasoning pattern of "Enzyme B is inhibited so [result] will happen" but they did not explain why. Students need opportunities to connect the process of feedback inhibition with the pathways themselves, rather than learning about intermolecular interactions independently and being expected to make the connection on their own when it comes to metabolism.

Third, activities should teach pathways as systems, not as step-by-step processes. Our data show that students tend to segment pathways and focus on the downstream products. Students need help to focus on all metabolic compounds in a pathway. They need to be instructed that a change in an individual chemical reaction will influence all metabolic compounds in the pathway and possibly other pathways. This could be handled, for example, by simply asking students to describe how the concentrations of each metabolic compound in a pathway changes due to negative feedback inhibition.

The proposed instructional innovations could be applied at the introductory biology or biochemistry level. Instructional level was not a critical distinction in our findings, although we saw some differences between the two samples, particularly in their selection of ideas about the dashed arrow and circled bar. Rather, our data suggest that students at both instructional levels would benefit from opportunities to refine and build on their intutions about metabolism. Evidence exists that students with relatively low or high background knowledge benefit equally from pedagogies that involve problem solving followed by instruction (Kapur, 2016; Toh and Kapur, 2017), so the instructional implications presented here can be applied at the beginning and intermediate levels of an undergraduate life sciences curriculum.

That being said, it is worth considering the typical learning objectives of introductory biology compared to biochemistry. Given the breadth of material introductory biology must cover, metabolism will likely be limited to the examples of cellular respiration and photosynthesis. There may simply not be time to allow students to deeply explore the concepts of metabolic pathway regulation or pathways as systems. However, introductory biology instructors can be mindful of our findings and shift their focus even in small ways, for example, by carefully considering their use of visual representations or linking negative feedback inhibition to related phenomena commonly considere in introductory biology (e.g., enzyme-substrate interactions and ligand-receptor binding). In contrast, metabolism is a central focus in biochemistry courses, and these courses will need to do the heavy lifting on metabolism. Many biochemistry instructors may assume that students enter their class with well-developed, stable ideas about metabolism, but more than likely most of their students resemble most of our biochemistry participants, entering biochemistry with numerous pertinent but independent ideas about metabolism that they will need help to refine.

Conclusion

Our research revealed students' knowledge elements about metabolism. Students utilized a diverse array of knowledge elements and assembled them in independent and fluctuating ways across problem-solving scenarios. Our findings show the impact of research on student thinking and point to the utility of the pieces framework. Our findings also provide a model for using basic research on student thinking to guide instruction. Instruction in metabolism can progress as data reveal the variety and rationale of the knowledge elements that comprise student thinking. Instructional materials should be

created to provide scaffolding that maximize students' knowledge refinement and integration.

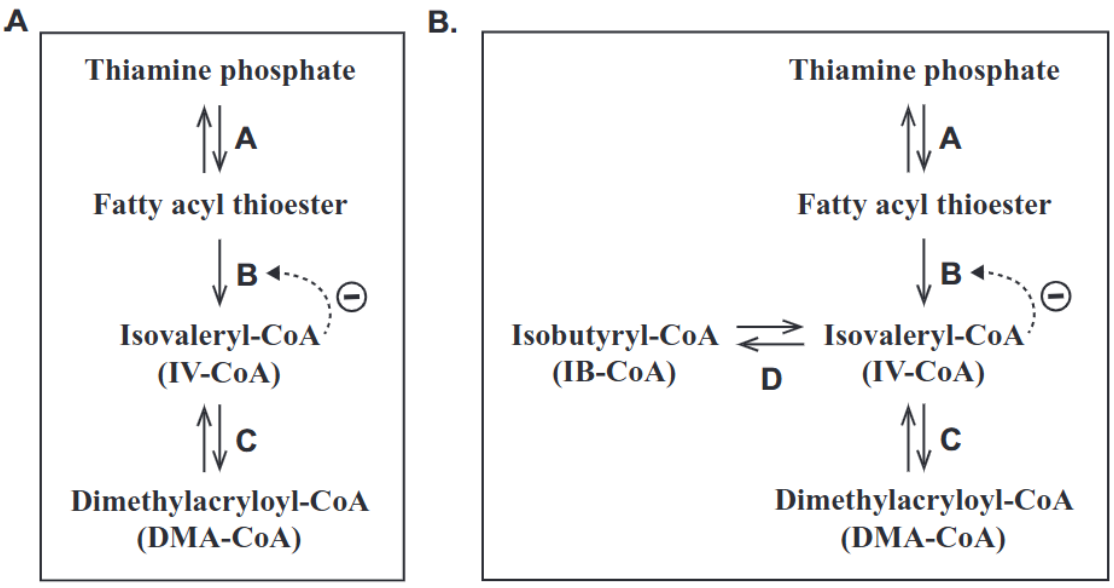


Figure 2.1: **The metabolic pathway referenced in the problem set students were asked to solve.** (A) Thiamine phosphate is converted over several steps to DMA-CoA by enzymes A, B, and C. If there is an excess of the intermediate compound IV-CoA, some of it can bind to enzyme B to prevent the enzyme from working via negative feedback inhibition. This slows down the rate of IV-CoA production. (B) This variation of the pathway introduces IB-CoA, which can be converted to IV-CoA (and vice versa) by enzyme D.

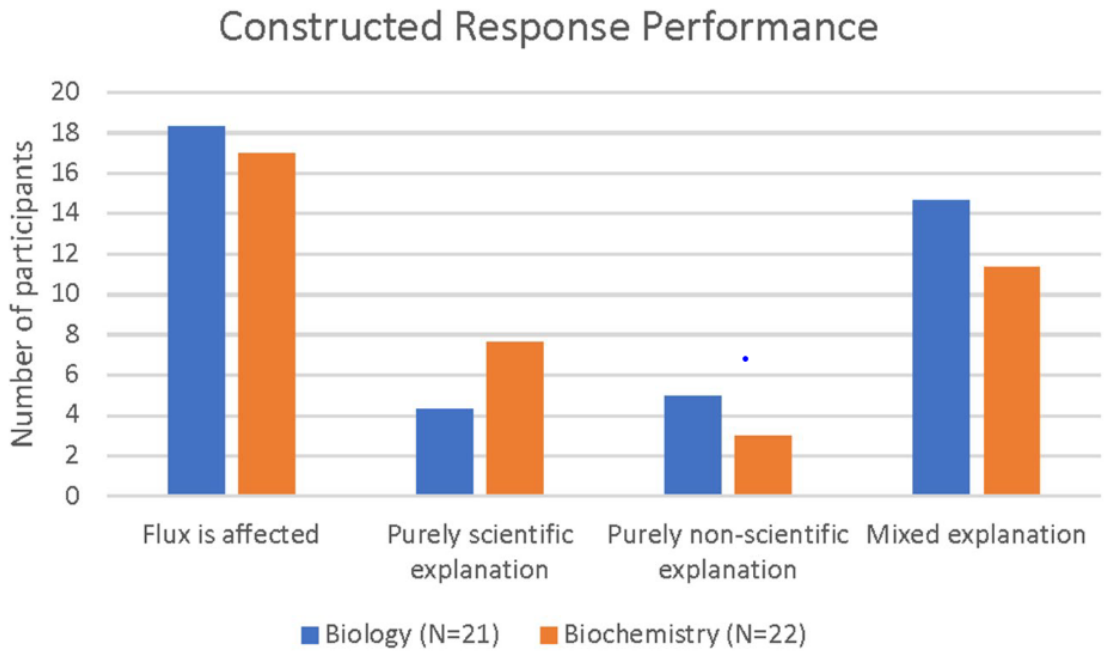


Figure 2.2: **Participant performance across contexts in a metabolism problem set.** Participants were asked to predict the outcomes of three perturbations to the metabolic pathway. They were asked to say whether or not overall pathway flux would be affected and scientifically explain their predictions. The leftmost set of bars shows the number of participants who predicted that flux would be affected by the perturbation. The remaining three sets of bars show the number of students who provided explanations that were purely scientific, purely nonscientific, or mixtures of both across all contexts. Blue bars correspond to biology participants (N=21) and orange to biochemistry participants (N=22).

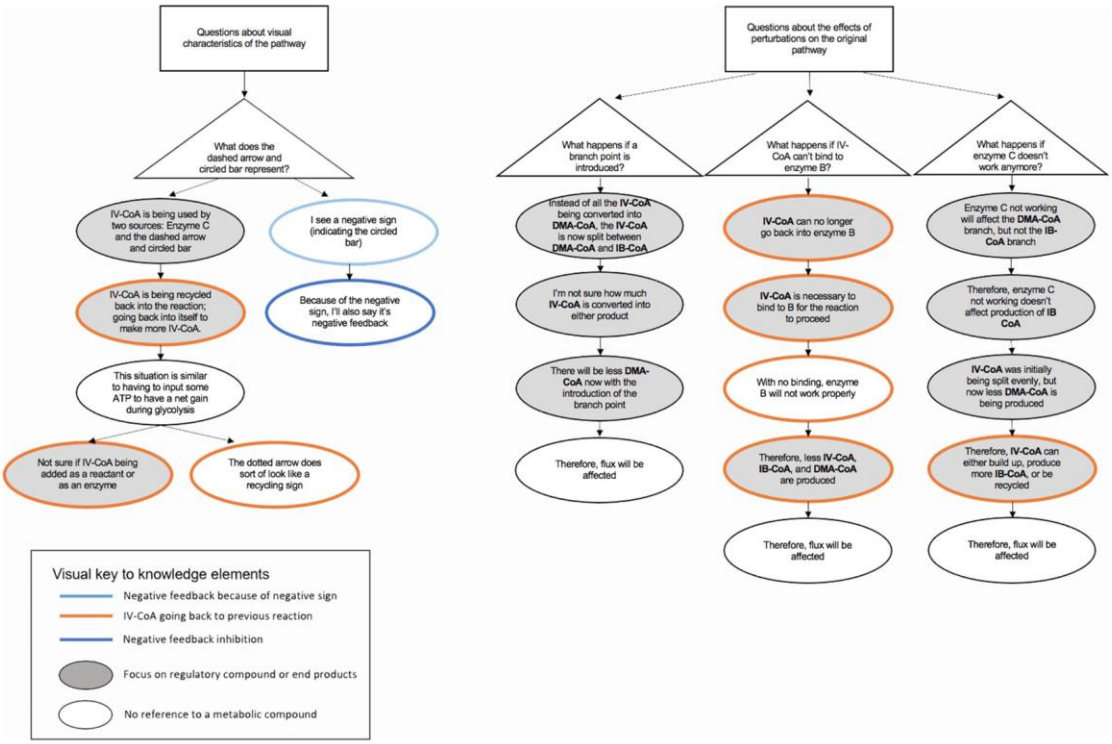


Figure 2.3: **Resource graph for Bailey.** White boxes indicate sections of the metabolism problem set that are the focus of this study. White triangles indicate specific questions within each section. Knowledge elements are shown as text within ovals. Arrows indicate the step-by-step flow of knowledge elements. Ovals with bold, colored outlines indicate different knowledge elements about the dashed arrow and circled bar: orange is for IV-CoA going back to be part of the previous chemical reaction, light blue is for negative feedback because of the negative sign, and dark blue is for negative feedback inhibition. The fill color of each oval represents different knowledge elements for student focus on metabolic compounds: gray fill is for focus on the regulatory compound or end products, and white indicates that the participant made no reference to a metabolic compound.

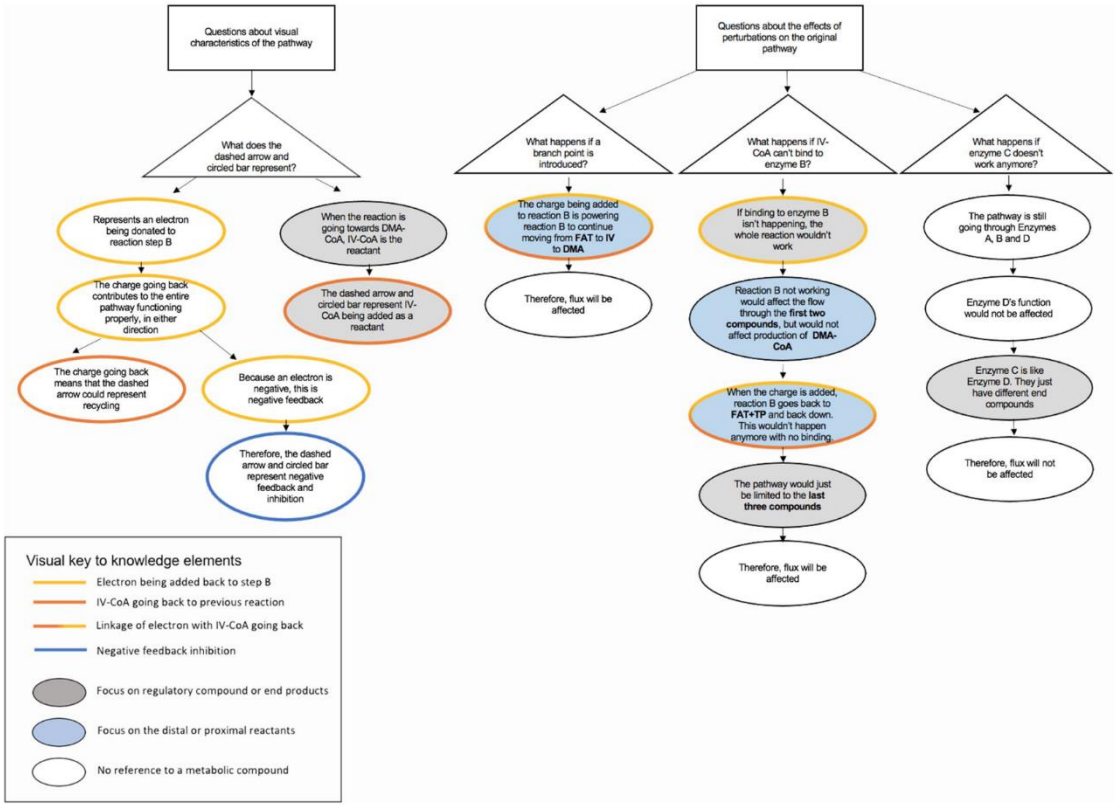


Figure 2.4: **Resource graph for Carson.** White boxes indicate sections of the metabolism problem set that are the focus of this study. White triangles indicate specific questions within each section. Knowledge elements are shown as text within ovals. Arrows indicate the step-by-step flow of knowledge elements. Ovals with bold, colored outlines indicate different knowledge elements about the dashed arrow and circled bar: yellow is for an electron being added back to step B, orange is for IV-CoA going back to be part of the previous chemical reaction (i.e., recycling or added back as a reactant), yellow-orange is for the linkage of the electron idea and the idea of IV-CoA going back, and dark blue is for negative feedback inhibition. The fill color of each oval represents different knowledge elements for student focus on metabolic compounds: gray fill is for focus on the regulatory compound or end products, blue is for focus on the distal or

proximal reactants, and white indicates that the participant made no reference to a metabolic compound.

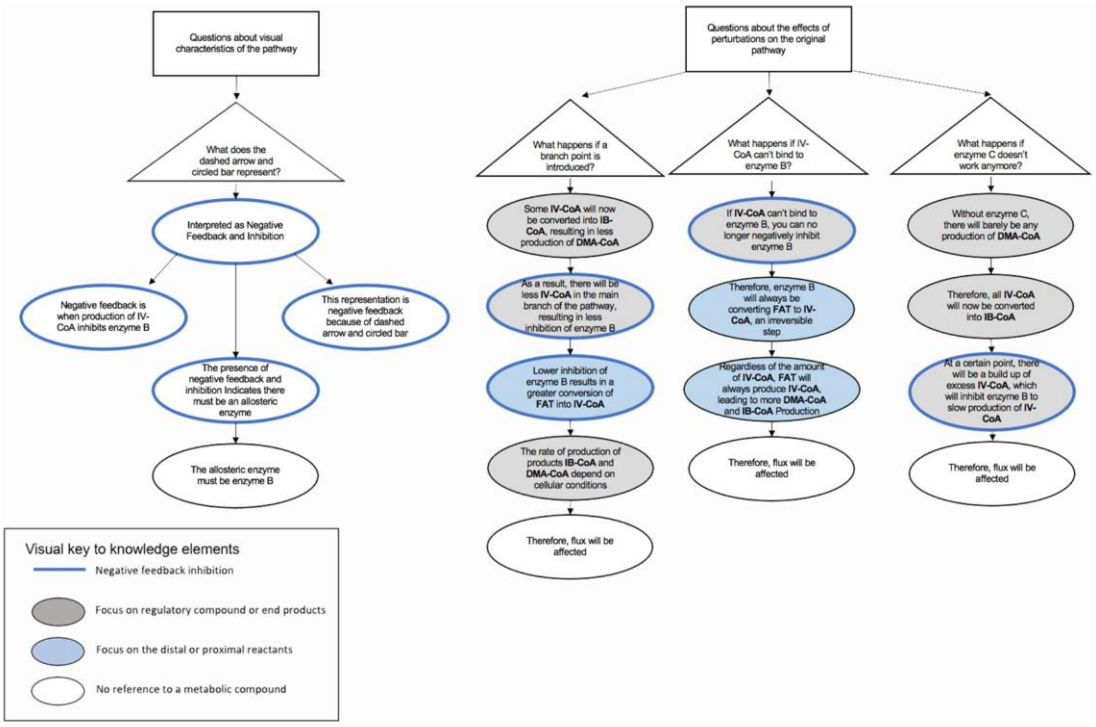


Figure 2.5: **Resource graph for Charlie.** White boxes indicate sections of the metabolism problem set that are the focus of this study. White triangles indicate specific questions within each section. Knowledge elements are shown as text within ovals. Arrows indicate the step-by-step flow of knowledge elements. Ovals with bold, colored outlines indicate different knowledge elements about the dashed arrow and circled bar: dark blue is for negative feedback inhibition. The fill color of each oval represents different knowledge elements for student focus on metabolic compounds: gray fill is for focus on the regulatory compound or end products, blue is for focus on the distal or proximal reactants, and white indicates that the participant made no reference to a metabolic compound.

Table 2.1: Participant performance on objective items of a metabolism problem set[a]

A. Participant responses to a multiple true-false prompt corresponding to Figure 1A: "What do the dashed arrow and circled bar represent?"[b]

Multiple true-false prompt	Biology participants (N = 22)	Biochemistry participants (N = 22)
Negative feedback or inhibition	**15**	**22**
Positive feedback or activation	4	0
Removal of IV-CoA	2	3
IV-CoA being added as a reactant	16	3
IV-CoA being recycled	18	6
Reverse reaction	3	3
Alternate pathway	11	3

B. Participant responses to a multiple-choice prompt corresponding to Figure 1A: "If enzyme C were inhibited, how might this eventually impact the activity of enzyme B?"[c]

Multiple-choice prompt	Biology participants (N = 22)	Biochemistry participants (N = 22)
The activity of enzyme B would not be affected	11	4
The activity of enzyme B would increase	2	6
The activity of enzyme B would decrease	**9**	**12**

[a]During the interview, participants selected responses to questions about the metabolic pathway represented in Figure 1.
[b]The bold prompt is true, while the other prompts are false. We indicate the number of biology and biochemistry participants who selected each statement as true.
[c] The intended answer is shown in bold. We indicate the number of biology and biochemistry participants who selected each answer.

Table 2.2: **Knowledge elements activated by the dashed arrow and circled bar with the number of biology and biochemistry participants who used each knowledge element**

Knowledge element, i.e., dashed arrow and circled bar represent …		Biology participants	Biochemistry participants
Negative feedback inhibition		7	17
Intermediate going back to be part of the previous chemical reaction	Intermediate being "recycled"	19	6
	Intermediate being "reverted"	8	5
	A reversible reaction	2	2
Negative feedback because of the negative sign		10	11
Intermediate binding to the enzyme		9	4
An electron donated to step B		0	1

Table 2.3: **Knowledge elements activated by the metabolic compounds with the number of biology and biochemistry participants who focused their attention on particular metabolic compounds during problem solving.**

Knowledge element, i.e., students focused their attention on the…	Biology participants	Biochemistry participants
Distal reactant: thiamine phosphate	3	7
Proximal reactant: fatty acyl thioester	5	9
Regulatory compound: IV-CoA	15	22
End product: DMA-CoA	16	21
End product: IB-CoA	17	22

Chapter 3

Introduction – Part 2

Cilia are membrane-bound, microtubule-based organelles on most mammalian cells, specializing in motility and signaling transduction. In lower-order eukaryotes, cilia are primarily used for locomotion within their aqueous environment, where they are more commonly known as flagella. They are also used for feeding or sensation of environmental cues, such as in *C. elegans* and other invertebrate worms, whose cilia are attached to the end of dendritic processes where they serve a sensory role for the organism (Bae & Barr, 2008). In humans, almost every cell type in the body is ciliated; some have a single, central primary cilium, while others are multi-ciliated cells. These different cilia phenotypes have structural differences, sometimes between different cell types, resulting in cilia that have a diverse range of specialized functions. For example, the male gamete has a single motile cilium that serves the function of locomotion (Afzelius, 1959). Cells that line our respiratory system are multi-ciliated to direct fluid and debris movement through and out of our respiratory system (Peabody et al., 2018) or cerebrospinal fluid in the nervous system (Eichele et al., 2020). During early embryonic development, mono-ciliated cells at the node direct fluid flow and early developmental signaling molecules (Blum et al., 2007; Nonaka et al., 2005; Wanner et al., 1996). In most vertebrates, most cells can possess primary immotile cilia. Primary cilia are the single, central cilium that can be the only cilia on a cell or the central cilia amongst many cilia on a multi-ciliated cell not dedicated to the movement of fluid (Sorokin, 1968). The

primary cilia typically serve as a critical regulator of signal transduction during development and homeostasis (Goetz & Anderson, 2010).

Conversely, multi-ciliated cells are cells decorated with dozens of motile cilia that beat in a coordinated fashion to direct fluid flow. These cells can be found in places like the spinal cord, where they drive fluid flow important for the circulation of cerebrospinal fluid and neuronal migration (Sawamoto et al., 2006), or in the airways, where they are important for mucus clearance to move debris out (Wanner et al., 1996). The function of cilia can be specific to cell or tissue type and has important functions in development and tissue homeostasis, primarily through its function as a signaling hub. Cilia also serve a role in the cell cycle, misregulation of which is linked to many cancers. Consequently, many developmental syndromes have been linked to defects in the formation and function of cilia, collectively called ciliopathies.

The Building of Cilia

Cilia are present on actively proliferating cells throughout the body, and ciliary assembly and disassembly are coordinated with the cell cycle. In most animal cells, the primary cilium is present in the G0 and G1 phases, when the centriole, a core aspect of the genesis of the cilium, is not required for mitotic functions, and is disassembled in the S and G2 phases when the centriole is required for mitotic entry and spindle function. Therefore, disassembly of the cilium is required for the centriole to detach from the plasma membrane, duplicate, and segregate through the corresponding phases of cell division (Goto et al., 2017). The cellular systems that control cilia must coordinate with cell cycle mechanisms to facilitate timely responses to cell cues.

Upon completion of cellular division, the basal body forms, usually from existing centrioles, migrates and becomes the base of a new cilium (Sorokin, 1962; Sorokin, 1968). One mechanism of ciliogenesis, observed in fibroblasts and smooth muscle cells, involves ciliary vesicles docking with the centriole inside the cell near the nucleus. Once combined, the ciliary vesicle/centriole complex slowly migrates to the cell membrane and merges with the cell membrane to generate a ciliary pocket and eventually, the cilium itself (Sorokin, 1962). Another mechanism observed in epithelial cells is the centriole independently migrating and fusing directly with the cell membrane, and ciliogenesis will start from the centriole directly (Molla-Herman et al., 2010; Sorokin, 1968).

The microtubule-based axoneme structure of the cilia is built from the primary centriole and consists of a ring of 9 microtubule doublets. The axoneme supports and shapes the cilium and ciliary membrane and acts as a scaffold for transporting cargo between the cytoplasm and the cilia. The tubulin of the outer doublets has the capacity for posttranslational modifications, which seem to alter ciliary assembly and potential motility (Thazhath et al., 2004; Pathak et al., 2007; Wloga et al., 2009; Kubo et al., 2010; Suryavanshi et al., 2010; Ikegami et al., 2010). These outer doublet structures also contain structural proteins, such as tektins and protofilament ribbons (Steffen & Linck, 1988; Linck & Norrander, 2003), that contribute to the high structural integrity of these axonemal microtubules. These axonemal microtubules are also decorated with dynein arms and radial spokes that allow for beat-like movement (Gudis & Cohen, 2010). Some axonemes built will have an additional central doublet of microtubules, denoted with 9+2, rather than a 9+0 description for axonemes lacking the central doublet. The differences in how these doublets are constructed help define different types of cilia. For example, a

9+2 axoneme composition describes motile cilia with the additional central pairs of microtubules responsible for the motility. A 9+0 axoneme composition describes non-motile primary cilia. The basal body from which this axoneme builds is not necessarily committed to form one type of conformation (Wheeler et al., 2015). So while the 9+2 and 9+0 cilia share fundamentally similar structures, it is likely dependent on cellular signals regarding which structure to build. The cilium remains dynamic once it has reached a desired length. There is constant turnover at the tip of the cilia where new microtubule molecules are continually incorporated to balance out the disassembly of microtubules. Motile and non-motile cilia likely share assembly and disassembly machinery.

Once built, there are a variety of posttranslational modifications that the axonemal microtubules can undergo. Higher acetylation levels during cilium assembly can help distinguish growing cilia from mature cilia undergoing steady-state turnover. Acetylation, catalyzed by αTAT1, is a marker for stability, as deacetylation of microtubules contributes to ciliary disassembly (Ran et al., 2015; Hernault & Rosenbaum, 1985; Portran et al., 2017). Microtubule glycylation and glutamylation promote microtubule polymerization, which appears to contribute to ciliary function and structure (Wloga et al., 2017). Glycylation positively regulates ciliary growth by marking and stabilizing long cilia (Gadadhar et al., 2017; Kubo et al., 2016). Glutamylation may negatively regulate cilia growth because the loss of glutamylation results in abnormally long cilia (Kubo et al., 2016). Glutamylation has also been thought to be critical for ciliary protein localization, kinesis motor speed, and sensory cilia function (O'Hagan et al., 2017). The question remains whether these posttranslational modifications independently or synergistically contribute to the stability of the microtubule axoneme, motility, and cargo transport.

The removal of cilia before division can be achieved by two main mechanisms - cleavage of the doublet microtubules that support the cilium away from the centriole (Parker et al., 2010) or the cilia can be reabsorbed through active disassembly from the ciliary tip (Pan et al., 2004; Pan & Snell, 2005). Mitotic kinase Aurora A (AurA) is a key regulator of ciliary disassembly, and several pathways converge on AurA activation (Pugacheva et al., 2007). Upstream regulators of cilia disassembly include polo-like kinase 1 (Plk1), disheveled 2 (Dvl2), Pitchfork, human enhancer of filamentation 1 (HEF1), and Nek2 (Korobeynikov et al., 2017; Kinzel et al., 2010; Pugacheva et al., 2007). Aurora A may exert some of its effect on ciliary disassembly by positively regulating the tubulin deacetylase histone deacetylase 6 (HDAC6) - decreasing tubulin acetylation, which reduces microtubule stability, leading to the disassembly of the axoneme (Pugacheva et al., 2007; Hubbert et al., 2002).

Intraflagellar Transport

The extension or building of the axoneme into cilia relies primarily on two significant complexes - Intraflagellar Transport (IFT) and the BBSome (Jenkins & Hernandez-Hernandez, 2015; Pedersen & Rosenbaum, 2008). Many components required for assembling and organizing the centriole and the genesis of the cilia are also involved in recruiting components responsible for IFT and the BBSome. IFT is the bi-directional movement of multiprotein complexes and components within the cilia along the axoneme (Kozminski et al., 1993). This process involves two major complexes, labeled IFT-A and IFT-B. The IFT-A is driven by dynein and is primarily responsible for retrograde transport. IFT-B is kinesin-II driven and is primarily responsible for anterograde transport (Cole et al., 1998). These complexes contain multiple, unique IFT proteins (Taschner et

al., 2012). IFT is responsible for constructing the axoneme and maintaining these axonemal structures throughout ciliogenesis, as well as the movement of cargo up and down these mature structures for cellular functions (Taschner & Lorentzen, 2016). This process is highly conserved among ciliated eukaryotes (Avidor-Reis et al., 2004; Li et al., 2004).

The IFT motor responsible for anterograde movement consists of heterotrimeric and homodimeric Kinesin-2 motor units and an associated accessory unit, kinesin-associated protein (KAP) (Cole et al., 1993). The heterotrimeric Kinesin-2 units are necessary to assemble and maintain cilia and flagella in many ciliated organisms. The heterotrimeric and homodimeric kinesin motor units may act complementarily when expressed in the same cilium. Heterotrimeric kinesin-2 facilitates ciliogenesis and IFT entry across the cilia's transition zone, and then homodimeric kinesin-2 picks up the cargo for faster transport to the ciliary tip (Mirvis et al., 2018). In *Chlamydomonas*, when one of the homologs for a kinesin-2 subunit (fla10, flagellar-assembly impaired 10) is knocked out, they cannot assemble flagella (Kozminski et al., 1995). The anterograde motor complex diffuses to the ciliary base following dissociation from the axoneme at the tip of the cilia (Wloga et al., 2017; Ishikawa & Marshall, 2017).

The IFT motor responsible for retrograde movement is dynein-2, a multiprotein complex whose primary function is to return IFT trains and associated cargo from the ciliary tip to the cell. Knockdown or knockout mutations in *Chlamydomonas*, *C. elegans*, and mice result in short cilia and cilia with inflamed, swollen tips that are filled with accumulated IFT components, a symptom consistent with the assumed role for motors responsible for retrograde IFT (Porter et al., 1999; Signor et al., 1999; Hou et al., 2004;

May et al., 2005). Without the primary method associated with bringing cargo back to the cell from the tip of the cilium, cargo and IFT trains responsible for anterograde transport will accumulate at the tip.

The complex that is moved via these motors comprises two distinct complexes, termed complex A and complex B. Complex A contains six known proteins - IFT43, IFT121, IFT122, IFT139, IFT140, and IFT144. Knockout mutations in IFT121, IFT139, or IFT144 usually result in normal or shorter cilia and typically have defects in retrograde trafficking or ciliary entry (Fu et al., 2016; Hirano et al., 2017; Veleri et al., 2014). Complex B contains 14 known proteins - IFT20, IFT22, IFT25, IFT27, IFT46, IFT52, IFT54, IFT57, IFT70, IFT74, IFT80, IFT81, IFT88, and IFT172. IFT81 binds to tubulin dimers for transport into the cilia (Bhogaraju et al., 2013). IFT25 is required for the trafficking of HH pathway components for proper signal transduction of the pathway (Keady et al., 2012). IFT172, IFT88, IFT20, and IFT54 result in the loss of cilia, indicating they are required for either the transport of structural components of cilia, docking with the base of the cilium, or interacting with the motor proteins used for movement (Berbari et al., 2011; Huangfu et al., 2003; Katoh et al., 2017). The majority of these IFT proteins are conserved among ciliated organisms.

Less is known about the BBSome complex. The BBSome complex consists of 7 proteins and an accessory protein, Arl6, which recruits the BBSome to the ciliary membrane (Maurao et al., 2014). The BBSome is required for early ciliogenesis during the centrosome-basal body transition, where it recruits Rab8a to the mother centriole to initiate ciliogenesis (Nachury et al., 2007; Westlake et al., 2011). The BBSome has been shown to have a role in regulating the movement of signaling components through the

transition zone of the cilia (Goetz et al., 2017). It has also been shown that BBSomes move on IFT trains through the cilia and are usually categorized as an IFT complex due to this association (Lechtreck et al., 2009; Liew et al., 2014; Williams et al., 2014). However, *bbs* mutations have minimal effects on cilia size (Nachury, 2018). In *Bbs4-/-* mice, structural defects do occur, leading to the degeneration of the ciliary membrane (Datta et al., 2015; Hsu et al., 2017). As a result, it is assumed that the BBSome serves a role in retrograde transport of cargo, likely linking cargo to IFT trains from the ciliary tip back to the cell body (Liew et al., 2014; Liu & Lechtreck, 2018).

Protein translation does not occur within the cilium. Thus, the protein composition of the cilium is determined by controlling access of proteins from the cytoplasm and transporting them through the cilia via IFT. For example, once molecules important for cilia building, such as tubulin, are synthesized, they must first be moved to the cilium along the cell's microtubule structures, moved through the transition zone into the cilia, and then moved to the site of assembly at the tip of the cilia via IFT. Entry of proteins into the cilium may occur passively through diffusion (Luo et al., 2017) or be strictly regulated entry via IFT (Ye et al., 2013; Wingfield et al., 2017; Lechtreck et al., 2017). The complexity of this process presents numerous potential points for ciliogenesis control. Parameters such as IFT train size and speed, frequency of cargo entry, and specificity of cargo transport by IFT are all potential parameters for cilia length regulation. Additional potential points of assembly/disassembly regulation include the regulation of the synthesis of precursors - synthesizing precursors when the cilia need them for assembly, repressing the synthesis of precursors when the cilia need disassembly, or regulating the speed or action of turnover within the cilia. With many

potential points of regulation, this raises the question of whether cells rely on a specific one or a combination of many of these regulatory valves to regulate the assembly and disassembly of cilia.

Growing cilia require more molecules moving into the cilia than leaving compared to steady state, implying a regulation valve on transportation for when a cilia needs to grow versus being at steady state (Marshall et al., 2005). In flagellar regeneration in *Chlamydomonas*, IFT train size increases disproportionately to flagella length, i.e., larger IFT trains in shorter, growing cilia than in longer cilia. In contrast, the frequency of these trains remains the same, independent of the flagella's length (Engel et al., 2009), indicating that the growing flagella are receiving more components per train to build with. Current models for length control are based on the observation that ciliary assembly at the tip not only occurs when the cilium is first assembled but continues after the cilium has reached final length (Marshall & Rosenbaum, 2001; Stephens, 1997; Song & Dentler, 2001; Stephens, 1995). The continuous assembly and disassembly of axonemal microtubules at the tip of cilia results in continuous turnover called steady state. Tubulin turnover in axonemal microtubules relies on a pool of cytoplasmic tubulin concentrated at the ciliary base and tip. Soluble tubulin is transported into the cilia as IFT cargo (Hao et al., 2011). IFT is required to maintain steady state, as when IFT is turned off in full-length cilia, the cilia immediately begin to shorten and reabsorb (Kozminski et al., 1995). In *Chlamydomonas* flagella, when IFT is inhibited, flagella cannot grow without restoring IFT function (Marshall et al., 2005). Therefore, IFT must carry axonemal components into the cilium/flagella and likely transport this cargo to the assembly site at the cilium's tip. The reabsorption during ciliary disassembly occurs

constantly throughout the process, implying that the disassembly rate at the tip is independent of ciliary length (Marshall & Rosenbaum, 2001). Assembly and disassembly are both occurring during steady state. It can be assumed that the rates of these two must be equal during steady state.

Cilia serve an important environmental sensory and response role, as many membrane proteins related to signaling pathways localize to the ciliary membrane. Many of these membrane proteins are moved into and throughout the cilium via IFT, which matches information of membrane proteins such as OSM-9, OCR-2, and PKD2 displaying IFT-like movement (Qin et al., 2005; Huang et al., 2007). These membrane proteins are also an example of the differential protein content of cilia versus the general cytoplasm, indicating a selection bias of what proteins enter at the ciliary base. Analysis of this diffusion barrier implicated a role for septins - membrane-associated proteins known to form barriers to diffusion during cytokinesis - in this transition zone barrier (Hu et al., 2010). In addition to a barrier, it was hypothesized that there are mechanisms to allow a correct subset of proteins to selectively bypass the barrier and enter the cilium, similar to the nuclear pore (Rosenbaum & Witman, 2002). In the nuclear pore, the selectivity of molecules that enter is determined by importins and associated machinery. Similar particles may perform selective cargo recognition or selection to transfer cargo through the diffusion barrier in the transition zone of the cilium.

Ciliary Role in Signaling

Studies suggest between 1200 and 1800 genes are required for primary cilium structure and function (Gherman et al., 2006; Wheway et al., 2015). Forward genetic screens were used to identify genes involved in early patterning and morphogenesis in

mice, and follow-up work showed that cilia machinery components are required for proper mouse development (Garcia-Garcia et al., 2005; Huangfu & Anderson, 2005). As noted earlier, cilia are important for a wide variety of physiological and developmental processes in mammals, as many human diseases have, as their root cause, defects in cilia. These diseases are collectively called ciliopathies (Reiter & Leroux, 2017). Many of the foundational developmental pathways have been linked to the cilia. In the following section, we recap many of the signaling pathways relevant during development and how they are linked to the cilium. Because all of these pathways are linked to the cilium and the localization of specific proteins, this highlights the importance of proper cilia construction and cargo transport in and out.

Sonic Hedgehog Signaling

The Sonic Hedgehog signaling pathway requires primary cilia for signal transduction (Caspary et al., 2007). Many of the core components of the Hedgehog pathway localize and are processed in the cilia, and the loss of cilia results in an inability to stimulate the pathway (Corbit et al., 2005; Goetz & Anderson, 2010; Rohatgi et al., 2007). In the absence of the Hedgehog ligand (Shh), the transmembrane receptor, Ptch, is localized near the base of the cilium. Unprocessed Gli transcription factors are sequestered and suppressed at the tip of the cilium (Haycraft et al., 2005; Zeng et al., 2010). Unstimulated Ptch represses and prevents Smoothened (Smo) from entering the cilium (Ocbina & Anderson, 2008). When the ligand Shh binds to the Ptch receptor, the inhibition of Smo is relieved, and Smo is transported into the cilium while Ptch leaves the cilium. (Corbit et al., 2005; Haycraft et al., 2005; Kim et al., 2009; Rohatgi et al., 2007). Smoothened entering the cilium represses Suppressor of fused (Sufu). The Gli

transcription factors are then processed and moved from the cilium into the nucleus, activating specific target genes (Chen et al., 2009; Haycraft et al., 2005). Loss of IFT leads to decreased Ptch expression and accumulation of Gli2 and Gli3 at the ciliary tip (Qin et al., 2011). Loss of IFT80 prevents Smoothened localization to the cilium, inhibiting canonical hedgehog signaling (Yuan et al., 2016). Defects in cilia or IFT result in loss of function hedgehog phenotypes in the neural tube (linked to Gli activators) and gain of function hedgehog phenotypes in limbs (linked to gli3 repressors) (Haycraft, 2005; Huangfu & Anderson, 2005). The hedgehog pathway may also regulate primary ciliogenesis and maintenance in a feedback loop. Several genes related to the hedgehog pathway are found to be knocked down in and as a result of processes leading to ciliary disassembly (Jacob et al., 2011).

Wnt Signaling Pathways

Wnt signaling pathways are necessary for embryonic development and adult tissue homeostasis. The canonical Wnt pathways involve the translocation of beta-catenin to the nucleus and activation of specific transcription factors that control cell proliferation. Primary cilia play important functions in the attenuation of canonical Wnt signaling pathways. Defects in cilia lead to an overactivation of the Wnt signaling pathway (Lin et al., 2003; Cano et al., 2004; Abdelhamad et al., 2013; Wheway et al., 2013). The Wnt ligand binds to the membrane receptor Frizzled and coreceptor LRP. The binding to Frizzled recruits the "destruction complex" to the cell membrane, which makes them lose the ability to degrade beta-catenin. This ultimately allows for the stabilization of beta-catenin by inhibiting its ubiquitination, thus allowing it to successfully be transported into the nucleus to activate target genes (Clevers & Nusse, 2012; Li et al.,

2012; Liu et al., 2022). *Ahi1* (an ortholog to Jouberin in humans) mutant mice show a loss of basal canonical Wnt signaling activity. Jouberin is a ciliary protein, linked to Joubert Syndrome, that remains sequestered in the cilium, away from the nucleus, keeping β-catenin away from the nucleus, which restricts downstream Wnt target genes (Lancaster et al., 2011).

The non-canonical Wnt pathways regulate cell polarity and migration. Normal ciliogenesis is essential for the planar cell polarity (PCP) non-canonical Wnt signaling pathway (Gomez-Orte et al., 2013). The basal body's positioning is both important for ciliogenesis and is part of the establishment of PCP through activation of disheveled, a stimulant of cytoskeleton reorganization (Jones et al., 2008; Park et al., 2008; Wallingford et al., 2000). The PCP pathway is required for the polarization of cell types in developing embryos and thus is important for many developmental features (Devenport & Fuchs, 2008; Montcouquiol et al., 2003). Defects in proteins regulating the initiation of ciliogenesis and basal body migration lead to complex PCP defects.

Notch Signaling

The Notch signaling pathway is also involved in many processes that determine cell fate during the development and maintenance of mature tissues. The mammalian canonical Notch pathway comprises four Notch receptors, Notch 1-4, and five transmembrane ligands -- Jagged 1 and 2 and Delta-like 1,3, and 4. Activation of the notch signaling pathway typically occurs following a notch receptor and ligand interaction that initiates a series of events resulting in the cleavage of the notch receptor. This results in a 'processed' Notch intracellular domain that is translocated to the nucleus for the activation of target genes (Oldershaw & Hardingham, 2010)

Notch pathway activation in the developing neural tube has been shown to lead to cilia elongation, resulting in more Smoothened expression and a stronger Hedgehog pathway response (Stasiulewicz et al., 2015). A key component of the Notch pathway, Presenilin, localizes to the basal body. In certain cell types, the Notch receptor also localizes to the basal body (Ezratty et al., 2011; Ezratty et al., 2016). The notch3 receptor and Notch processing enzymes colocalize with cilia. The primary cilium regulates spatial localization of Notch signaling intermediates during differentiation (Ezratty et al., 2016). Loss of cilia leads to diminished Notch activation (Grisanti et al., 2016).

Hippo

The Hippo signaling pathway is composed of mammalian STE20-like kinase 1/2 (MST1/2), Salvador homologue 1 (SAV1), MOBKL1A/B (MOB1A/B), large tumor suppressor kinase 1/2 (LATS1/2), Yes-associated protein 1 (YAP), WW-domain-containing transcription regulator 1 (TAZ), and the transcriptional enhanced associated domain (TEAD) family (Cheng et al., 2020). The Hippo pathway plays an important role in the development and homeostasis of organs. Various upstream signals, such as cell polarity and stress signals, modulate the Hippo pathway (Harvey et al., 2013; Yu et al., 2012; Misra & Irvine, 2018). MST1 and 2 localize to the basal body and are required for ciliogenesis. Loss of MST1 and 2 or the activator of MST1 and 2, *SAV1*, impairs ciliogenesis (Yu & Guan, 2013). MST1 and 2 are also required for Aurora Kinase A (AurA) phosphorylation to prevent it from complexing with HDAC6 to disassemble cilia. MST1/2 and SAV1 are also associated with the NPHP-transition zone complex, which regulates ciliary loading of cargos onto IFT transport machinery at the transition zone (Kim et al., 2014).

mTOR

Mammalian target of rapamycin (mTOR) is involved in many signaling pathways that regulate cell growth, metabolism, and autophagy (Stanfel et al., 2009). The mTOR signaling pathway involves two distinct complexes, the mammalian target of rapamycin complex 1 and 2 (mTORC1 and 2). mTORC1 is involved primarily in cell growth and metabolism, while mTORC2 is involved in cell proliferation and cell survival (Chantranupong et al., 2015; Unni & Arteaga, 2019). The bending of cilia caused by fluid flow results in the downregulation of the mTOR pathway to control cell growth, possibly through liver kinase b1, Lkb1, a tumor suppressor protein localized to the primary cilium (Boehlke et al., 2010). Folliculin (FLCN) is a ciliary protein that plays a role in this process. FLCN recruits Lkb1 to the primary cilia to activate AmpK, which causes downregulation of mTORC1 (Zhong et al., 2016).

GPCR Signaling

G-protein coupled receptors, or GPCRs, are a large family of cell membrane receptor proteins important for initiating signal transduction cascades in cells. GPCRs are essential for primary cilia function (Marley & von Zastrow, 2012; Wheway et al., 2015). Ciliary GPCRs such as EP4 and HTR6 (Brodsky et al., 2017) stimulate cAMP production, promoting cilia elongation (Besschetnova et al., 2010). Reduction of G-αi GPCRs, such as DRD2 and MCHR1, promotes cilia shortening (Alhassen et al., 2022). Overexpression of GPCRs such as SSTR3 or 5HT6 causes abnormal ciliogenesis with longer and branched cilia. This overexpression is associated with an overexpression of IFT proteins such as Kif3a, cytoplasmic dynein D1, IFT88, and GPCR trafficking protein, TULP3. An overexpression of 5HT6, not SSTR3, prevents ciliary localization of

ACIII (Guadiana et al., 2013), a GPCR which is typically localized to cilia of neurons and is part of signal transduction cascades in the ciliary membrane. Other GPCRs have been linked to promoting shorter cilia, such as MCHR1 (Hamamoto et al., 2016). The loss of cilia can lead to impaired GPCR signaling, and the loss of GPCRs can lead to loss of cilia or cilia function.

Cilia Length Regulation

Many molecular mechanisms are used for length regulation. For example, calcium levels affect ciliary length depending on concentration, dynamics, and cellular location (Avasthi & Marshall, 2012). Increased levels of calcium upstream of AurA are associated with reduced length (Hu et al., 2021; Pugacheva et al., 2007). Calcium-dependent protein kinase (PKC) can increase ciliary length via MAPK (Abdul-Majeed et al., 2012). Levels of soluble tubulin available can also impact ciliary length. Increased levels of soluble tubulin lead to increased ciliary length, and conversely, decreased levels of soluble tubulin lead to short ciliary length. Microtubule stabilization reduces the pool of soluble tubulin, resulting in short or absent cilia (Sharma et al., 2011). Histone deacetylase, HDAC6, acts downstream of AurA to deacetylate ciliary microtubules, promoting disassembly of the axoneme and a reduction in ciliary length.

Cell Cycle-Related Kinase

Genetic screens in *Chlamydomonas* have identified mutations that alter the length of flagella and cilia. These mutations are grouped into 'short flagella' (*shf)* and ‘long flagella’ (*lf)* mutants. Two of these ‘long flagella’ mutants are kinases with orthologs in mammals. One is a mitogen-activated protein (MAP) kinase family member - *lf4.* The

mammalian orthologue of *lf4* is a male germ-cell-associated kinase (MAK). The other is a cyclin-dependent kinase (CDK) family member called *lf2.* Null mutations in *lf2 (lf2-6)* cause flagella of various lengths (Tam et al., 2007). *Lf2* mutants also have defects in the transport of molecules to and within the cilia (Sequrira et al., 2017). The mammalian orthologue of *lf2* is called cell cycle-related kinase (CCRK), which has a partner protein called broad-minded or *Bromi* (Omori et al., 2010; Ko et al., 2010). Broad-minded contains a GTPase domain similar to other Rab proteins but does not interact with other Rabs known to be involved in ciliogenesis (Ko et al., 2010). CCRK shows characteristics of a traditional cyclin-dependent kinase, but its role in the cell cycle or ciliogenesis is poorly understood (Qiu et al., 2008; Liu et al., 2004; Wohlbold et al., 2006). However, when knocked out in mice, there are noticeable developmental defects (Snouffer et al., 2017). Like in *Chlamydomonas*, CCRK knockout fibroblasts also have a broad distribution of cilia lengths compared to wild-type fibroblasts (Snouffer et al., 2017). CCRK mutants also display abnormal phenotypes when it comes to ciliary cargo import. CCRK mutants display an accumulation of cargo at the tip of the cilium (Yang et al., 2013). We also see a delay in the import of specific ciliary cargo into the cilium (Snouffer et al., 2017). These results predict that CCRK may have a role in IFT function, ciliary cargo import, and/or ciliary cargo transport. In the following chapters of this thesis, we further investigate the role of cell cycle-related kinase in mammalian cells.

Chapter 4

Understanding the Role of Ccrk in Cilia Maintenance

A previous member of the lab performed a SILAC proteomics experiment on Ccrk wild-type and Ccrk knockout IMCD cells to identify a list of potential downstream targets of Ccrk by identifying proteins that were differentially phosphorylated in the two cell lines. Of the differentially phosphorylated proteins, two targets in particular were identified as proteins of interest due to their links to ciliogenesis: Map4 and MyosinIIa.

Map4, or microtubule-associated protein 4, is a member of the MAP family. Proteins in the Map family regulate the stability, dynamics, and trafficking function of microtubules throughout the cell. Maps can regulate microtubule motors by directly competing for binding on the microtubules, thus promoting or inhibiting motor attachment (Dixit et al., 2008; Vershinin et al., 2008; Tymanskyj et al., 2018; Hooikaas et al., 2019; Nabti et al., 2022). Maps are highly regulated, usually via isoform expression and/or phosphorylation (Wang & Mandelkow, 2016; Stern et al., 2017). Map4 has both actin and tubulin binding domains, and when bound to actin, it reshapes and elongates actin filaments (Matsushima et al., 2012). Map4 localizes along the ciliary axoneme. siRNA silencing of Map4 results in cilia elongation and recruitment of SEPT2, a ciliogenesis promoter. Map4 interacts with SEPT2 at the transition zone to control microtubule binding accessibility (Ghoussaub et al., 2013). Map4 stabilizes microtubules and is negatively regulated by SEPT2 binding (Kremer et al., 2005). In wild-type Ccrk

cells, there are low to no phosphorylation levels on Map4. This lack of phosphorylation allows Map4 to function as an actin/tubulin bridge, facilitating cargo movement to enter cilia unimpeded. With cells lacking functional Ccrk, there is an increase in phosphorylation of Map4 at Serine-1493 and Serine-787. Map4 is phosphorylated by CDK1 at Serine-787 via a CAK-mediated interaction with Ccrk (Ookata et al., 1997; Wholbold et al., 2006). This phosphorylation inhibits its association with microtubules, meaning it can no longer serve as the actin/tubulin bridge, impacting cargo transport into the cilium (Ebneth et al., 1999; Kitazawa et al., 2000). The interaction between Cdk1 and Ccrk is not well understood, so there remains the possibility that Ccrk could be phosphorylating Map4 at a different residue that either leads to dephosphorylation or a lack of phosphorylation at Serine-761. A noted possibility is a phosphorylation at Serine-901, as this area has a CDK recognition motif that might bind Ccrk.

MyosinIIa (MyoIIa) is a myosin involved in the bundling of actin filaments (Raab et al., 2012). Through its actin-bundling activity, MyoIIa inhibits ciliogenesis, leading to restricted actin dynamics and cargo transport (Dulyaninova et al., 2005; Rao et al., 2014). Ck2, a direct target of Ccrk, phosphorylates MyoIIa at Serine-1943, an inhibitory phosphorylation that dissociates MyoIIa from actin filaments and relieves bundling, resulting in easier cargo transport (Dulyaninova et al., 2005). In cells lacking functional Ccrk, we see less phosphorylation, but not an elimination of phosphorylation, of MyoIIa. This decrease in phosphorylation should lead to MyoIIa associating with actin filaments, leading to more actin bundling. This actin bundling should reduce cargo transport, including proteins and molecules responsible for building and maintaining cilia.

With two potential downstream targets of Ccrk identified, we sought to confirm that these proteins were indeed differentially phosphorylated when functional Ccrk was present in the cell versus when Ccrk was knocked out, indicating whether or not Ccrk has a role in the phosphorylation of these proteins. A former lab member had generated truncated versions of Map4 and MyoIIa that contained the phosphorylated and functional domains for easier detection on western blot gels. These truncated versions also included a V5-His tag incorporated to facilitate easier isolation. We transfected these truncated versions of Map4 and MyoIIa in two cell lines, one with wild-type Ccrk (IMCD:IFT88YFP) and one with Ccrk knocked out (IMCD:71.12.A2). These cell lines are IMCD-3 (inner medullary cell duct) cells transgenic for a YFP-tagged IFT88 protein. These transfected cells expressing the truncated forms of Map4 and MyoIIa were expanded, and protein extracts were harvested and isolated 2-3 days post-transfection. These protein extracts were analyzed via a standard western blot gel protocol to confirm the presence and correct size of the truncated proteins (Figure 4.1).

Once we confirmed that these cells were expressing the truncated, tagged forms of Map4 and MyoIIa, we looked to confirm that these proteins were differentially phosphorylated based on the presence of Ccrk. These protein extracts were then run on Phosphate-Tag gels—a specialized gel emphasizing the separation of proteins based on their phosphorylation state. Hyperphosphorylation of Map4 was present in knockout Ccrk cells (Figure 4.2), and lower levels of phosphorylation of MyoIIa were present in knockout Ccrk cells (Figure 4.3).

Once we confirmed that these proteins were differentially phosphorylated in cells with Ccrk and those without, our next goal was to establish whether the differential

phosphorylation states of Map4 and MyoIIa play a role in the ciliary phenotypes we saw when we knocked out Ccrk, answering whether Ccrk's effect on Map4 and MyoIIa is responsible for the ciliary defects that we are seeing. We explored the phosphorylation sites on Map4 and MyoIIa mediated by Ccrk. We designed specific point mutations via homology-directed repair via CRISPR/Cas9 on Map4 and MyoIIa to change the serine that typically gets phosphorylated. This serine could be changed into a glutamic acid or aspartic acid, mimicking the negative charge received from phosphorylation, meaning that the protein would act like it was phosphorylated (a phosphomimetic mutation), regardless of whether Ccrk was present or not. The serine could also be changed into an alanine. This amino acid cannot be phosphorylated, meaning that the protein would always act like it was unphosphorylated, whether Ccrk was present or not.

These mutations aim to generate cell lines with Map4 that are constantly phosphorylated with and without functional Ccrk and a cell line with Map4 that cannot be phosphorylated with and without functional Ccrk. The process would be repeated to generate cell lines for MyoIIa. After generating these cell lines, we would then assay the ciliary phenotypes of these eight cell lines. By comparing the phenotypes we see to the phenotypes of our wild-type and knockout Ccrk cell lines with normal Map4 and MyoIIa, we can determine whether Map4 or MyoIIa phosphorylation state mediated by Ccrk is responsible for the ciliary phenotypes we see between wild-type and Ccrk knockout cell lines.

When we mutate Map4 to a constitutively non-phosphorylated state in wild-type cell lines, we expect no significant difference in ciliary phenotype, as Map4 is usually unphosphorylated in Ccrk wild-type cells. In Ccrk knockout cell lines, we typically see

an increase in the phosphorylation of Map4. Therefore, we expect the ciliary phenotype to be rescued when we mutate Map4 to a constitutively non-phosphorylated state in Ccrk null cell lines. Map4 in a constitutive phosphomimetic state in Ccrk knockout cell lines is expected to show a more severe ciliary phenotype than typically seen in Ccrk knockout cell lines. Map4 in Ccrk knockout cell lines is observed to increase phosphorylation, but unphosphorylated Map4 is still present. If all Map4 present is phosphorylated and contributes to cargo transport within cilia, we expect a more severe ciliary phenotype. When phosphomimetic Map4 is generated in wild-type cell lines, we should see phenotypes similar to or more severe than those of cell lines with mutant Ccrk. Even if wild-type Ccrk is present, Map4 will always act as if it is phosphorylated, leading to mutant ciliary phenotypes. Constitutively non-phosphorylated MyoIIa should lead to a more severe ciliary phenotype than what we already see in Ccrk knockout mutant cell lines, as instead of only a portion of the protein being non-phosphorylated, all protein present should be. We also expect non-phosphorylated MyoIIa to induce mutant ciliary phenotypes in wild-type cells, as Ccrk can no longer phosphorylate MyoIIa. Phosphomimetic MyoIIa in Ccrk wild-type cells should result in ciliary phenotypes similar to what has already been seen and measured. Phosphomimetic MyoIIa in Ccrk mutant cells should rescue the ciliary phenotype observed.

Unfortunately, after many attempts, we could not generate stable cell lines with these mutations of interest. A combination of low transfection efficiency and low cell survivability post-selection resulted in a very low number of cells for analysis that potentially had our mutations of interest. Of the few cells that survived selection, we had no homozygous mutations that would have resulted in completely phosphomimetic or

unphosphorylatable proteins. As a result, while we could confirm that Map4 and MyosinIIa are differentially phosphorylated between cells with and without functional Ccrk, we could not confirm whether they were responsible for the ciliary phenotype we saw in Ccrk mutant cell lines.

The movement of cargo into and through the cilium is essential for many of the signaling and cellular processes that rely on the cilium. We have seen that downstream effectors of Ccrk, such as Map4 and MyoIIa, are proteins that may impact cargo transport to, into, and/or through the cilium. Ccrk mutants are known to have a ciliary length phenotype where lengths are longer on average, but have a wider range of lengths seen (Snouffer et al., 2017). This indicates that cells seem unable to regulate the length of the cilium. There is also evidence that in Ccrk knockout cells, there is a buildup of cargo at the tip of cilia (Figure 4.4; Snouffer et al., 2017). The goal of the experiments in this chapter is to investigate whether the rates of IFT and cargo import and transport differ in wild-type and mutant Ccrk cells to tease apart the effects Ccrk has.

Previous work in the lab indicated changes in the speed of anterograde IFT transport and a slight decrease in the speed of retrograde IFT transport in Ccrk mutant cells compared to wild-type Ccrk cells. There was also a detection of reduced frequency of anterograde IFT transport, indicating that Ccrk might be causing potential issues at the transition zone for IFT machinery to enter the cilia or attach to the microtubule axoneme for transport. We generated kymographs utilizing our wild-type and knockout Ccrk cell lines expressing YFP-fluorescent IFT88YFP. Our data observed a similar phenomenon, with Ccrk mutant cells displaying fewer instances of IFT movement than wild-type Ccrk

cells (Figure 4.5). This leads us to predict that Ccrk is impacting the import or attachment of the IFT machinery.

To further investigate Ccrk's impact on the ability of IFT machinery to enter the cilium, we utilized fluorescence recovery after photobleaching (FRAP). This protocol works by bleaching the current fluorescence levels in a defined region, such as the YFP-labeled IFT machinery in a cilium, using a short burst from a high-intensity laser. By measuring the average fluorescence in our defined ciliary region post-bleaching, an increase in the average fluorescence levels should indicate a new instance of our YFP-labeled IFT machinery entering the cilium. By analyzing the rate at which fluorescent IFT machinery enters the bleached cilia, we can gain insight into the rate at which IFT particles enter the cilia. The expectation would be that fluorescence in wild-type Ccrk cells will return to pre-bleach fluorescent levels much faster than in Ccrk knockout cells.

Using TIRF microscopy, in photobleached cilia from Ccrk wild-type cells, we see fluorescence recovery to approximately one-fourth to one-third of the initial fluorescence levels one to two minutes post-bleaching event (Figure 4.6). In Ccrk knockout cells, we see minimal fluorescence recovery one to two minutes post-bleaching event (Figure 4.7). We believe that IFT machinery still enters the cilium in Ccrk mutant cells, as these still build a cilium, and IFT machinery is necessary for this behavior. However, this supports the idea that IFT machinery entering cilia is negatively affected in cells lacking functional Ccrk. This data looks at cilia in short periods post-bleaching, due to limitations of the experimental design we utilized. Therefore, we cannot tell how long it takes for the Ccrk knockout cells to mimic similar levels of fluorescence recovery to their Ccrk wild-type counterparts. There is an expectation that, given a long enough time frame, the

fluorescence of cilia in both wild-type and Ccrk knockout cells would recover to the pre-bleach levels.

Cargo Import

With the conclusion that IFT machinery entry into the cilium is impacted by knocking out Ccrk, we set out to interrogate whether this disruption extended to proteins known to localize to the cilium. These are a variety of cargo proteins that are differentially moved and transported to and within the cilia. We set out to generate Ccrk wild-type and Ccrk knockout cell lines that co-expressed fluorescent IFT88YFP and fluorescent cargo of interest. With these cell lines, we can perform similar experiments to the ones we performed on fluorescent IFT88, where we bleach the fluorescent cargo within wild-type and knockout Ccrk cilia and assay phenotypes such as the rate of entry or speed of movement of the cargo within the cilium.

We selected four proteins of interest: tubulin, arl13b, sstr3, and smoothened. Tubulin is a protein in two forms: free tubulin and tubulin actively being organized into the microtubule cytoskeleton structure in the cell and the cilia. Arl13B is a GTPase associated with ciliary formation, function, and regulation of the composition. Somatostatin receptor type-3 (Sstr3) is a G-protein-coupled receptor that localizes to cilia that mediates the effects of somatostatin on cells. Smoothened (Smo) is a transmembrane protein trafficked to the ciliary membrane upon hedgehog pathway activation. By assaying these different classes of ciliary proteins that serve various roles within cilia, we can gain insight into whether Ccrk affects all classes of cargo protein entry, whether the effect is specific to certain classes of cargo protein, or whether Ccrk has no impact on ciliary cargo import.

Due to the issues we had with generating homozygous mutations in previous experiments, we decided to use a lentiviral transfection protocol to label our cargo proteins of interest (Figure 4.6). Utilizing the PLEX lentiviral packaging vector system, we generated lentiviral plasmids for each of our cargo of interest containing an mCherry-tag for visualization. We generated lentiviral plasmids for arl13b-mCherry and tubulin-mCherry. These plasmids were transfected into our IMCD:IFT88YFP and IMCD:71.12.A2 cell lines. These plasmids are stably integrated into the genome randomly, generating stable Ccrk wild-type and mutant Ccrk cell lines expressing IFT88YFP and the respective cargo labeled with mCherry. This cargo is still expressed and localizes to the expected locations within the cell (Figure 4.9, Figure 4.10). Moving forward, these cell lines can be used to perform similar experiments to assay movement of the cargo into and within the cilia to further interrogate Ccrk's effect on the activity of cargo import and movement into and within the cilia.

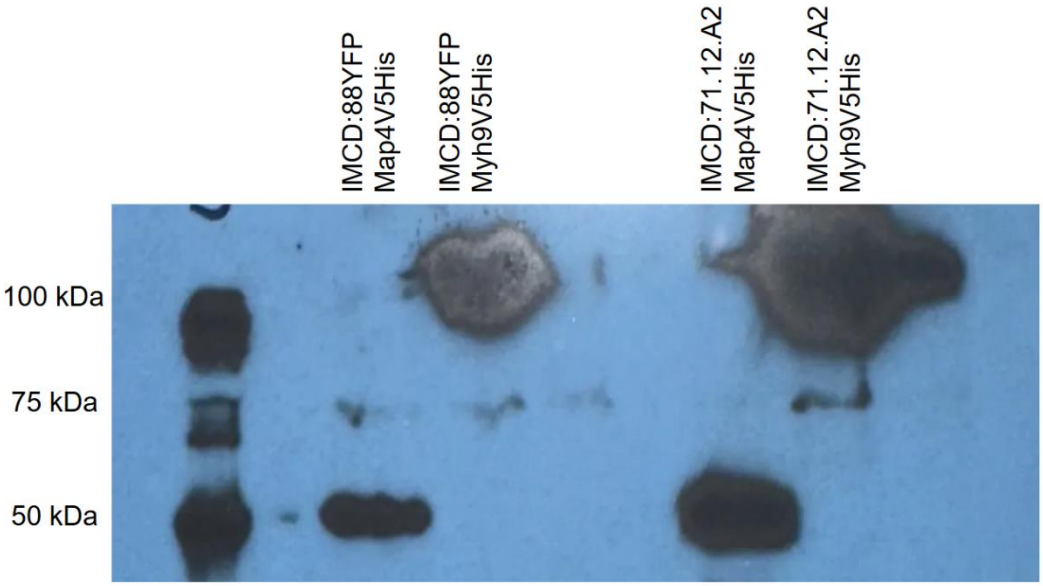


Figure 4.1: Validation of Map4V5His and Myh9V5His truncated protein expression in IMCD:IFT88YFP wild-type Ccrk and IMCD:71.12.A2 knockout Ccrk cells. The truncated proteins were transfected into these cell lines, harvested, and run on a 7.5% SDS-page gel and analyzed via standard PVDF western blot analysis and X-ray film exposure. Truncated Map4V5His is expected to be ~47kDa. Truncated Myh9V5His is expected to be ~130kDa.

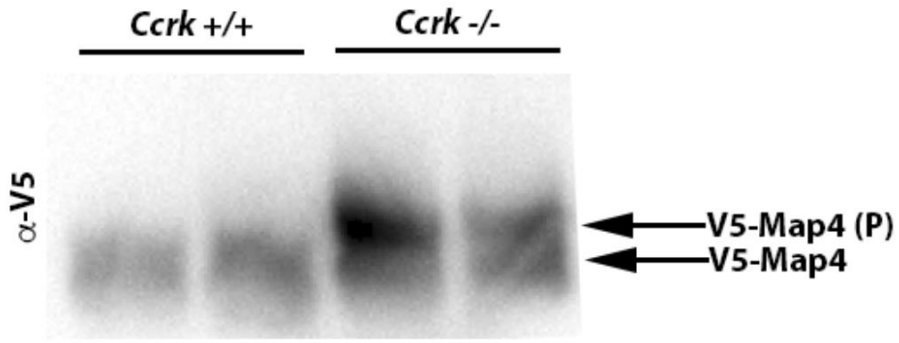


Figure 4.2: Phos-tag analysis of Map4V5His truncated proteins in IMCD:IFT88YFP wild-type Ccrk and IMCD:71.12.A2 knockout Ccrk cells. The higher band in Ccrk mutant cell lines indicates increased phosphorylation levels of the Map4V5His protein.

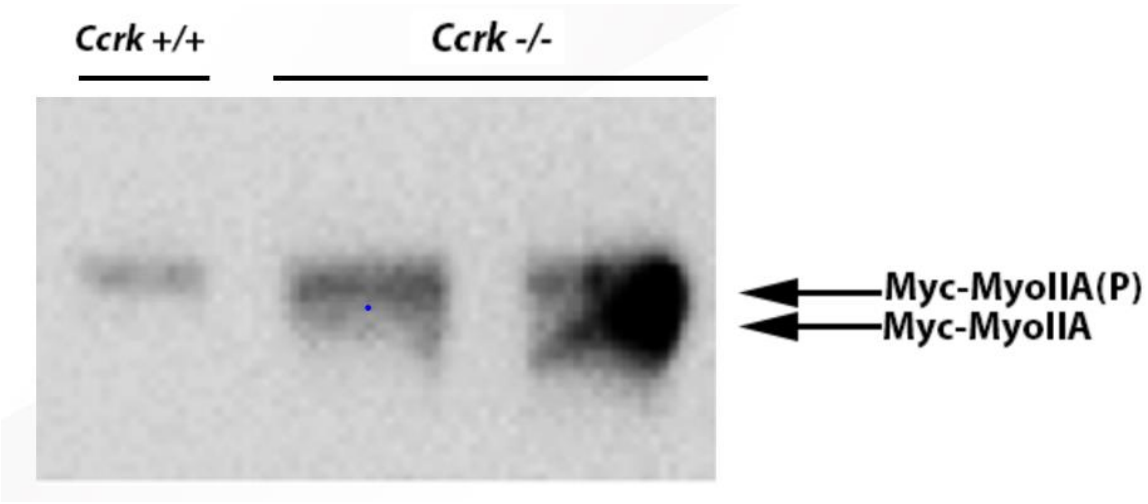


Figure 4.3: Phos-tag analysis of MyoIIa truncated proteins in IMCD:IFT88YFP wild-type Ccrk cells and IMCD:71.12.A2 knockout Ccrk cells. The lower bands in the Ccrk knockout lanes indicate reduced phosphorylation of the MyoIIa protein.

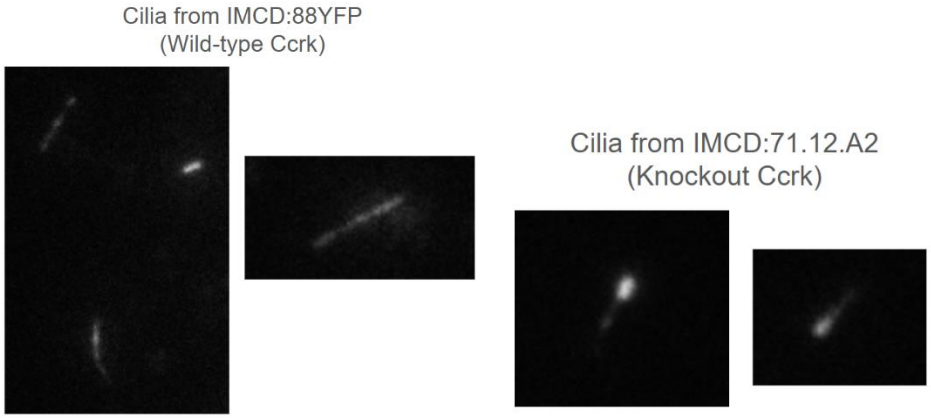


Figure 4.4: Total Internal Reflection Fluorescence (TIRF) microscopy images of wild-type and knockout Ccrk IMCD cells expressing fluorescent IFT88. The two left panels are cilia of wild-type Ccrk IMCD cells. The right two panels are cilia of knockout Ccrk IMCD cells. The cilia of knockout Ccrk cells have more intense fluorescence at the tip, likely indicating an accumulating of IFT particles at the tip of the cilia.

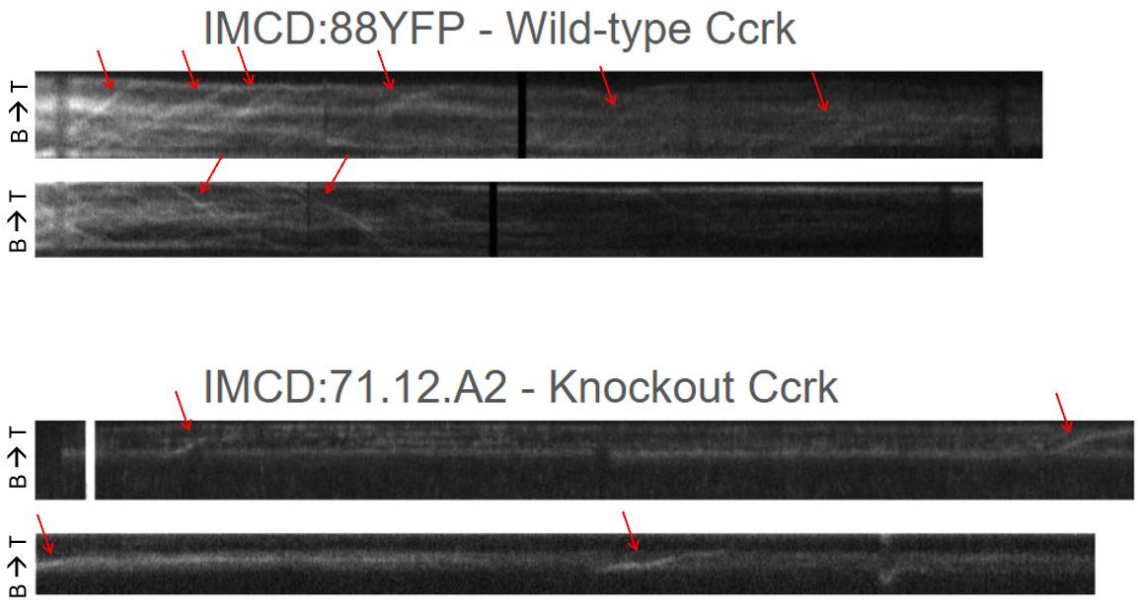


Figure 4.5: Kymographs of IFT88 particle movement in wild-type and knockout Ccrk IMCD cell lines. Images generated via FIJI/ImageJ. The red arrows indicate instances of IFT particle movement. The top two kymographs represent IFT88YFP particle movement within the cilium of a wild-type Ccrk IMCD cell. The bottom two kymographs represent IFT88YFP particle movement within the cilium of a knockout Ccrk IMCD cell. The white lines with positive and negative slopes indicate anterograde and retrograde transport respectively of IFT88YFP particles tracked within the cilia. We see more instances of IFT88YFP movement within wild-type Ccrk cells compared to knockout Ccrk cells.

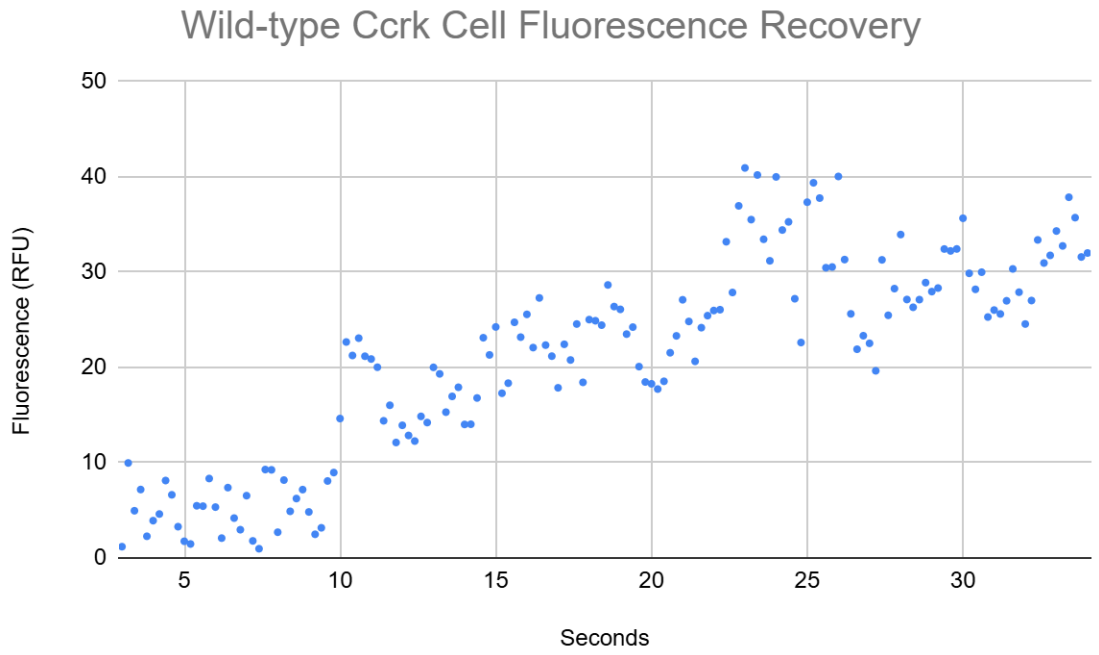


Figure 4.6: Fluorescence Recovery after Photobleaching (FRAP) graph displaying the fluorescence recovery of IFT88YFP in a wild-type Ccrk cilia post-bleaching event. FRAP fluorescence data were recorded by FIJI/ImageJ. Measurements were set for the area and mean gray value. The plugin Plot Z-Axis Profile then generated the average fluorescence levels within the defined area of our cilia as a function of time/frame. Standardized pre-bleach levels were in the range of 84-90 RFU. The bleaching event had an intensity of ~5,500-7,000 RFU. The video analyzed was 5fps. Fluorescence recovers to ~30 RFU within the imaging window.

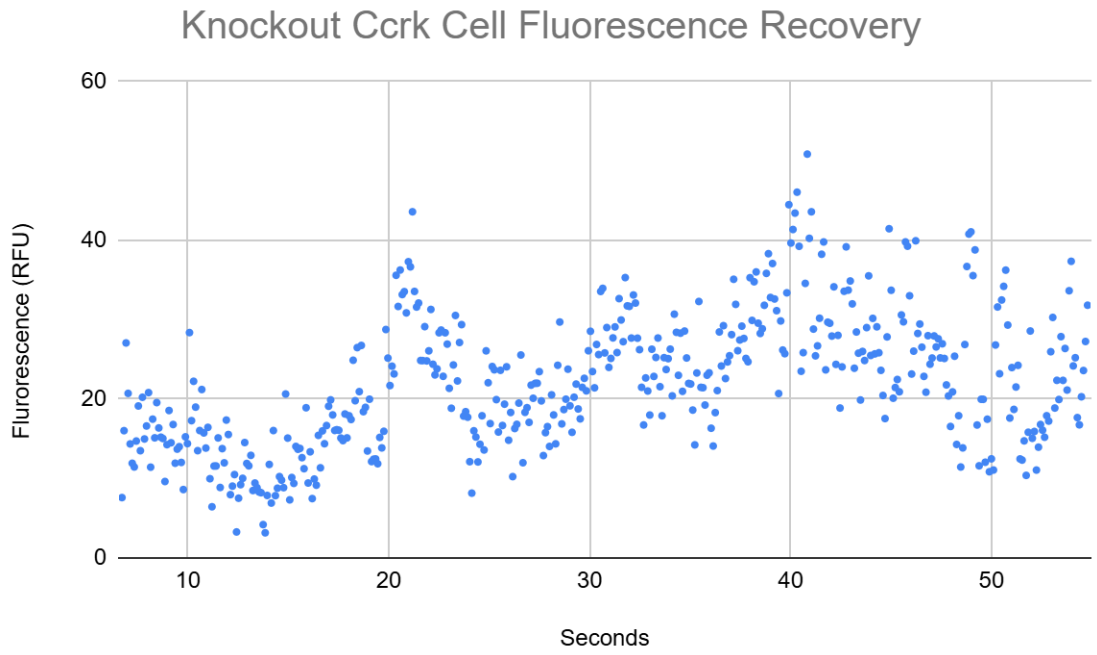


Figure 4.7: Fluorescence Recovery after Photobleaching (FRAP) graph displaying the fluorescence recovery of IFT88YFP in a knockout Ccrk cilia post-bleaching event. FRAP fluorescence data were recorded by FIJI/ImageJ. Measurements were set for the area and mean gray value. The plugin Plot Z-Axis Profile then generated the average fluorescence levels within the defined area of our cilia as a function of time/frame. Standardized pre-bleach levels were in the range of 90-110 RFU. The bleaching event had an intensity of ~1,200-4,000 RFU. The video analyzed was 5fps. Fluorescence recovers to ~25 RFU within the imaging window.

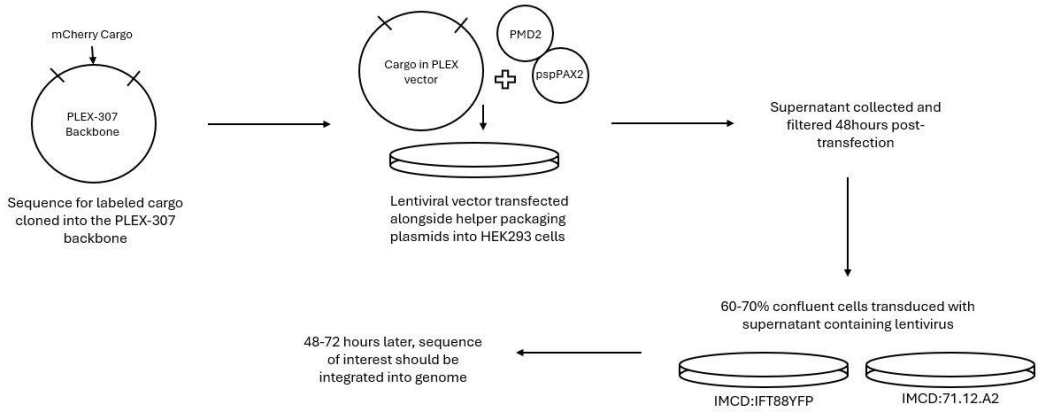
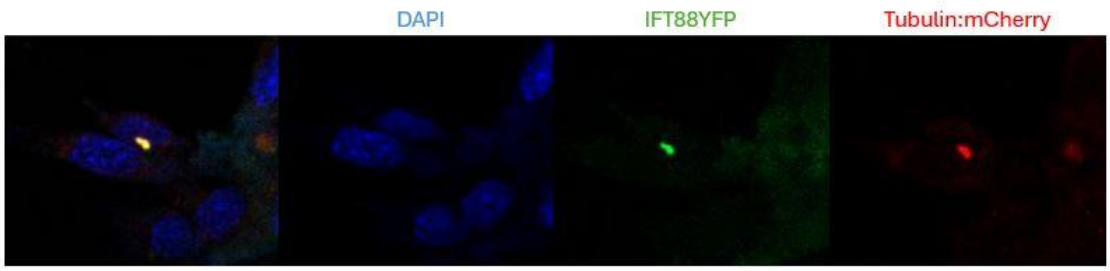


Figure 4.8: Lentiviral Transfection Workflow: Our tagged cargo of interest is inserted into our PLEX-307 vector backbone. The two helper plasmids, pMD2.G and pspPax2, were combined with our cargo/PLEX plasmid at a 1:1:1 molar ratio. This DNA complex was transfected into HEK293 Lenti-X Now Cells. The supernatant of these transfected cells was collected and filtered after forty-eight hours. These cells contain our lentivirus. This supernatant is then added to our cell lines of interest to transduce them with the lentivirus. After forty-eight hours, it is expected that our cell lines have integrated the sequence for our cargo of interest.

A.



B.

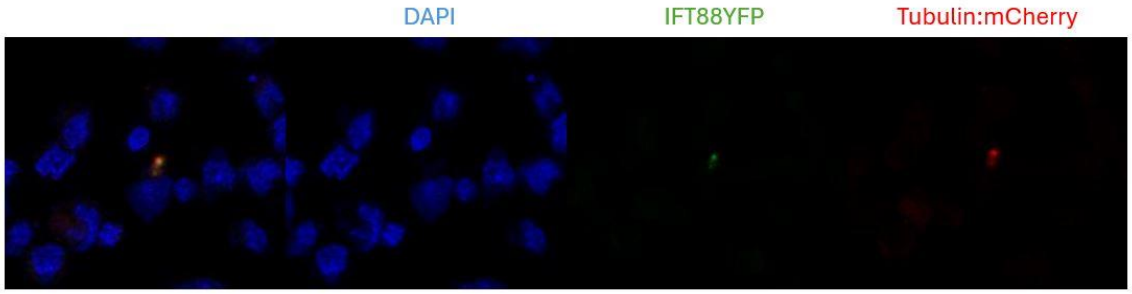
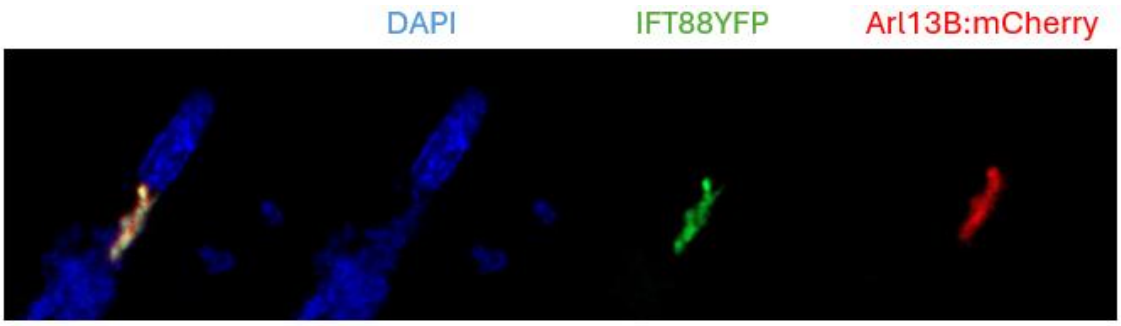


Figure 4.9: Wild-type and knockout Ccrk cell lines expressing IFT88YFP were transduced with lentiviruses containing mCherry-tagged tubulin. After integrating the tagged cargo into the genome of our cell lines, these cells were expanded and passaged multiple times. These cells were then placed under forty-eight hours of serum starvation to stimulate cilia production, fixed, and imaged using confocal microscopy (LSM 900, Biomedical Microscopy Core, UGA) to verify that these sequences stably integrated into our cell lines. Panel A shows a wild-type Ccrk cell line co-expressing IFT88YFP and Tubulin:mCherry at 20x magnification. Panel B shows a knockout Ccrk cell line co-expressing IFT88YFP and Tubulin:mCherry at 20x magnification.

A.



B.

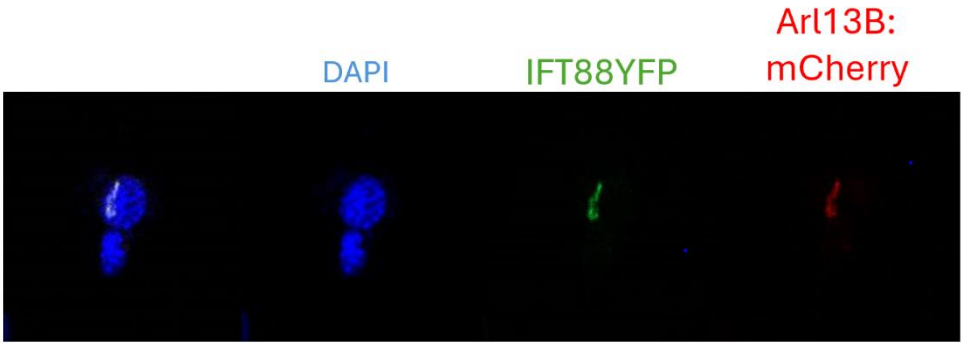


Figure 4.10: Wild-type and knockout Ccrk cell lines expressing IFT88YFP were transduced with lentiviruses containing mCherry-tagged Arl13b. After integrating the tagged cargo into the genome of our cell lines, these cells were expanded and passaged multiple times. These cells were then placed under forty-eight hours of serum starvation to stimulate cilia production, fixed, and imaged using confocal microscopy (LSM 900, Biomedical Microscopy Core, UGA) to verify that these sequences stably integrated into our cell lines. Panel A shows a wild-type Ccrk cell line co-expressing IFT88YFP and Arl13b:mCherry at 40x magnification. Panel B shows a knockout Ccrk cell line co-expressing IFT88YFP and Arl13b:mCherry at 20x magnification.

Table 4.1: Expected outcomes of phosphomimetic and unphosphorylatable mutations at Serine-760 of Map4

Map4	Wild-type Ccrk cells	Ccrk Knockout cells
Normal phenotype seen	Low to no phosphorylation of Map4; Normal ciliary phenotype	Some phosphorylation of Map4; mutant ciliary phenotype
Phenotype expected with phosphomimetic mutation	Map4 *always* phosphorylated; more severe mutant ciliary phenotype	Map4 *always* phosphorylated; more severe mutant ciliary phenotype
Phenotype expected with non-phosphorylatable mutation	Map4 *never* phosphorylated; normal ciliary phenotype	Map4 *never* phosphorylated; rescue of wild-type Ccrk ciliary phenotype

Table 4.2: Expected outcomes of phosphomimetic and unphosphorylatable mutations at Serine-1943 of MyosinIIa

MyosinIIa	Wild-type Ccrk cells	Ccrk Knockout cells
Normal phenotype seen	MyoIIa is phosphorylated; Normal ciliary phenotype	Less phosphorylation of MyoIIa; mutant ciliary phenotype
Phenotype expected with phosphomimetic mutation	MyoIIa *always* phosphorylated; normal ciliary phenotype	MyoIIa *always* phosphorylated; rescue of wild-type Ccrk ciliary phenotype
Phenotype expected with non-phosphorylatable mutation	MyoIIa *never* phosphorylated; more severe mutant ciliary phenotype	MyoIIa *never* phosphorylated; more severe mutant ciliary phenotype

Materials and Methods

Cell Culture:

IMCD:IFT88YFP cells were a gift from Jagesh Shah to the lab (Besschetnova et al., 2009). IMCD:71.12.A2 cells with knockout Ccrk generated in the lab by Jonathan Walsh. These cell lines were cultured in DMEM/F12 (50:50) media containing 10% FBS, 1% Pen/Strep, and 1% glutamine growth supplement. Immortalized mouse embryonic fibroblasts heterozygous or homozygous for a mutant Ccrk allele were also experimented with. These cell lines were cultured in DMEM, 10% FBS, 1% Pen/Strep, and 1% glutamine growth supplement.

Map4V5His and MyoIIaV5His Protein Isolation:

Plasmids expressing truncated versions of Map4V5His and MyoIIaV5His were transformed into Dh5α *E. coli*. These bacterial cells expanded for 16 hours overnight. A maxi-prep kit was utilized to isolate the plasmid DNA for transfection into IMCD:IFT88YFP and IMCD:71.12.A2 mammalian cell lines. The plasmids were transfected into these cell lines using the standard Lipofectamine 3000 protocol. Forty-eight hours post-transfection, cells are harvested and treated with RIPA buffer containing protease and phosphatase inhibitors. We then used HisPur Ni-NTA Resin (ThermoScientific #88221) to purify our His-tagged proteins from our protein extracts using the established gravity-flow column protocol from ThermoScientific.

Western Blot:

Purified protein extracts were run on BioRad PROTEAN precast gels and transferred to PVDF membrane using the semi-dry blotting method (BioRad Trans-Blot

SD Semi-dry Transfer Cell). A monoclonal mouse-antiV5 antibody (ThermoFisher #R960-25) was used to bind to our V5His truncated proteins (1:5000 concentration at 4C overnight). An anti-mouse HRP secondary antibody (1:10000 concentration) was used. The membrane was treated with Clarity Western ECL substrate (BioRad 170-5061) and developed on X-ray film.

Phos-tag Gel Analysis:

The same concentrations of purified protein extracts of truncated Map4V5His and MyoIIaV5His were run on 7.5% SuperSep Phos-tag gels (WakoChemicals #198 17981). Before transfer to PVDF membrane, the run gel is soaked in transfer buffer containing 10 mM EDTA for two fifteen-minute washes to chelate out the zinc ions in the phos-tag gels. The gel is then transferred onto a PVDF membrane using the semi-dry blotting method (BioRad Trans-Blot SD Semi-dry Transfer Cell). A monoclonal mouse-antiV5 antibody (ThermoFisher #R960-25) was used to bind to our V5His truncated proteins (1:5000 concentration at 4 °C overnight). An anti-mouse HRP secondary antibody (1:10000 concentration) was used. The membrane was treated with Clarity Western ECL substrate (BioRad 170-5061) and imaged on a BioRad bioimager.

CRISPR Mutagenesis and Clone Generation:

Cas9 enzyme, our guideRNA template for the gene of interest (Map4 or MyoIIa), and the template for mutation were transfected into 70% confluent IMCD:IFT88YFP and IMCD:71.12.A2 cell lines at a 1:6:6 concentration using the TransIT-X2 Dynamic Delivery System reagents and protocols. They were also incubated with Scr7 at 10 μM concentration (MedChemExpress #HY-107845), a small molecule used to inhibit non-

homologous end joining, increasing the number of instances of homology-directed repair. There were a total of four transfections of interest (Map4 S→E; Map4 S→A; MyoIIa S→E; and MyoIIa S→A). Forty-eight hours post-transfection, cells are put under puromycin selection at 1ug/mL. After one week under selection, cells are relieved of selection for forty-eight hours. The surviving cells were then single-cell sorted via the MoFlo Astrios Cell Sorter (CTEGD Cytometry Shared Resource Laboratory at UGA) into 96-well plates for clonal colonies to form.

Screening for Homozygous Mutations:

PCR amplification of the region being targeted was used. Map4 mutants were screened by cutting PCR products with the AvaII restriction enzyme. Map4 mutants have an AvaII site eliminated, resulting in two products, rather than three. MyoIIa mutants were screened by cutting PCR products with the SalI restriction enzyme. MyoIIa mutants have a SalI site eliminated, resulting in one product, rather than two.

Transwell Culture Protocol and TIRF Microscopy Imaging:

To image cilia in live cells using TIRF microscopy, we culture our cells upside down on transwells (Sigma-Aldrich #CLS3470-48EA; Stemcell Technologies, #38024) situated in a 24-well cell culture plate using a protocol adapted from Ott & Schwartz, 2012. Cells cultured on 10cm plates are resuspended, and 100 μL of the diluted cells are applied directly to the membrane of the inverted transwell. These cells are incubated inverted for two to six hours for the cells to adhere to the membrane. After this incubation, we flip the dish and add 1.5 mL of media to the outside of the transwell (bottom of the multi-well dish) and 0.75 mL of media to the inner well of the transwell

(opposite side of the membrane). Once cells are 75% confluent on the transwell membrane, the growth media is replaced with serum-starved media (Opti-MEM with 1% FBS) for 24-48 hours to stimulate cilia production. Live imaging was performed using TIRF microscopy (Lechtreck Lab) at 60x magnification at five frames per second.

Kymograph and FRAP Image Generation:

Videos analyzed using FIJI (ImageJ). The ciliary region of interest is covered with the segmented line tool. The plugin MultipleKymograph is utilized to generate kymographs. FRAP fluorescence data were recorded by setting measurements for the area and mean gray value. The plugin Plot Z-Axis Profile will then generate the average fluorescence levels within the defined area of our cilia as a function of time. This data is then exported to Excel to clean up the spikes in fluorescence from the bleaching and standardize the fluorescence levels to make visual representation easier.

Lentiviral Plasmid Generation

All cargo and PLEX-307 plasmids were expanded using standard transformation protocols in dH5α *E. coli*. Plasmid DNA was purified using a plasmid DNA Maxi Kit (Omega Bio-Tek #D6926-03). hsp70:Arl13B-mKate2 (Addgene #105964) amplified using forward primer 5'-GCTAGCTGCTAGCTCCAGTCTGATGGCCAACTG-3' (introduces Nhe1 restriction enzyme site) and reverse primer 5'-CGATCGATACGCGTGTAAACGACGGCCAGTGAAT-3' (introduces Mlu1 restriction enzyme site) and inserted into PLEX-307 plasmid (Addgene #41392) utilizing Nhe1 and Mlu1 restriction sites. Proper insertion was diagnosed using the EF1a-F promoter sequence (5'–TCAAGCCTCAGACAGTGGTTC–3') present on the PLEX-307 plasmid

and the 5'–GGCTTTCCTGATATCCTCGG–3' primer sequence containing the EcoRV restriction site present within the Arl13B sequence. mCherry-Smoothened1-N-18 (Addgene #55134) amplified using forward primer 5'-GGTTTAGTGAACCGTCAGATC-3' and reverse primer 5'-CATGCATACGCGTACTAGATCTCAGCGCCGGC-3' (introduces Mlu1 restriction enzyme site) and inserted into PLEX-307 plasmid utilizing Nhe1 and Mlu1 restriction sites. Proper insertion was diagnosed using the EF1a-F promoter sequence (5'–TCAAGCCTCAGACAGTGGTTC–3') present on the PLEX-307 plasmid and the 5'–GGCATTCCGGAGGCCGG–3' primer sequence containing the BspEI restriction site present within the Smoothened sequence. mCherry-SSTR3-N-17 (Addgene #55135) amplified using forward primer 5'-GCTACGTGCTAGCCTCGAGACCGCCATGGCCAC-3' (inserts Nhe1 restriction enzyme site) and reverse primer 5'-GATCGATACGCGTAATACTAGATCTCAGCGCCG-3' (inserts Mlu1 restriction enzyme site) and inserted into PLEX-307 plasmid utilizing Nhe1 and Mlu1 restriction sites. Proper insertion was diagnosed using the EF1a-F promoter sequence (5'–TCAAGCCTCAGACAGTGGTTC–3') present on the PLEX-307 plasmid and the mCherry-R primer sequence 5'–TTGGTCACCTTCAAGCTTGG–3' present within the SSTR3 sequence. mCh-alpha-tubulin (Addgene #49149) amplified using forward primer 5'-GGTTTAGTGAACCGTCAGAT-3' and reverse primer 5'–CGTACGTACGCGTTCAGTTATCTAGATCCGGTG–3' (introduces Mlu1 restriction enzyme site) and inserted into PLEX-307 plasmid utilizing Nhe1 and Mlu1 restriction sites. Proper insertion was diagnosed using the EF1a-F promoter sequence (5'–

TCAAGCCTCAGACAGTGGTTC–3’) present on the PLEX-307 plasmid and the mCherry-R primer sequence 5’–TTGGTCACCTTCAAGCTTGG–3’ present within the tubulin sequence.

Lentiviral Transfection Protocol

The two helper plasmids, pMD2.G and pspPax2, were combined with our cargo/PLEX plasmid at a 1:1:1 molar ratio. This DNA complex was transfected into HEK293 Lenti-X Now Cells (Gift from Pengpeng Bi Lab; TakaraBio #632180) using standard lipofectamine transfection reagents and protocols. Forty-eight hours post-transfection, the supernatant media was collected and filtered. Combined with normal IMCD cell growth media at a two-to-one ratio, this media was applied to our IMCD:IFT88YFP and IMCD:71.12.A2 cell lines. After forty-eight hours, these cells should be stably expressing fluorescent cargo.

Chapter 5

Conclusions

This chapter describes the contributions of the studies within the two parts of this dissertation and plans for future research.

Chapter two of this dissertation examines student thinking and problem-solving about metabolic pathway dynamics and regulation, a threshold concept in biochemistry education. This work revealed evidence of independent knowledge elements for student thinking about metabolism and how these knowledge elements interact within a problem-solving context. Many students exhibit behavior of drawing knowledge elements independently, and this behavior fluctuates through the problem contexts we present. Students have good reasons for thinking the way they do. “Incorrect” ideas are just misapplied or a result of ambiguous instructions or visuals prompting inappropriate usage. When looking across our students, we see students who draw upon similar knowledge elements but differ in their usage throughout the problem-solving process. We have a student, Charlie, who is evidence of a student who can express coherent and stable use of knowledge elements and possesses representational competence. This is evidence that students can refine varied, fluctuating, and fragmented knowledge elements into a unified set of scientific ideas.

This work revealed two major categories of student knowledge elements – the dashed arrow and the circled bar, and the scientific concept it represents, and the primary

focus of students on the end results or products of a pathway. Students display various knowledge elements to describe the dashed arrow and circled bar. This dashed arrow and circled bar was intended to represent negative feedback inhibition, and many participants eventually gravitated towards this idea. However, students generally could not display representational competence to link this visual to the underlying scientific concept. Most biochemistry students, but only a few biology participants, noted negative feedback inhibition when looking at the dashed arrow and circled bar. Many ideas were presented in contrast, including that the intermediate was returning to be a part of the previous chemical reaction. Participants' reference to "negative feedback because of the negative sign" aligns with the WYSIWG (what you see is what you get) knowledge element (Elby, 2000). We designed the visual representation with a bar because it signals "negative." However, participants must recruit knowledge elements beyond "bar means negative." For many participants in our sample, the dashed, curved arrow simply was not a strong enough cue to indicate an entirely different process than the process indicated by the solid, straight arrows. Students who heavily rely on this WYSIWYG knowledge element need to build representational competence to instead draw upon knowledge of the molecular interactions that underlie negative feedback inhibition. Moving forward, there are broader questions about visual representations brought to light by this study. Simple visuals are typically used to represent complicated processes. Would changes to instruction and instructional material design to reduce the cognitive load of learning visual representations help students build a more coherent understanding of metabolism? Are students more likely to construct a more cohesive set of ideas about concepts such as metabolism if they are explicitly instructed about the common visual representations used

and how to interpret them? Are there visual representations to be designed that lead to easier representational competence for students?

The second category of student ideas was their uneven focus on the end products of the pathway presented in the problem, rather than considering the importance of all components within the pathway. The ultimate aim of our problem set was for participants to discuss how each metabolic compound would be affected by changes to a pathway component. However, as most participants made predictions about pathway dynamics, most of them focused on how it would impact the end reactants of the pathway. They rarely dealt with the impacts on the upstream components of the pathway. The usual presentation in instruction of pathways in a stepwise pattern (fatty acyl thioester gets turned into IV-CoA, which gets turned into DMA-CoA) may play upon students' natural biases (i.e., this pathway exists to make DMA-CoA). Metabolism is normally taught by walking through multiple essential pathways, such as photosynthesis, cellular respiration, or glycolysis. These pathways are often talked about in a goal-oriented manner – for example, the point of photosynthesis can be talked about as turning carbon dioxide into oxygen. This could lead to a constant underlying notion that every pathway has tangible goals, and the end products are the only thing that matters.

Representational competence lies beneath the surface as a constant influence on the knowledge elements students utilize. The question is how we get students from appropriate representational competence to understanding what knowledge elements to utilize and how to apply them to construct understandings of questions. Future research is needed that investigates the knowledge elements cued by different visual representations, especially those commonly used in undergraduate biology and chemistry instruction.

Instructional materials for biochemistry should then be designed based on those findings and investigated to see if using those materials results in greater representational competence. There are also potential avenues for future research on these topics. Rather than teaching metabolism through step-by-step processes, teaching metabolism as a focus on pathways as systems may impact student knowledge element activation and application. Focusing on teaching metabolism as systems may also help students integrate knowledge elements of processes and better representational competence. A focus on all metabolic compounds in a system and how one perturbation can affect all of the compounds in the pathway and even other related pathways is the goal.

Ultimately, the work presented in chapter two focuses on a single concept in biochemistry in a single context. While a starting point for research on knowledge elements activated in metabolism problem solving, this work is just a small drop in the bucket of potential research in this field. These findings are also just about metabolism problem-solving and cannot be generalized to other topic areas.

The work presented within chapter four presents a model for the regulation of ciliary length by the cell. Our work leads us to believe that Ccrk is responsible for the phosphorylation of two downstream effector proteins – Map4 and MyosinIIa. The specific phosphorylations on these proteins are linked to controlling actin filament and microtubule dynamics, linking them to the movement of proteins, including cargo and IFT particles to the cilia, and thus, impacting ciliogenesis. We believe this may be a contributing factor in the IFT movement phenotypes in *Ccrk* knockout cells. In these knockout Ccrk cell lines, we see fewer instances of IFT anterograde transport and retrograde transport. The lack of retrograde transport observed can be linked to the

bulbous tip of the cilia. This bulbous tip is likely the proteins responsible for IFT machinery and their cargo that are unable to be transported back to the cell. The phenotype we see of decreased anterograde transport can be linked to the impairment of movement along the skeletal structure of the cell. This impairment of movement includes decreased levels of IFT proteins being moved to the cilia. Future experiments were designed to explore whether these downstream effectors themselves are linked to the ciliary phenotype we see when we knockout Ccrk. By changing the specific site that is differentially phosphorylated and comparing the phenotypes of these generated cells with the presence of Ccrk or knockout Ccrk, these experiments can confirm a link between these effector proteins and the ciliary phenotypes we see.

Another piece of evidence supporting this hypothesis can be observed when examining the fluorescence recovery of YFP-tagged IFT proteins into the cilia. When Ccrk is knocked out in the cell, we observe a decrease in the import of new IFT machinery into the cilia. This provides support for a previously observed phenotype, where we saw fewer instances of anterograde IFT movement within cilia. The decrease in the import of new IFT machinery into the cilia could be linked to the impaired movement of IFT proteins to the cilia, which we believe is a result of Map4 and MyosinIIa differential phosphorylation. Further experiments should be done to confirm the phenotypes discussed here, as these conclusions are based on experiments with a small sample size.

With cell lines we generated that express both fluorescently-tagged IFT machinery and cargo, future experiments can explore whether cargo proteins follow a similar phenotype to IFT machinery when Ccrk is knocked out. Four different categories

of cargo proteins were utilized in order to assess whether all categories of cargo protein that we selected have their import affected, or whether only certain categories, or none of the significant categories of cargo protein movement are affected.

Ultimately, the work done in chapter four has led to a proposed model for how knocking out cell cycle-related kinase leads to the ciliary phenotype we see of a range of ciliary lengths and the cilia having a bulbous tip. Ccrk regulates ciliogenesis through the modification of actin and microtubule dynamics in the cytoskeleton and at the base of the cilium. Future experiments can analyze the extent to which Ccrk affects IFT machinery and cargo movement. An exploration of the other proteins differentially phosphorylated without functional Ccrk may be useful in helping fill out the molecular model of how Ccrk affects ciliogeneis.

Chapter 6

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Chapter 7

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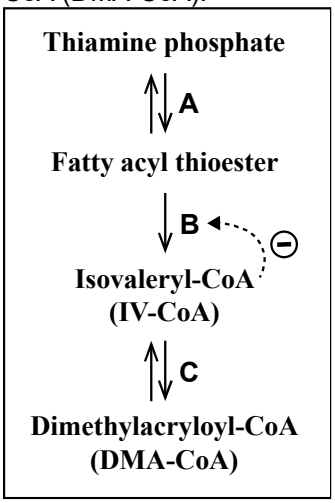
Appendix A

Supplemental Data for Part 1

A.1: Complete problem set used in the study. Correct answers are available from the corresponding author upon request.

The metabolic pathway below shows the conversion of thiamine phosphate to dimethylacryloyl-CoA (DMA-CoA).



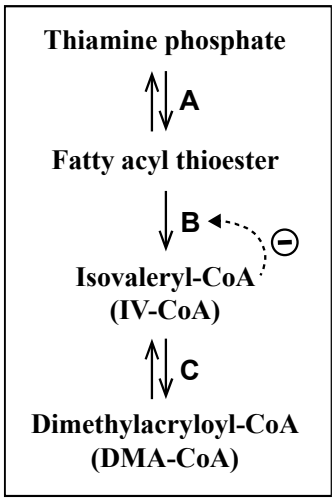
1. How many of each kind of molecule are shown in the figure?

	0	1	2	3	4	5	6	7	8	9	10
Enzymes	○	○	○	○	○	○	○	○	○	○	○
Metabolic compounds	○	○	○	○	○	○	○	○	○	○	○

2. What do the dashed arrow and circled bar represent?

	True	False
negative feedback or inhibition	○	○
positive feedback or activation	○	○
removal of IV-CoA	○	○
IV-CoA being added as a reactant	○	○
IV-CoA being recycled	○	○
reverse reaction	○	○
alternate pathway	○	○

The metabolic pathway below shows the conversion of thiamine phosphate to dimethylacryloyl-CoA (DMA-CoA).



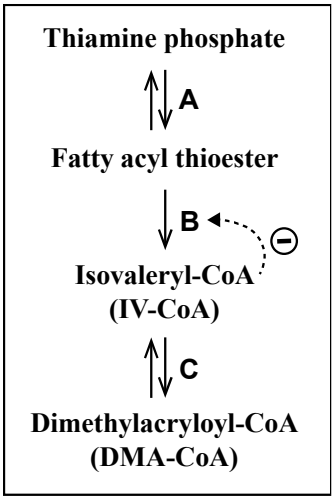
3. Do you predict that Enzyme A could convert fatty acyl thioester to thiamine phosphate?

- ○ Yes, enzymes are generally reversible.
- ○ No, enzymes are generally irreversible.

4. If enzyme C were inhibited, how might this eventually impact the activity of enzyme B?

- ○ The activity of enzyme B would not be affected.
- ○ The activity of enzyme B would increase.
- ○ The activity of enzyme B would decrease.

The metabolic pathway below shows the conversion of thiamine phosphate to dimethylacryloyl-CoA (DMA-CoA).



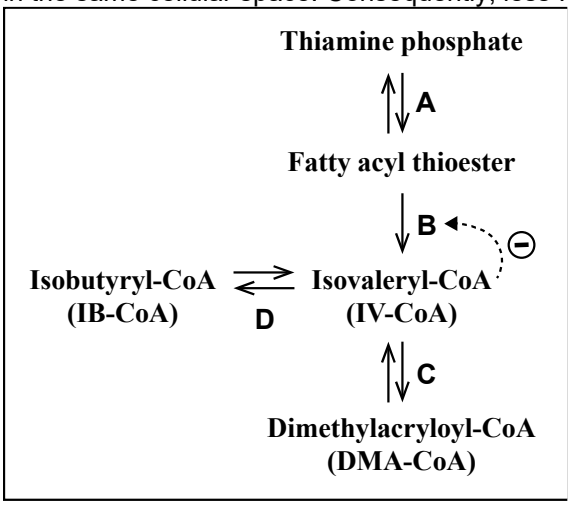
5. Assuming a constant concentration of all enzymes, which are fully functional, what controls how much DMA-CoA is made by this pathway?

	True	False
concentration of thiamine phosphate	○	○
concentration of fatty acyl thioester	○	○
concentration of IV-CoA	○	○
inhibition by IV-CoA	○	○
activation by IV-CoA	○	○
binding of IV-CoA to enzyme B	○	○

6. Under what conditions would it be possible for enzyme C to convert DMA-CoA into IV-CoA?

	True	False
the ratio of DMA-CoA to IV-CoA > the ratio at equilibrium	○	○
the conversion of DMA-CoA into IV-CoA has a negative delta G value	○	○
the reaction is coupled to another favorable reaction	○	○

As shown below, isovaleryl-CoA (IV-CoA) can also be consumed by another pathway that occurs in the same cellular space. Consequently, less IV-CoA is available for DMA-CoA production.

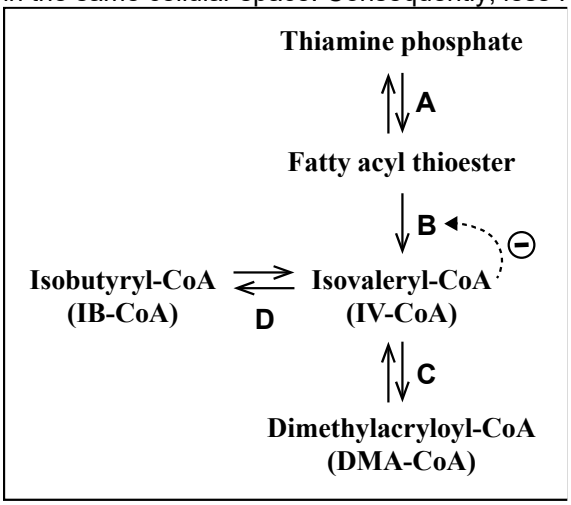


7a. Assume that both branches of the pathway are in place, and there are no other changes. Do you predict that having a branch point in this pathway will affect flux through the pathway as a whole, including both branches, as opposed to when only the main branch was in place?

- ❍ Yes, flux will be affected.
- ❍ No, flux will not be affected.

7b. Provide a scientific explanation to support your prediction.

As shown below, isovaleryl-CoA (IV-CoA) can also be consumed by another pathway that occurs in the same cellular space. Consequently, less IV-CoA is available for DMA-CoA production.

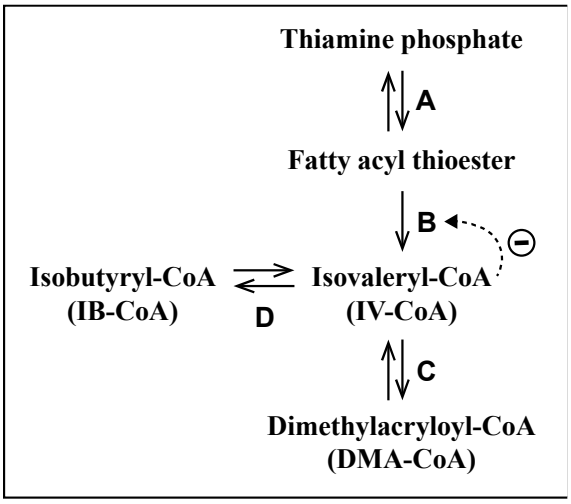


8a. Assume that both branches of the pathway are in place, and the only other change is that IV-CoA can no longer bind to enzyme B. Do you predict that this will affect flux through the pathway as a whole, including both branches, as opposed to when only the main branch was in place?

- ❍ Yes, flux will be affected.
- ❍ No, flux will not be affected.

8b. Provide a scientific explanation to support your prediction.

As shown below, isovaleryl-CoA (IV-CoA) can also be consumed by another pathway that occurs in the same cellular space. Consequently, less IV-CoA is available for DMA-CoA production.

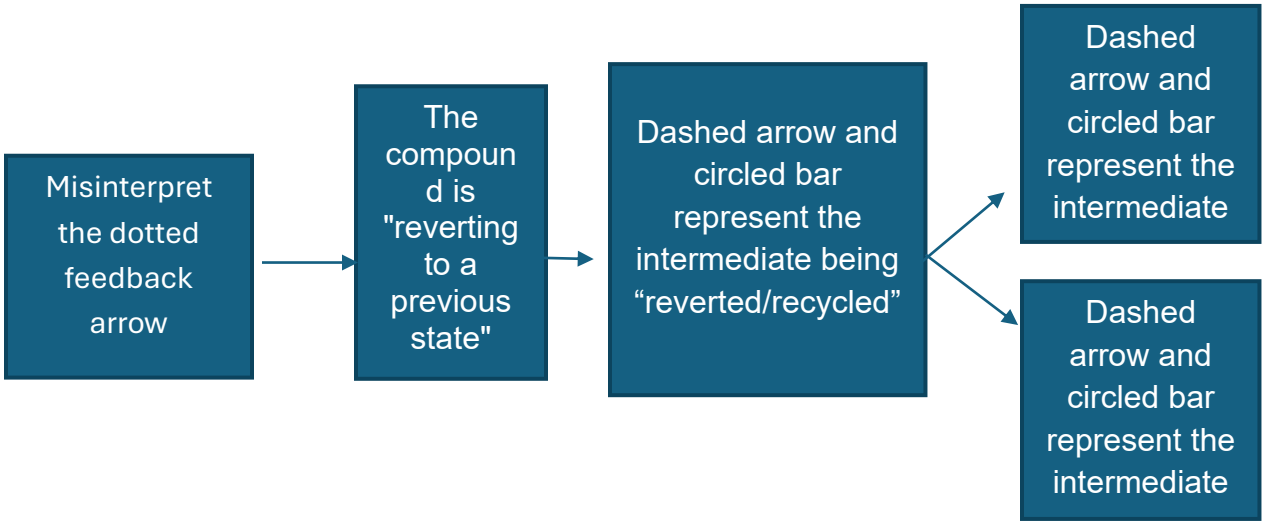


9a. Assume that both branches of the pathway are in place, and the only other change is that enzyme C doesn't work anymore. Do you predict that this will affect flux through the pathway as a whole, including both branches, as opposed to when only the main branch was in place?

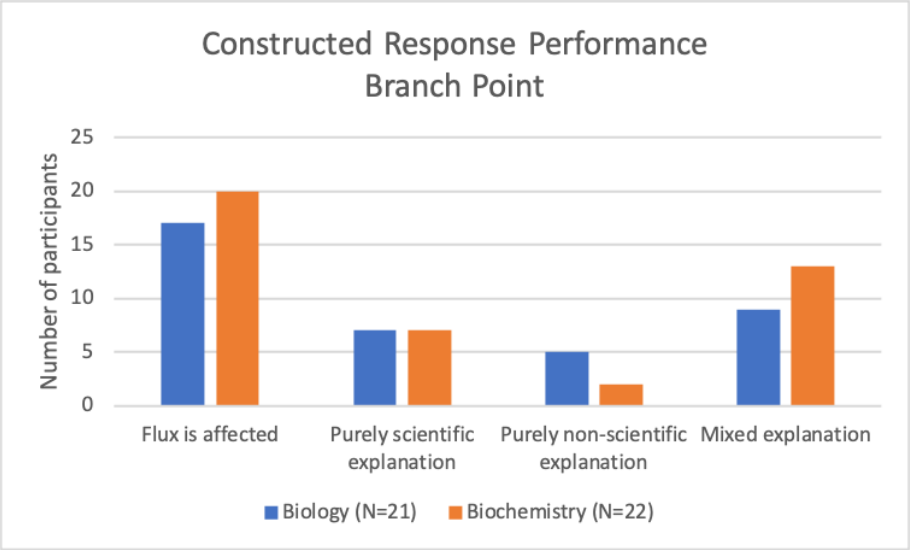
- ❍ Yes, flux will be affected.
- ❍ No, flux will not be affected.

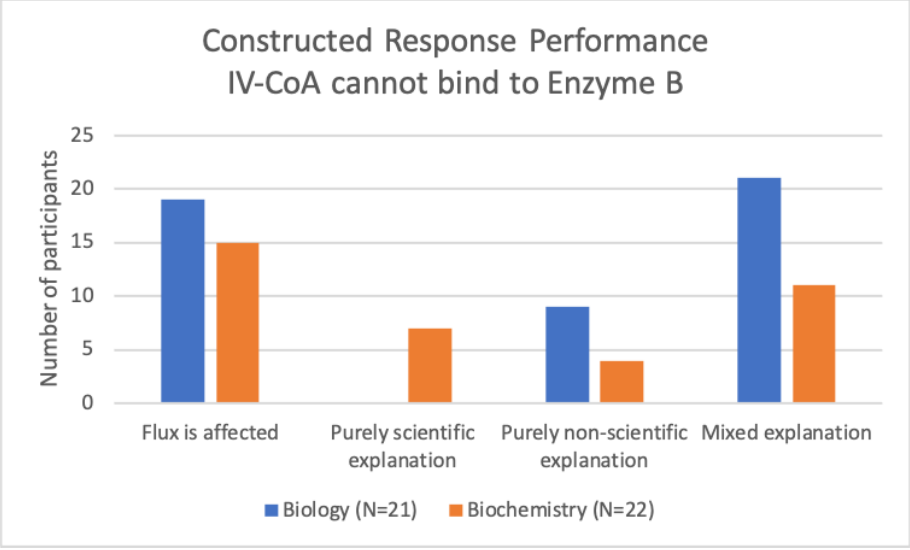
9b. Provide a scientific explanation to support your prediction.

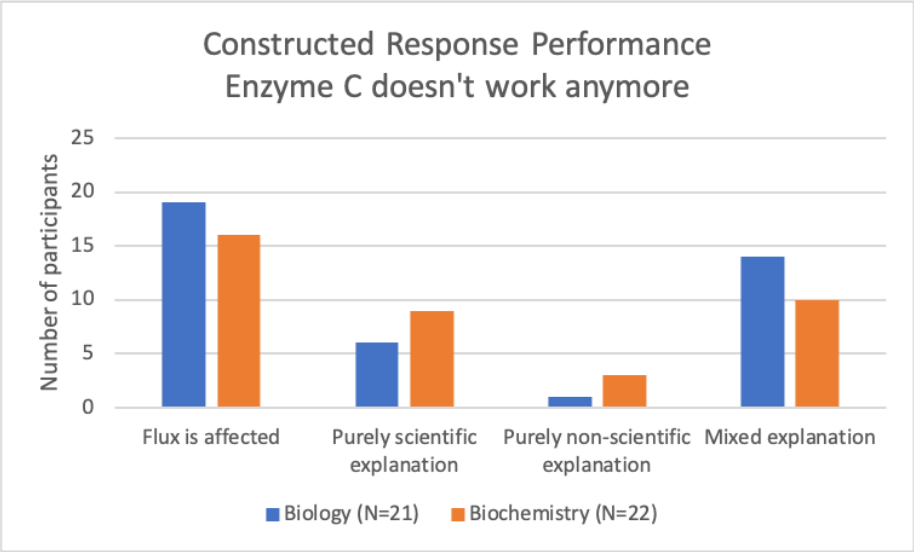
A.2 Coding process for qualitative content analysis. The *a priori* codebook was based on prior research and was refined to provide a final codebook of twelve codes which are presented in Tables 2 and 3 of the main paper. This figure illustrates the process. The original codebook contained the *a priori* code “Misinterpret the dotted feedback arrow.” In round 2 coding, we revised this code to “The compound is ‘reverting to a previous state’” in order to represent the dataset with greater precision. During a review of coding, the code name was revised yet again to indicate multiple ideas present in the dataset. In the final round of analysis, we split this code into two distinct knowledge elements students expressed about the dashed arrow and circled bar: recycling and reversion.



A.3: Participant performance on individual constructed response items. Participants were asked to predict the outcomes of three perturbations to the metabolic pathway (items 7-9 in Figure S1). Figure 2 in the main paper shows the average performance across all three problems. The figures below show student performance on each item. A) Item 7: Predict the impact of adding a branch point to the pathway; B) Item 8: Predict the impact if IV-CoA can no longer bind to enzyme B; C) Item 9 (next page): Predict the impact if enzyme C doesn't work anymore. The leftmost set of bars shows the number of participants who predicted that flux would be affected by the perturbation. The remaining three sets of bars show the number of students who provided explanations that were purely scientific, purely non-scientific or mixtures of both across all contexts. Blue bars correspond to biology participants (N=21) and orange to biochemistry participants (N=22).






Constructed Response Performance
Enzyme C doesn't work anymore
Number of participants
25
20
15
10
5
0
Flux is affected
Purely scientific explanation
Purely non-scientific explanation
Mixed explanation
Biology (N=21)
Biochemistry (N=22)

A.4: Lesson designed to improve student learning about metabolism

The following activity was designed to address the results reported in this article. The activity provides scaffolding for students to refine their intuitions of the visual representations commonly used to depict metabolism, the principles of reaction reversibility, metabolic pathways as whole systems instead of individual reactions, and predictions of the dynamics of a pathway in response to changes in the cellular environment. A key for the activity is available from the corresponding author upon request.

This activity addresses fourteen learning objectives and is taught over two weeks of a four-credit course.

Metabolic Pathway Dynamics and Regulation

Learning Objectives

1. State the cause, symptoms, and treatment for Hereditary Orotic Aciduria
2. Explain what it means to say that a metabolic pathway is a system and why it is important to think of them as systems
3. Interpret visual representations of metabolic pathways
4. Define “flux” in a chemical pathway
5. Explain the following ways that enzymes are regulated: substrate-level control, feedback control, heteroallostery
6. Use the pyrimidine synthesis pathway to illustrate the fact that enzyme regulation allows metabolic pathways to be sensitive and responsive to an organism’s environment
7. Use the example of aspartate transcarbamoylase to describe the noncovalent interactions and structural changes that happen when allosteric molecules bind to allosteric enzymes
8. Note that metabolic pathways include near-equilibrium and far-from-equilibrium reactions.
9. Distinguish among standard, actual, and equilibrium conditions and ΔG values.
10. Use the following equation to calculate Q: $\Delta G = \Delta G^{\circ\prime} + RT \ln Q$.
11. Distinguish between Q and Keq.
12. Define steady state.
13. Provide examples of the fact that steady state levels of metabolic compounds are influenced by the regulation of numerous biological reactions.
14. For a given scenario, predict how changes in one metabolic pathway can influence flux through another pathway.

To this point in the semester we have studied the structure and function of macromolecules like proteins, considered how enzymes (i.e., one class of

proteins) work to change the kinetics of chemical reactions. We have also considered that enzymes can be inhibited. We have only considered isolated chemical reactions. Now we will shift our thinking to systems of chemical reactions that we call metabolic pathways. Metabolic pathways show us how biological molecules connect to each other in complex networks and that a change in one component of the network can affect many other components.

1. **Read the case study of Orotic Aciduria posted to eLC.**

   A. What enzymes are deficient in Orotic Aciduria?

   B. What are the symptoms or Orotic Aciduria?

   C. Why does orotic acid buildup in these patients?

   D. What is the treatment and why does it work?

2. **Use your textbook Figure 19.9, de Novo biosynthesis of pyrimidine, as a reference for this question. In this case, we are considering the pyrimidine biosynthesis pathway as an example that illustrates the importance and features of metabolic pathways. Work your way through the questions below to make sure you know how to "read" the pathway.**

A. How many enzymes are in this pathway? How are they visually represented?

B. How many distinct substrates are in this pathway? Not all substrates are visually represented in the same way. What are the different ways the substrates are visually represented?

C. The reactions shown are all visually represented with unidirectional arrows. Should we assume that the reactions can only go in the forward direction? Why or why not? What additional information would we need to determine the favored direction of each reaction?

D. How many enzymes in the pathway are heteroallosterically regulated? What are the allosteric molecules and do they activate or inhibit the enzymes? How is heteroallostery visually represented?

E. Tying back to question 1c, state how problems with heteroallosteric regulation lead to orotic aciduria?

NOTE: Biochemists do not have good conventions for visually representing metabolic pathways. Enzymes, substrates, arrows, and heteroallostery are visualized in different ways across textbooks and even within the same textbook. Beware of this. Ask if you are unsure how to interpret the representation. Now let's try to understand more about the concepts that the visual representations attempt to convey.

3. **Explain how substrate-level control could influence pyrimidine synthesis.** Use step 4 (dihydroorotate → orotate) as your example for thinking about substrate-level control. *Note: In this scenario, assume the enzyme is functional.*

4. Feedback control and heteroallostery

A. Consider enzyme 2 in pyrimidine biosynthesis. This enzyme is aspartate transcarbamoylase (ATCase), and it catalyzes the conversion of aspartate + carbamoyl phosphate to carbamoyl aspartate. CTP is a heteroallosteric regulator of ACTase; CTP participates in feedback inhibition of ATCase. Explain what it means for CTP to be a feedback inhibitor and why cells benefit from feedback inhibition.

B. Summarize the mini-lecture you heard about the reasons that ATP is a heteroallosteric activator of ATCase.

C. Imagine a scenario in which CTP is inhibiting ACTase. How will this affect the concentrations of the following molecules?
   - Aspartate -
   - Bicarbonate -
   - Carbamoyl aspartate -
   - Orotate -
   - Uridine monophosphate -
   - CTP –

D. Use Figure 8.37 from your textbook to describe how CTP and ATP affect the structure and activity of ATCase.

E. Consider Figure 8.38 from your textbook. Noncovalent interactions are enabling ATP, CTP, UTP, and Mg^{2+} to interact with ATCase. Based on the structures of these heteroallosteric molecules, propose what types of ATCase R groups they may be interacting with. Name at least two types of R groups that may be involved.

Now that we have studied some principles of metabolism using the pyrimidine biosynthesis pathway, let's look at another pathway that is more familiar to you, glycolysis.

5. **Near- and Far-From-Equilibrium Reactions.** Examine Figure 16.1 and Table 16.1 from your textbook. These figures describe glycolysis, which we will spend more time on in Unit 3. For now, we want to use glycolysis as an example of metabolic pathway control and steady state.

   A. Name two glycolysis steps that are near equilibrium and two that are far from equilibrium. Explain which delta G value you used to make your decision and why.

   B. Explain the relationship between the driving force of a reaction and the distance of that reaction from equilibrium.
   C. Compare the near- and far-from-equilibrium steps of glycolysis. Are near- or far-from-equilibrium steps more likely to go in reverse? Explain.

D. Based on your answer to #4, do you think near- or far-from-equilibrium steps are more likely to be the control points in a metabolic pathway? Explain.

6. **Distinguish among the ΔG of reactions under various conditions.** We have established that cells tend to exert control over flux through a pathway at one or a few steps, not every step. We have also established that these steps tend to be far-from-equilibrium steps. The questions below help you distinguish the relationships among ΔGs values at standard, steady state, and equilibrium conditions. We will do this by considering step 2 in glycolysis: the interconversion of glucose-6-phosphate and fructose-6-phosphate.

	Concentration of Glucose-6-phosphate	Concentration of Fructose-6-phosphate	Mass Action Ratio ([F6P]/[G6P])	Free Energy
Standard conditions				**ΔG°'= 1.7 kJ/mol**
Steady state conditions				**ΔG = -2.5 kJ/mol**
Equilibrium conditions				**ΔG =**

A. Under standard conditions, what is the concentration glucose-6-phosphate? Fructose-6-phosphate? The mass action ratio? Write these numbers in the table.

B. Calculate the concentrations and mass action ratio for this reaction under steady state conditions. Use this equation: ΔG = ΔG°' + RT ln Q. *Note that you are solving for Q. R = 0.008314 kJ/(mol*K) ; T = 298K*

i. What is the mass action ratio?
ii. Assuming there is 1 mM of fructose-6-phosphate in the cell under steady state conditions, what would be the concentration of glucose-6-phosphate?
iii. Write these numbers in the table.

C. Equilibrium conditions:
   i. What is the ΔG at equilibrium?
   ii. Use the following equation to calculate the equilibrium concentration and mass action ratio at equilibrium: ΔG = ΔG°' + RT ln Q. Note that you are solving for Q again.

iii. Under equilibrium conditions Q has a special name. What is it?

iv. Write this information in the table.

v. Is the interconversion of glucose-6-phosphate and fructose-6-phosphate near- or far-from-equilibrium? Is this step likely to be regulated? Explain.

vi. ***For practice on your own,*** create and complete a similar table for step 1 of glycolysis: glucose + ATP → glucose-6-phosphate + ADP + H^+

7. **Maintenance of steady state.** Given our work so far, we know we need to consider the following to understand flux through a metabolic pathway:
   - What are the control points?
   - What are the near- and far-from-equilibrium steps?
   - How do the concentrations of reactants and products compare among standard, steady state, and equilibrium conditions?
   - Given all these considerations, let's think about steady state and how cells maintain it.

   A. Explain steady state in your own words.

   B. In this case, we have considered pyrimidine biosynthesis and glycolysis. These two pathways may seem disconnected, yet figure 13.17 from your textbook shows linkages between them. Consider three questions:

      i. Explain how glycolysis could influence steady state levels of the metabolic compounds in pyrimidine synthesis. Remember that the end product of glycolysis is pyruvate.
      ii. Imagine that pyrimidine biosynthesis slows down (e.g., through inhibition of ATCase). How might this impact flux through glycolysis? Explain.
      iii. Imagine a person who has just consumed a glucose-rich meal. As a result, flux through glycolysis increases. Could this impact flux through the pyrimidine biosynthesis pathway? Explain.
      iv. Given these three scenarios, generalize to describe how steady state levels of metabolic compounds are influenced by the regulation of numerous biological reactions.

Appendix B

Supplemental Data for Part 2

B.1: Lentiviral Sequence Design

Smoothened:

Forward Primer: **5' – GGTTTAGTGAACCGTCAGATC – 3'**
Amplification upstream of Nhe1 restriction site

Reverse Primer: **5' – CATGCATACGCGTACTAGATCTCAGCGCCGGC – 3'**
7bp of plasmid + Mlu1 RE site + BP Tail

Arl13b:

Forward Primer: **5' – GCTAGCTGCTAGCTCCAGTCTGATGGCCAACTG – 3'**
Bp Tail + Nhe1 RE site + 20bp of plasmid

Reverse Primer: **5' – CGATCGATACGCGTGTAAACGACGGCCAGTGAAT – 3'**
Bp Tail + Mlu1 RE site + 20bp of plasmid

Tubulin:

Forward Primer: **5' – GGTTTAGTGAACCGTCAGAT – 3'**
Amplification upstream of Nhe1 RE site

Reverse Primer: **5' – CGTACGTACGCGTTCAGTTATCTAGATCCGGTG– 3'**
20bp of plasmid + Mlu1 RE site + bp tail

Sstr3:

Forward Primer: **5' – GCTACGTGCTAGCCTCGAGACCGCCATGGCCAC – 3'**
Bp tail + Nhe1 RE site + 20bp of plasmid

Reverse Primer: **5' – GATCGATACGCGT AATACTAGATCTCAGCGCCG – 3'**
Bp tail + Mlu1 RE site + 20bp of plasmid