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Structural dynamics in the PriA DNA helicase govern the initiation of the PriA/PriB/DnaT replication restart pathway

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Biochemistry)

at the

UNIVERSITY OF WISCONSIN-MADISON

2026

Date of final oral examination: May 5th, 2026

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Appendix III. A personal journey into DNA replication and repair

I would like to dedicate this chapter to my dad, James Deorio. Thank you for always guiding me, supporting me, and encouraging me to live curiously. Without you, I would not be the woman and scientist I am today.

My scientific curiosity has been shaped by personal, real-life experiences that compelled me to ask the questions of how, what, when, and why. While these moments are countless, there are several that stand out that I now recognize as pivotal in shaping me into the scientist I am today. From elementary school through graduate training, science has remained central to my identity. I have learned to embrace challenges, ask the difficult questions, pursue demanding paths, explore new disciplines, and build a career despite adversity. This chapter reflects on my journey through science, and the profound influence my father had in shaping that path. I will revisit the formative memories that I hold most closely. These moments where I spent discussing science with him have fundamentally shifted my perspective on the world and my place within it. I will also describe the scientific contributions I have made through my doctoral research, emphasizing a new lesson that has defined my growth: failure and hardship are not obstacles to success, but essential components of it. This chapter is written for the Wisconsin Initiative for Science Literacy (WISL) driven by a goal to enhance science communication for non-scientists. Thank you to Rodney Schreinder, WISL Associate Director, Bassam Shakhashiri, William T. Evjue Distinguished Chair for the Wisconsin Idea and Director, Elizabeth Reynolds, editor, and Cayce Osborne, WISL outreach coordinator.

The strawberry

As I reflect on my scientific journey over the last several years, I am lucky enough to remember the first moment in which I began to question life on Earth. Sometimes our memories change, they shift and soften over time, shaped by feelings, and often become more vivid than the photographs we try to preserve. This one, however, remains

especially alive. This memory is full of color, the feeling of the warm sun on my neck, dirt pressed beneath my fingernails, and the image of my father's tan face and brown eyes. It is a memory I hold so closely that I sometimes worry it may one day fade. Writing it here relieves me of that fear, anchoring it into permanence. This memory will be forever fixed in words, if not in time.

It begins with me walking along the garden that my mom and dad planted in my childhood home in Stratford, Connecticut. I remember the growing and tangled vines of cucumbers and tomatoes, sprawling within a makeshift cage designed to protect them from wandering animals. I would check on the garden constantly, watching and waiting for signs of growth, often disappointed that our vegetables never seemed to reach the size of those in the grocery store. I can feel the sharp spikes on the outside of the cucumber, the fuzz wrapped around its vine, and the deep craters of the tomatoes. But the fruit that held my attention the most was the strawberry. It was not my favorite fruit, yet something about its color and its sweetness set it apart from everything else.

My dad was a handy man, a do-it-yourself guy, and was unbelievably smart. On this day, he was in the garden, hands deep in the dirt removing weeds. This was often a task that he would assign to me or my other siblings, focusing our labor to the rose garden or along the stone pebble driveway. But on this day, he took on the task himself. I can still remember his dirty and scuffed-up Timberland boots, speckled with drips of paint and concrete. He often wore dark blue demin jeans, also speckled with dirt and paint, and his light grey Comprehensive Masonry T-shirt. I opened the latch to allow myself into the garden and went to check on my strawberries. Digging my fingers into the dirt, I discovered a tiny, slightly off-white berry among its vibrant red-berry siblings. I turned to

my dad and questioned him about its odd color. The details of our conversation have been lost with time, but I remember him pausing his weeding and taking the strawberry from my hands. He explained that this strawberry, like me, was made up of millions of cells that were controlled by a large molecule called DNA. This molecule could be found in all living things and holds the instructions for life. He explained that the DNA instructed the strawberry on when to change color, likely influenced by the sun and garden nutrients. Its color was a visual cue that the strawberry was ripe and ready to attract animals to help spread its seeds. It was there, in the garden of my childhood home, alongside my dad, where I was first introduced to DNA. This memory, morphed by time, aging, change, and loss, will forever mark the day in which I decided to let my curiosity guide me. It is the day when I began to question the existence of our world, not be afraid of its complexities, and to share that spark with those around me.

The lightbulb

My dad was a very skilled stone mason. In the basement of my childhood home, he had a workshop, where he stored all his tools, built firewood boxes, and sketched the designs for his projects. The floor was covered with saw dust, dirt from his boots, and paint splatter. The room smelled of freshly cut wood and paint and was lit by a single dangling light bulb in addition to the sun from a tiny side window overlooking the back driveway. One evening I was sitting on a stool in his workshop as he organized his bench, when suddenly, the dangling lightbulb turned off. He stopped what he was doing and looked right at me, expecting that I was the reason the light had turned off. To his amazement, I sat in the dark on my stool, far from the light. He went to the lightbulb, pulled the metal

string, and with a click, the light turned back on. For the next several minutes, he focused his attention on the string and the bulb itself, trying to replicate the light suddenly turning off. His curiosity as to how this light turned off, his knowledge of how a lightbulb became bright, and how much force was needed to pull the string to hear the click, excited me. Together, we played with the string while he taught me about electricity, gravity, and force. This was one of my first exposures to the scientific method: observe, question, predict, test, analyze, repeat. From that day forward, every action I took became an iteration of the scientific method, guided by curiosity, shaped by questions, and refined through experience.

The pandemic

At the start of the Covid-19 pandemic, I had just graduated from Penn State University with a degree in biochemistry and molecular biology and had begun working in a new research lab on campus. As the situation rapidly escalated, campus operations were suspended, uncertainty grew, and the first wave of lockdowns took effect. During this time, I was frequently checking in with my parents, who lived just outside of New York City in Connecticut. I learned that their local stores had been entirely depleted of essential supplies, including toilet paper, paper towels, hand sanitizer, masks, and basic medications. In contrast, State College, Pennsylvania had not yet experienced the same level of shortage. I therefore purchased and gathered items to bring back to my family in Connecticut.

I remember pulling into my dad's driveway with a box of gloves, hand sanitizer, and masks in the backseat. We met outside, both wearing masks, keeping our distance. He asked me about the virus, and I explained its basic life cycle and why it posed a heightened threat to older and immunocompromised individuals. At the time, he was undergoing treatment for his liver, so I emphasized the importance of social distancing, frequent handwashing, and avoiding public spaces. We parted ways with a hug, despite the recommendation for social distancing, and he stood in the driveway, waving goodbye as I drove back to Pennsylvania.

Throughout my time in college and after graduation, my dad often asked me questions about DNA, physics, chemistry, and medicine. As I grew into my role as a scientist and as he grew older, our conversations evolved; he became the one asking questions, and I became the one teaching. Unlike anyone else in my family, my dad shared my deep curiosity about our world, galaxy, and universe. It became our common language; a way we connected and understood each other. He shared unique bonds with each of my siblings – history with Jack, architecture with Sam, and the love of Christmas movies with Mina. With me, it was always science.

The last time I saw my dad was in August of 2020. I left his hospital bedside leaving behind a bouquet of red roses for him to smell and touch, reminiscent of the rose garden he planted at my childhood home. Just four months after I spoke with him that day in his driveway about the virus, his health, plans for future family gatherings and holidays, and my intention to pursue my doctorate, he passed away. During one of the most difficult periods of my life, and of so many others that year, I made a promise to myself: I would continue to follow my curiosity, ask difficult questions, and become the scientist he always

encouraged me to be. I committed not only to myself, but to him and to my family, that despite uncertainty and hardship, I would honor the sacrifices my parents made. I would carry forward the long conversations we shared about the universe and genetic traits that connect us and the countless hours spent studying, working, and pushing myself to grow. I decided that I would be the first in my family to pursue graduate education. I would become a scientist.

Defining the regulatory mechanism of DNA replication restart

In August of 2021, I packed up my belongings and moved to Madison, Wisconsin, to pursue my PhD in biochemistry. I was drawn to research that focused on understanding the mechanisms used to copy and protect our genetic code. I joined Dr. James Keck's lab in the Department of Biomolecular Chemistry, where his research focuses on using high-resolution structural techniques to reveal atomic-level details of protein machines that copy DNA. My project centered on understanding how these molecular machines regulate their activities to ensure faithful DNA duplication.

In every cell, the genetic information that determines traits, such as a cat's fur length or our eye color, is encoded by DNA (1). This large molecule is made up of four letters (nucleotides = A, T, C, G) which provides the instructions that guide cellular function. Like the 26 letters of the English alphabet, unique combinations of the four nucleotides provide the information the cell needs to produce molecular machines (proteins). Each letter has its own structure and complementary partner (A:T, C:G), allowing the sequence to be accurately read and copied (2, 3). Every living organism

contains a library (genome) full of books (genes) that use this four-letter alphabet to describe all types of stories (proteins). This genetic library is wrapped tightly in the center of the cell, forming the chromosomes.

Unlike bacteria, which are single celled organisms, humans and others (such as plants, fungi, archaea, and mammals) are made from millions of cells that communicate to one another. Each cell contains an identical genetic library, ensuring that every cell carries the same instructions to perform its specific functions. All organisms can produce new cells from pre-existing ones through a process called cell division. A critical step in cell division is the precise copying of all genetic information from the old (parental) cell to the new (daughter) cell. This process, known as DNA replication, relies on specialized protein machines that unwind the DNA, exposing the code, and uses the same A, T, C, G letters to write a copy of the sequence to produce a new chromosome (4, 5). In humans, each cell contains 46 chromosomes arranged into 23 pairs. DNA replication in human cells typically takes ~ 6 to 8 hours due the large size of the human genome, which consists of roughly 3.2 billion letters. In contrast, bacterial genomes are much smaller, with a range of 4 – 13 million letters, thus allowing DNA replication to occur more rapidly.

Our cells stress the importance of accurately copying the genetic code. Single letter changes (point mutations) can lead to genetic diseases such as sickle cell anemia and cystic fibrosis (6). This evolutionary pressure has shaped the development of protein machines that stabilize and support the replication machinery. In addition to the copying of the DNA, the DNA molecule itself is vulnerable to damage from internal toxins and external agents that can alter the structure of the nucleotides, thereby threaten the stability of DNA and compromise the integrity of the genetic code.

For decades, scientists have studied simple single-celled organisms to gain insight into the complex molecular processes that govern cellular function. These fundamental mechanisms by which DNA is copied and protected are remarkably conserved from these simple organisms to complex ones, including humans (7). Just as all car engines share the fundamental components and principles needed to make the car move, the basic requirements for each part is conserved across all car types. Variations typically arise in specific features that manufacturers have refined or optimized over time. Evolutionarily, the high degree of conservation in these molecular processes makes it possible to investigate how bacteria and other simple organisms replicate and safeguard their genetic information. Insights gained from these studies can then be applied to more complex organisms, allowing us to anticipate and predict similar mechanisms at work in human cells.

In James Keck's lab at the University of Wisconsin-Madison, we study the model organism *Escherichia coli (E. coli)* to better understand the molecular mechanisms that safeguard the genetic code during DNA replication. *E. coli* is a small, 2-micron rod-shaped bacterium naturally found in the lower intestines where it contributes to gut health, although some strains can cause serious disease. In the laboratory, *E. coli* divides every 20 minutes and is easy to handle and experimentally manipulate, making it an ideal model organism. For decades, scientists have investigated DNA replication and repair in simple model organisms, which rely on evolutionarily conserved mechanisms that are shared with more complex systems, like humans. These organisms, like *E. coli,* have been foundational to our understanding of DNA replication, offering critical insight into how genome integrity is preserved in multicellular life.

Like all living organisms, *E. coli* must accurately replicate its genetic information during cell division to ensure that each daughter cell inherits a complete copy of the genome. However, during DNA replication, the highly complex replication machinery, the replisome, can encounter damaged nucleotides or roadblocks. When this occurs, the replisome can become destabilized and disassemble from the chromosome. At these stalled sites, the DNA is left vulnerable, risking incomplete chromosomal duplication and potentially cell death (Figure 6.1A). To counteract these threats, *E. coli* have evolved backup protein machines that detect when replication has failed and reloads the replisome at these sites. This process, known as replication restart, is critical for ensuring complete and faithful DNA replication (8–11). By studying how *E. coli* preserve their genomic DNA and ensure faithful DNA replication, we can use our findings to better help predict and understand similar mechanisms employed in more complex organisms, which often become dysregulated or damaged in cancer and disease.

The primary replication restart pathway in *E. coli* is the PriA, PriB, and DnaT pathway. Collectively, these three proteins engage with the DNA at the stalled site, build a multi-protein complex called the primosome, and recruit the replication proteins back to the DNA to resume DNA replication (Figure 6.2). Similar to a LEGO set, the addition of specific pieces in a set order must occur for the structure or design to be accurately made. The PriA/PriB/DnaT complex is assembled in a specific order, in which PriA binds to the DNA first, followed by the recruitment of PriB then DnaT (9–11). This ordered assembly is likely evolutionarily essential to ensure that the formation of this complex only occurs at sites where replication has failed.

To understand how proteins bind to each other and to DNA, researchers have developed structural approaches that capture highly detailed snapshots of these complexes. Techniques such as cryo-electron microscopy (cryo-EM) and x-ray crystallography use electrons and x-rays, respectively, to resolve the molecular architecture of proteins with atom-level detail (Figure 6.3). This field, known as structural biology, has been instrumental in defining the key features of the PriA, PriB, DnaT replication restart proteins (Figure 6.4). While these approaches are extremely powerful, they only provide a static snapshot and are often insufficient to fully describe the molecular details of complex pathways. Like a photograph captures a single moment in time, it misses the sounds, scents, and textures that a video can bring to life. To overcome this, scientists often combine these imaging methods with other approaches, like intentionally mutating proteins and performing tests both outside and inside the cell to see how these changes affect protein function. This enables a more comprehensive, multi-perspective understanding on the mechanisms of these biological processes.

My doctoral research primarily focused on using cryo-EM, protein mutagenesis (modification of protein composition), and genetics to describe the molecular mechanisms that govern the sequential assembly of the PriA/PriB/DnaT primosome. Much like how a key's shape is specific to its lock, proteins possess unique molecular structures that dictate their cellular roles. PriA, the first protein to begin assembling the primosome, has a structure tailored to recognize and engage with specific DNA configurations present at failed replication sites. PriA binds to three-way forked DNA structures, using multiple regions of the protein to stabilize and protect the DNA. Past high-resolution structures of

PriA revealed the atomic interactions of specific regions of the protein that engage and stabilize the abandoned DNA replication fork.

Using Cryo-EM, my graduate research revealed that variations in the DNA structure trigger movements within a small region of PriA (called a domain) which exposes the docking site for the second and third primosome components, PriB and DnaT. Analogous to a key turning a lock, this structural rotation ensures that PriB and DnaT binding occurs in a precise and regulated manner (Figure 6.1B). Using protein mutagenesis (Figure 6.5), I then demonstrated that disrupting this rotation of the small PriA domain prevented PriB from engaging with the complex. When tested within the cell, disruption to the rotation of PriA's domain impairs restarting DNA replication. This highlights that molecular dynamics within PriA are critical for proper function of the primosome.

My research revealed that *E. coli* has tightly regulated systems to ensure DNA is faithfully replicated. It is critical that the cell initiates replisome reloading strictly to DNA replication forks, as aberrant DNA replication initiation can jeopardize genome integrity. My work illustrates a dynamic dance between the stalled DNA replication fork, PriA's engagement with the DNA, and PriA's internal rearrangements that expose the binding sites for PriB and DnaT. This work expands our understanding of how *E. coli* maintain the accuracy of the genetic code by rapid DNA detection, DNA replication fork binding and remodeling, and domain rotations in PriA, which collectively ensure accurate replication restart.

Absence of any of these steps inhibits the continuation of the pathway, acting as a safeguard to ensure appropriate replication restart. Despite these findings, many questions remain regarding the regulation of the PriA/PriB/DnaT replication restart pathway: which of the three proteins (PriA, PriB, DnaT) recruits and directs the loading of DnaB to the abandoned replication fork? What are the molecular mechanisms controlling DnaB recruitment and reloading? After DnaB reloading, how is the primosome disassembled so DNA synthesis can continue? These questions will prompt the next wave of scientific research into the regulation of replication restart in *E. coli*.

Although these experiments contributed to advancing the field of replication restart, they were often accompanied by failure, late nights in the lab, missed holidays with family, and moments of intense self-doubt. I frequently found myself reflecting on the time before graduate school, when my sense of drive, confidence, and joy in science felt effortless. During my graduate school interviews, I was often asked “Are you resilient?” At the time, I did not think graduate school could be more challenging than the personal hardships I had faced. But it was through repeated failure, uncertainty, and the challenge of teaching myself complex techniques, that I came to understand the meaning of resilience. My success was not achieved alone. It was made possible by the unyielding support of my advisor, the motivation and guidance from my lab mates, the long phone calls with my mother, and the steady love of my life partner. I came to Wisconsin as a confident scientist, yet personally feeling lost. Through the many ups and downs of this journey, I have gained clarity in what I value and how I want to move through life. I have learned to trust and care for myself, to stay true to who I am, and to reclaim my confidence.

During the years of my doctoral training, I often thought of those memories I shared with my dad, the memories that brought me to the bench. I had never imagined that a simple conversation about the DNA in an unripe strawberry would one day lead me to studying proteins that bind, unwind, and stabilize complex DNA structures at atomic resolution. With every experiment, seminar, exam, and presentation, he was there alongside me, asking the same questions. I have come to appreciate the privilege of understanding, in such molecular detail, the features that govern life itself. This journey has taught me not only about the biophysical properties of DNA and proteins, and the intricate mechanisms our cells employ to faithfully replicate and protect our genetic code, but also about the importance of trusting myself and pursuing a life driven by curiosity. You never know where a single question will lead. You cannot predict the opportunities you will encounter or the people you will meet. That is what makes the pursuit of science endlessly rewarding.

Thank you, dad.

FIGURES

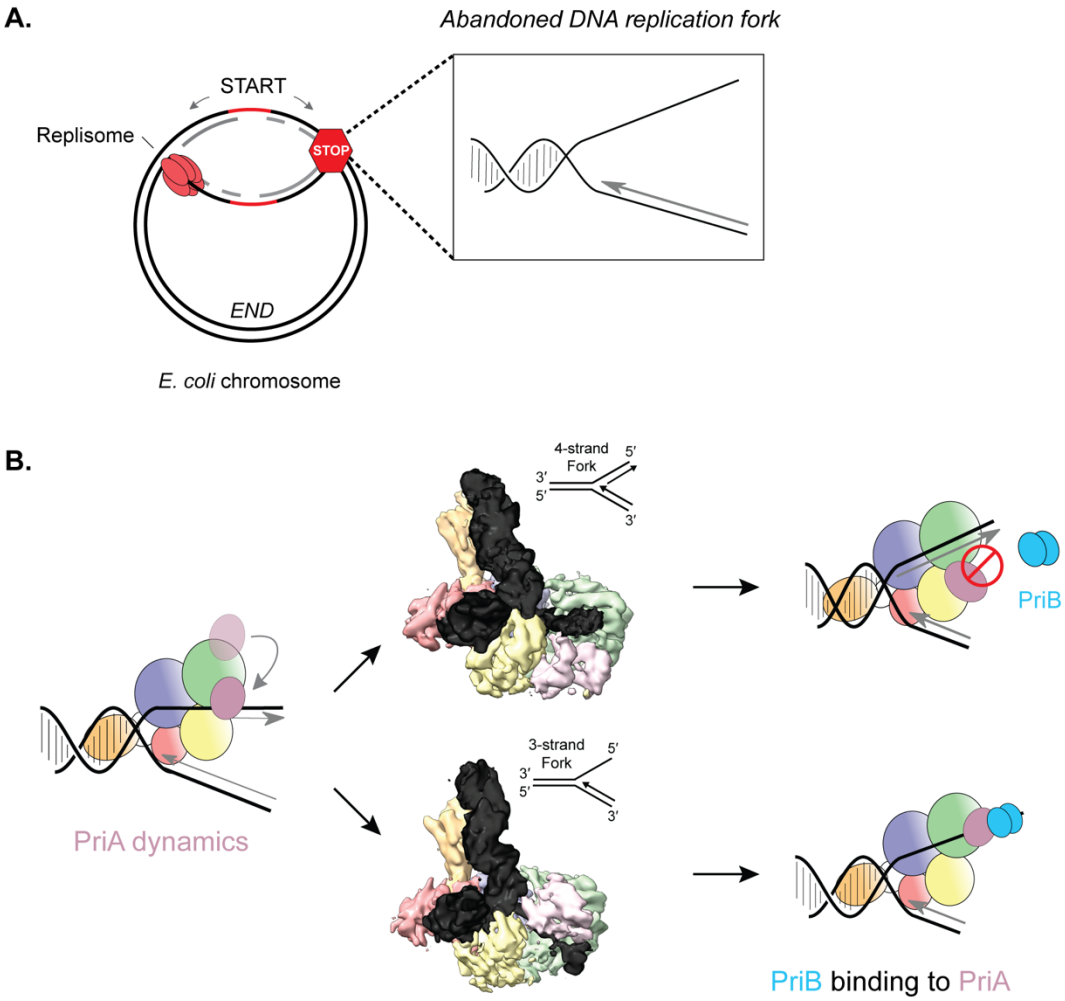

Figure 6.1. Restarting failed DNA replication in *E. coli* through domain dynamics in PriA.

A. *E. coli* chromosomal DNA replication. Upon encountering a roadblock, the right-most replication machinery dissociates, leaving behind an abandoned DNA replication fork. The left-most replication fork continues until it reaches the end (terminus) of the chromosome. DNA replication remains incomplete until both replication forks reach the terminus. **B.** PriA (colored by its different functional domains) binds to the abandoned DNA replication fork. Dynamics of PriA (pink) differ depending on the structure of the DNA.

When on the correct structured replication fork, PriA rotates to expose the PriB docking site.

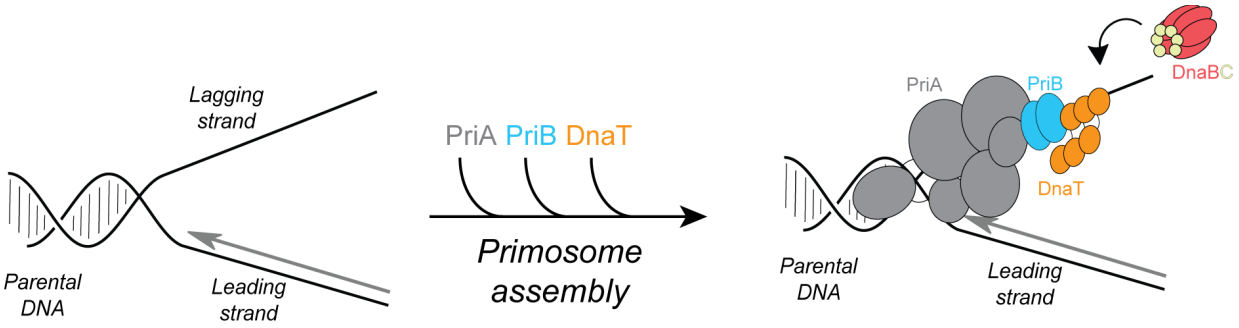

Figure 6.2. Primosome assembly on abandoned DNA replication forks to restart DNA replication.

An abandoned DNA replication is a three-way branched DNA molecule, with the double-stranded unreplicated arm known as the “parental DNA” and the two exiting strands comprising the “lagging” and “leading” strand. These two strands can either be single-stranded (as visualized on the lagging strand) or double-stranded (as visualized on the leading strand). The ordered assembly of PriA, PriB, DnaT on the DNA substrate forms the multi-protein primosomal complex. This complex recruits and promotes the loading of DnaB by DnaC to restart DNA replication.

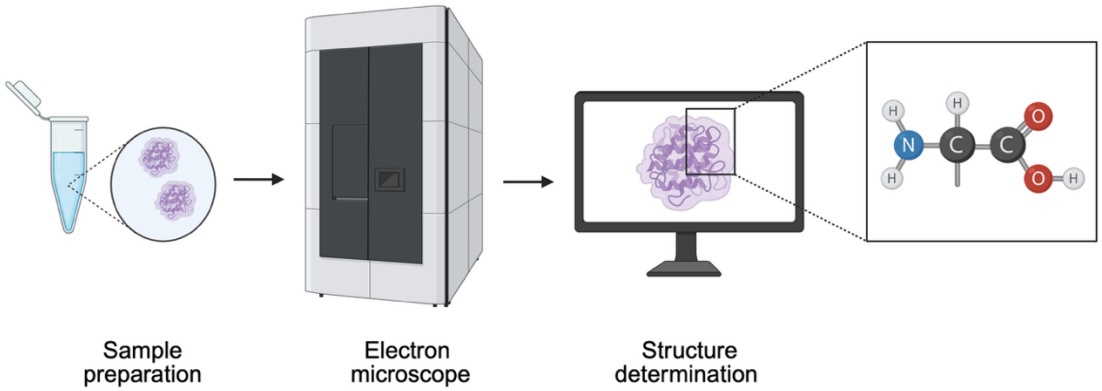

Figure 6.3. Solving high-resolution structures of proteins using cryo-EM.

A highly purified protein sample is imaged using a high-resolution cryo-EM microscope. Upon exposure to an electron beam, electrons are scattered by the sample and captured by a detector. Thousands to millions of images are then computationally processed and averaged using software to reconstruct a high-resolution three-dimensional structure, revealing details into the unique features of the protein.

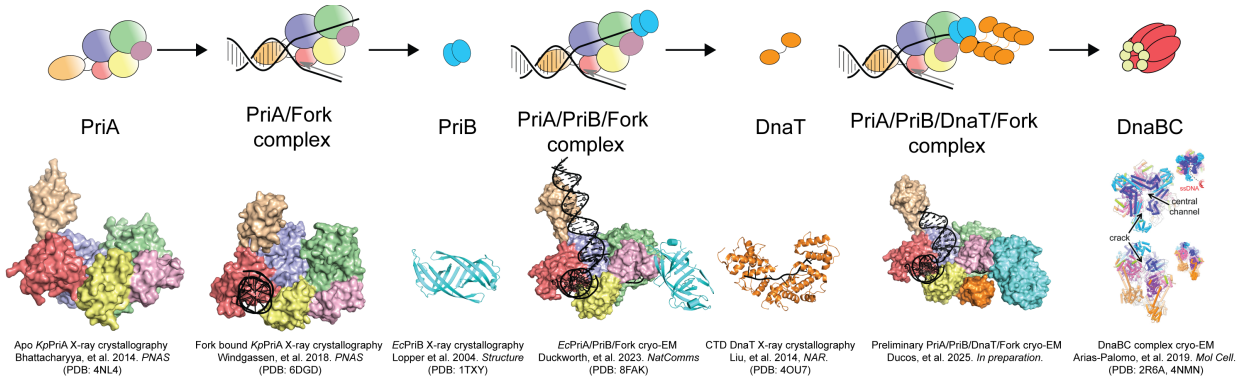

Figure 6.4. Solved high-resolution structures of the PriA/PriB/DnaT replication restart pathway proteins.

PriA recognizes and binds to stalled DNA replication forks, initiating assembly of the restart machinery. PriB associates with PriA and the DNA, stabilizing the complex and facilitating DnaT recruitment. The formation of the primosome, the multi-protein complex with PriA, PriB, and DnaT bound to the DNA replication fork, enables the loading of DnaB to restart DNA replication.

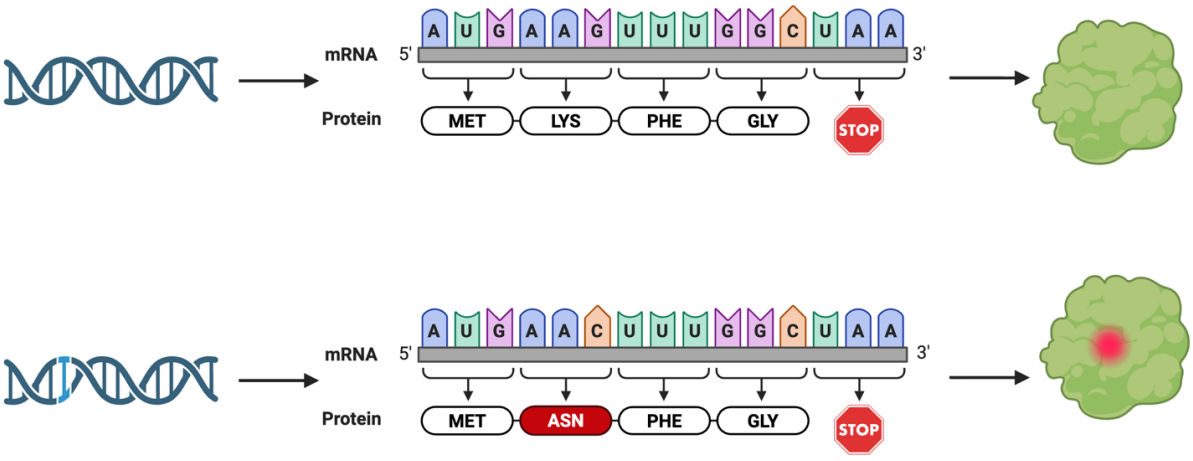

Figure 6.5. Protein mutagenesis to interrogate changes in protein function.

DNA encodes the sequence that is passed down in the form of a messenger molecule (mRNA) that is used to produce a protein. A combination of three bases (A, U, G, C) in the messenger molecule provide information to the protein-production machinery on what pieces are needed to assemble the protein. (Top). Changes to the DNA sequence (light blue) results in a change in the messenger molecule sequence (Lys → Asn) and resultantly modifying the composition of the protein. (Bottom).

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