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Tanner Dean's Scholar Program Summer 2019

Elucidating the Checkpoint Signaling Functions of the RAD9A Mammalian DNA Damage Response Protein

Abstract

Proper genome maintenance is required for the survival and function of cells in all organisms. To respond to DNA damage, cells have developed an elaborate mechanism known as the DNA damage response (DDR). The DDR monitors the genome and upon insult to DNA, activates a protection response through a series of phosphorylation events and other post-translational modifications. Checkpoint activation halts cell cycle progression to ensure that DNA damage is properly repaired before DNA replication or chromosome segregation is attempted. A main player in the DDR is the RAD9A-HUS1-RAD1 (9-1-1) complex, a heterotrimeric clamp with a toroidal molecular structure that serves as a molecular scaffold at the site of DNA damage. Through interaction between RAD9A and TOPBP1, 9-1-1 indirectly activates ATR, one of the main regulators of checkpoint signaling and also directly participates in DNA repair by recruiting a variety of DNA repair proteins. Given the dual function of the 9-1-1 complex, my project this summer will focus on a model utilizing a RAD9A separation of function mutant (S385A) that specifically disrupts the checkpoint signaling 9-1-1/TOPBP1/ATR axis but leaves the DNA damage repair functions of 9-1-1 intact. Conducting a phosphoproteome analysis of genotoxin treated and untreated wildtype and RAD9A S385A mutant MEFs will provide insight into the phosphorylation events dependent on 9-1-1 signaling but independent of ATR. These studies will help elucidate novel protein interactions of the checkpoint signaling functions of the 9-1-1 complex.

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Biographical Sketch

Growing up in Northern Virginia, I had the privilege to conduct research in Dr. Yihong Ye's laboratory at the National Institute of Diabetes, Digestive, and Kidney Diseases through the National Institutes of Health Summer Internship Program. That summer I studied the misfolded protein secretion pathway implicated in neurodegenerative diseases like Parkinson's. Working at the NIH was one of the most intellectually stimulating environments I had been in, surrounded by a constant flow of creative ideas, strong collaboration, and new questions. My time at the NIH helped me realize that I want to be in a career field that is always changing with new challenges.

At Cornell, I have continued to pursue studying the biomedical sciences. I joined Dr. Robert Weiss' laboratory as a freshman to complement my courses and have found that the research I am doing is can answer deeper questions I had after taking classes like biochemistry. My research in the Weiss lab focuses on elucidating the roles of the DNA damage response pathway. It has helped me see the connections between basic science and advancements in the medical field. Given that I have worked in a laboratory for several years and my interest in the biomedical sciences, after graduation, I hope to attend medical school and remain connected with current research to better treatments by serving as a physician who enrolls patients in clinical trials.

Outside of my research, I am involved with Cornell MEDLIFE (Medicine, Education, and Development for Low Income Families Everywhere) and Cornell Club Swim, serving as President of both organizations. My work with these organizations has taught me the value in connecting with people of different backgrounds. I also serve as the Philanthropy Chair of the Cornell chapter of Phi Delta Epsilon International Medical Fraternity, as a Biology Student Advisor, and as a Teaching Assistant for BIOG1445: Introductory to Comparative Anatomy and Physiology, Individualized Instruction.

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Statement of Purpose

DNA is constantly exposed to damaging agents from both endogenous sources, such as replication stress and reactive oxygen species, and exogenous sources, such as UV radiation and chemical mutagens. Unrepaired DNA damage can result in genomic instability, errors in transcription, and tumorigenesis. As a result, cells have developed DNA damage response (DDR) mechanisms to maintain genomic integrity. The DDR consists of signal transduction pathways that have evolved to sense DNA damage and trigger downstream activation of a plethora of proteins to halt cell cycle progression and repair the damage or initiate apoptosis if the damage is too severe [1].

A key player involved in the DDR is the RAD9A-HUS1-RAD1 (9-1-1) heterotrimeric checkpoint clamp complex. Upon DNA damage, the 9-1-1 complex is loaded to the damage site at the junction between double-stranded DNA and RPA-coated single-stranded DNA. Independently, ATR is recruited by the ATR-interacting protein ATRIP to the site of damage. The phosphorylated C-terminal tail of RAD9A interacts with TOPBP1, allowing for the activation domain of TOPBP1 to bind and activate ATR. Once activated, ATR phosphorylates many downstream targets, including the kinase CHK1. Phospho-CHK1 can then phosphorylate several downstream factors, notably CDC25, initiating cell cycle arrest through checkpoint signaling to allow for DNA repair or the initiation of apoptosis [2,3,4].

Much is understood regarding the interactions of ATR with downstream signaling substrates, but little is currently known about which phosphorylation events are strictly dependent on 9-1-1-mediated ATR activation. Recently, another protein ETAA1 was discovered to activate ATR independently of the 9-1-1/TOPBP1 axis [5]. Because dual loss of ETAA1 and TOPBP1 ATR activating domains displays synthetic lethality in vitro, it can be hypothesized that ATR

activation through both axes has similar but distinct functions [5]. Studying these pathways is key to understanding the specific checkpoint signaling functions of 9-1-1 mediated by ATR activation as a response to DNA damage.

As a DNA clamp, the 9-1-1 complex has a toroidal structure with an inner ring that allows it to interact with DNA. The outer ring of the clamp not only contains the TOPBP1-interacting C-terminus tail of RAD9A, but also serves as a molecular scaffold for numerous DNA repair factors involved in a variety of DNA repair pathways such as base excision repair, mismatch repair, and homologous recombination [6]. Deletion of any of the 9-1-1 subunits in mice results in embryonic lethality, highlighting the importance of the clamp [7]. Interestingly, *Hus1* is upregulated in many cancers, suggesting a role for the complex in modulating stress tolerance in cancer cells [8]. Because genomic instability is a hallmark of cancer and can lead to the progression of a benign tumor to a malignant one, studying the function of the 9-1-1 complex in genome maintenance can lead to a better understanding of the role of DNA damage repair in tumorigenesis.

Previous work in our lab studying *Hus1*-null cell lines has shown that the loss of HUS1 leads to numerous cellular defects such as an increase in genomic instability and stress hypersensitivity [9]. Conditional knockout mouse models with germ cell-specific *Hus1* deletion also show that the loss of HUS1 leads to subfertility in male mice [10]. However, given that the 9-1-1 complex has roles in both activating checkpoint signaling and recruiting DNA damage repair factors, it is necessary to study to each effector function independently to better understand the dual role of the complex. Therefore, to further study the specific interaction between the 9-1-1 complex and ATR, a graduate student in my lab, Catalina Pereira, generated a mouse model using CRISPR/Cas9 to make an S385A point mutation in RAD9A that disrupts the key physical interaction between RAD9A and TOPBP1 that positions TOPBP1 at the site of damage and allows

it to activate ATR [11]. This separation-of-function mutant leaves the outer clamp surface interactions with repair proteins intact while inhibiting its ability to indirectly stimulate ATR activity via TOPBP1. My project this summer will focus on characterizing this model to elucidate the function of the TOPBP1-dependent interaction between 9-1-1 and ATR in checkpoint signaling.

Preliminary work I have done with this model has shown that inhibiting the activation of ATR through the 9-1-1/TOPBP1 axis results in increased sensitivity to treatment with hydroxyurea (HU), a genotoxin that induces replication stress by inhibiting the production of deoxyribonucleotides. Compared to the wild-type control, RAD9A S385A mouse embryonic fibroblasts (MEFs) displayed decreased short-term survival and decreased long-term proliferation following the treatment. My work has been corroborated by others in the lab who have done similar experiments not only with HU, but also with other genotoxins such as mitomycin C and aphidicolin that induce replication stress through various mechanisms. These results indicate that our RAD9A S385A model is unable to cope with genotoxic stress. However more work is needed to ascertain the specific mechanisms affected to elucidate the role of 9-1-1 in the checkpoint signaling pathway.

Therefore, this summer, the focus of my project will be to identify key factors regulating checkpoint signaling in both WT and defective TOPBP1-ATR mediated checkpoint signaling. To accomplish this goal, I will perform a phosphoproteome analysis comparing *Rad9A*$^{+/+}$ and *Rad9A*$^{SA/SA}$ MEFs utilizing immobilized metal affinity chromatography (IMAC) in combination with mass spectrometry (MS). The two cell lines will be grown in either light or heavy isotope SILAC DMEM media, and then either treated with genotoxin or left untreated. The genotoxin treatment will induce ATR activation through 9-1-1 in the wild-type MEFs but not in the *Rad9A*$^{SA/SA}$ MEFs. The cell lysate will be collected and enriched for phosphopeptides. Comparison of the phosphorylation levels of proteins between wildtype MEFs left untreated, wildtype MEFs

treated with HU, S385A MEFs left untreated, and S385A MEFs treated with HU through MS will highlight proteins with differential phosphorylation in the absence of 9-1-1 mediated ATR activation. Since only the 9-1-1/TOPBP1/ATR axis has been disrupted in our model, the results of this experiment will tell us which phosphorylation events occur in the absence of ATR stimulation in response to DNA damage. Understanding which ATR phosphorylation targets are or are not dependent on 9-1-1 will allow for better understanding of the checkpoint signaling function of the 9-1-1 complex in response to DNA damage.

I will conduct my research this summer in Dr. Robert Weiss' laboratory at the Veterinary Research Tower in the Department of Biomedical Sciences of the Cornell University College of Veterinary Medicine. Our lab will provide me with the necessary supplies and equipment such as experiment reagents, cell lines, and machinery. Materials that my lab does not possess are available through shared equipment with the Department of Biomedical Sciences. My mentor, Catalina Pereira, a graduate student in the lab, and the principal investigator, Dr. Robert Weiss, Professor of Molecular Genetics, will guide me through my project this summer. As a Biological Sciences major, I hope to use my findings from my research this summer to guide my senior honors thesis.

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