Abstract

Identifying novel HUS1 interactors that play a role in genome maintenance

Maintenance of the genome is essential for proper functioning and survival of all organisms. Cells experience constant DNA damage, due to both endogenous and exogenous stresses. To monitor and combat DNA damage, cells have evolved a mechanism known as the DNA damage response (DDR). The DDR includes elaborate signaling cascades that activate checkpoints. Checkpoint activation results in halting cell cycle progression to ensure the fidelity of the replicating and transcribing DNA. Checkpoint activation also initiates DNA repair pathways or the apoptotic machinery depending on the extent of the damage. Two of the major regulators of the DDR are the phosphoinositide 3-kinase (PI3K)-related protein kinases ataxia-telangiectasia mutated (ATM), and ATM and RAD3 related (ATR); both playing a major role in maintaining genomic integrity. Disruptions in ATR/ATM signaling can lead to genomic instability. Another element of the DDR is the RAD9-RAD1-HUS1 (9-1-1) complex, a heterotrimeric clamp that has a toroidal structure similar to that of Proliferating Cell Nuclear Antigen (PCNA). 9-1-1 is loaded
onto damaged DNA and acts as a molecular scaffold. By interacting with TOPBP1, 9-1-1 promotes the activation of the ATR kinase; it also directly participates in DNA repair by associating with repair factors.

The focus of my project is on the HUS1 subunit of 9-1-1 and seeks to identify novel interactors of HUS1 that are important for its functions in DNA repair. Although the role of HUS1 in ATR-mediated checkpoint signaling is well known, its function in DNA repair is poorly understood. Preliminary studies done in the Weiss laboratory suggest that HUS1 interacts with several proteins in the neddylation pathway, which is a process in which ubiquitin-like protein NEDD8 is conjugated to target proteins for activation. My project this summer will focus on uncovering the interactions between 9-1-1 and proteins that mediate neddylation, and their functional importance in DNA repair. I will first confirm physical interaction between HUS1 and neddylation pathway proteins such as UBE2M and CUL4A using co-immunoprecipitation. I also plan to perform immunofluorescence assays to investigate the localization of neddylation proteins in Hus1−/− and Hus1+/− mouse embryonic fibroblasts in order to test if HUS1 plays a role in recruiting the neddylation machinery to sites of DNA damage. In addition, I plan to continue with identifying HUS1 residues that are important for protein-protein interactions and testing their functional importance through genotoxin sensitivity assays. Through this project, we can obtain a more complete picture of the DNA damage response. One of the most common characteristics of human cancers is genomic instability, and there has been a connection shown between a dysfunction in DNA damage response to both a neoplastic phenotype and sensitivity to particular therapeutic strategies. By more fully understanding HUS1 and the DNA damage response, these data can be utilized for targeted antitumor therapies that disrupt the DDR by interfering with interactions between HUS1 and downstream effectors.
Biological Sketch

When I walked into Dr. Chand Khanna’s laboratory as a sixteen-year old teenager, all I could wonder was how I could last the entire summer in such a boring and repetitive environment. Not only was it tedious work, I was discouraged by how little I knew, and that nothing was working. However, I realized soon enough that it was only uninteresting because I was approaching it in such a way—I learned that one way to make lab work exciting was to constantly ask myself what I could improve on so that the next trial would work better, and that I should also be asking my mentor questions that started with “why” or “how”.

My time in the Khanna lab at the NIH steered me towards my decision to pursue research as a career. At Cornell, I joined Dr. Robert Weiss’s laboratory as a freshman, and was happy to find that I still loved doing research for an extended period of a time of a school year, and not just during the summer. I also found that a more thorough background in the biology of the research I was doing, with classes such as biochemistry, helped me to think more about the importance of my research, and understand more about how the protocols worked, rather than just doing them blindly.

After graduating, I hope to attend medical school to obtain a MD/PhD and become a physician scientist, conducting translational research in cancer biology. I am particularly interested in finding ways to improve the current methods of early diagnosis of cancer. Having worked in both the Khanna lab and the Weiss lab, I have learned the importance of always thinking back to the larger picture, and of validating and verifying in vitro data translationally in vivo.

At Cornell, I am very involved in classical music at Cornell, as a clarinetist in part of chamber music ensembles and previously as part of the Cornell Symphony Orchestra. I am also a Biology Student Advisor and part of the Becker House Council. I am also President of Cornell’s chapter of MEDLIFE (Medicine, Education, and Development for Low Income Families Everywhere), an organization that strives to provide primary medical care to impoverished people.
II. Statement of purpose (3-5 pages). Please describe your project in detail. Explain the significance of your research and your reasons for wishing to engage in it, and tell us where you will carry out this research and what resources you will use. Discuss briefly how your project relates to existing research in your field on the same subject. Be sure to include a bibliography.

**Statement of Purpose**

DNA constantly undergoes damage from both endogenous and exogenous sources, such as stress caused by replication, reactive oxygen species, or ultraviolet light. To combat these damages, cells have evolved a mechanism to maintain genome stability, known as the DNA Damage Response (DDR). The DDR is a signal transduction pathway that senses DNA damage and stimulates a response to repair the damage. The phosphoinositide 3-kinase related protein kinases (PIKKs) are the main regulators of the DDR, which include the Ataxia-Telangiectasia Mutated (ATM) and ATM and Rad3 related (ATR) kinase. ATR is essential for the viability of replicating human and mouse cells, and is activated during the synthesis phase (S-phase) to regulate the firing of replication origins, repair of damaged replication forks, and to prevent the premature onset of mitosis (1).

One of the key players in the DDR is a heterotrimeric checkpoint clamp composed of three subunits, RAD9-RAD1-HUS1, known as the 9-1-1 complex. The 9-1-1 complex is loaded at the junction between duplex DNA and single-stranded DNA coated by RPA at the site of damage by the clamp loader RAD17-RFC2-5. The phosphorylated C-terminal tail of RAD9 then interacts with TOPBP1 to activate ATR kinase, which is localized to the site of damage by ATRIP. Upon activation, ATR phosphorylates and activates many downstream targets, including CHK1. CHK1 phosphorylates and inactivates the cyclin-dependent kinase-specific phosphate CDC25, resulting in halting cell-cycle progression in order to surveillance the DNA for repair, or if the damage is too severe, initiate apoptotic machinery (2).

Structurally, the 9-1-1 complex is similar to another DNA clamp known as Proliferating Cell Nuclear Antigen (PCNA), which has a well-established role as a processivity factor for DNA replication and repair. Both have a toroidal structure that has an inner ring that allows interactions with DNA (3).
The 9-1-1 complex has two independent functions, to activate ATR-mediated checkpoint signaling and DNA repair. Two hydrophobic pockets in HUS1 have been suggested to be important for genome maintenance—a PCNA-like hydrophobic pocket and a novel pocket with conserved residues topologically equivalent to the PCNA secondary binding site (4).

9-1-1 also acts as a molecular scaffold, interacting with numerous DNA repair factors in multiple DNA repair pathways, including base excision, mismatch, nucleotide excision repair, homologous recombination, non-homologous end joining, and translesion synthesis (4). The focus of my project revolves around understanding the role of HUS1, one of the subunits of 9-1-1, in mediating DNA repair as a molecular scaffold and recruiting repair factors to the site of damage. Loss of HUS1 leads to a number of cellular defects, such as embryonic lethality, subfertility in conditional knockout models of mice with germ cell-specific Hus1 deletion, genomic instability in cell lines, and cancer resistance in cancer mouse models (5 and unpublished data). Many mutations and genomic changes in cancers are a result of DNA injury or abnormal genome maintenance. Genome instability can lead to progression the progression of a tumor from benign to malignant (6). By uncovering the role of HUS1 in DNA repair, we hope to further elucidate the relationship between the DDR and cancer development and identify novel interactors that can be targeted for therapeutic benefit.

Preliminary work done by a graduate student in the lab, Darshil Patel, has suggested that HUS1 might be interacting with proteins from the neddylation pathway. In an initial proteomic screen using Stable Isotope Labeling of Amino acids in Culture (SILAC), 293T cells were labelled with heavy isotope, and transfected with a pCMV14 plasmid overexpressing HUS1-3XFLAG. A large-scale immunoprecipitation (IP) using anti-FLAG beads was conducted, and these IP samples were used to do a Liquid Chromatography Mass Spectrometry (LC-MS/MS) analysis. In this initial screen, several proteins of the neddylation protein were identified as potential novel interactors. Neddylation is a post translational modification that allows the target proteins to be conjugated with ubiquitin-like protein
NEDD8 (Neural precursor cell Expressed Developmentally Downregulated protein 9), which requires neddylation specific E1, E2, and E3 enzymes. The Cullin family proteins, which are parts of the E3-RING ubiquitin ligases (CRL), are the primary targets of neddylation. CRL proteins play a role in proteosomal degradation by poly-ubquitinating target proteins. The activation of CRL is dependent on neddylation, and CRL has been shown to play a role in cell cycle checkpoint responses after DNA damage (7). The literature suggests that MLN492, an inhibitor of the NEDD8 E1 NAE1, when applied to Hus1 knockdown cells, leads to synthetic lethality (8). Given these data, I predict that HUS1 interacts with the neddylation pathway proteins as a molecular scaffold to coordinate the degradation of repair proteins after repair.

My project this summer will focus on continuing the work I am currently doing in the laboratory. One of the first priorities is to replicate the preliminary results demonstrating physical interaction between HUS1 and UBE2M (NEDD8 E2) and CUL4A, a Cullin family substrate of NEDD8. To do this, I will transiently transfec HEK293T cells with the pCMV14-Hus1-Flag (pCMV) vector, which has a 3X-FLAG epitope tag for ease with immunoblotting assays. I will perform a co-immunoprecipitation assay (co-IP) in order to verify physical interaction. Next, I plan on conducting immunofluorescence (IF) assays on neddylation proteins using Hus1\(^+/\) and Hus1\(^{-/-}\) mouse embryonic fibroblasts to test if HUS1 plays a role in recruiting the neddylation machinery to sites of DNA damage. I hope to see that neddylation proteins and HUS1 co-localize to sites of DNA damage, which would verify the role of neddylation proteins in the DDR, and suggest that HUS1 could be recruiting the neddylation machinery to the site of damage. These experiments will help to lay the foundations for future experiments that will involve knocking down UBE2M in Hus1\(^+/\) and Hus1\(^{-/-}\) mouse embryonic fibroblasts and then treating with different genotoxins. Survival assays will be conducted with these treatments in hopes of identifying the specific repair pathway that HUS1 and neddylation are involved in.

I will conduct my research in Dr. Robert Weiss’s laboratory at the Veterinary Research Tower at Cornell University, which will provide me with all the supplies and equipment necessary for my
experiments. All the cell lines, reagents, and machinery such as a microscope, are all available either in the Weiss laboratory or is shared equipment that is part of the Department of Biomedical Sciences. My mentor, Darshil Patel, a graduate student in the lab, will guide me, as well as the principal investigator of the laboratory, Dr. Robert Weiss, Professor of Molecular Genetics. With the Tanner Dean's Summer Research Grant, I hope to be able to conduct lab work full-time in the summer, continuing on and expanding on my work during the semester, when I did not have the time to work in the laboratory from 9AM-5PM every day. In addition, I plan on making this project a part of my senior honors thesis for the biological sciences major, using the results to inform and guide my honors thesis.

References


