PURE Final Report

“The Role of RAD51 Paralogs and Their Interactions in the RAD51- Mediated Homologous Recombination DNA Repair”

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Introduction:

One of the most toxic forms of DNA damage is double-stranded breaks (DSBs) that occur endogenously during DNA replication or exogenously by DNA damaging agents. DSBs are fixed through two major pathways: non-homologous end joining (NHEJ) or homologous recombination repair (HRR) and failure to repair these breaks leads to genome instability, a hallmark of cancer. RAD51 and the human RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) are important proteins for HRR and mutations to these genes are linked with breast and ovarian cancer, and Fanconi anemia[1]. In humans, the RAD51 recombinase enzyme assembles into a helical nucleoprotein filament around 3' single-stranded DNA overhangs after the processing of the DSB ends to perform strand invasion and homology search. The human RAD51 paralogs form two unique complexes during HRR: RAD51C-XRCC3 (CX3) heterodimer and RAD51B-RAD51C-RAD51D-XRCC2 heterotetramer[1]. The roles of these complexes in HRR are poorly understood. The focus of this research project was on the CX3 complex, which is possibly involved in the stabilization of the RAD51-DNA filament and in the resolution of Holliday junctions during later HRR steps[1].

The purpose of this summer project was to use proteomic methods to determine and validate interaction partners with the stable maltose binding protein tagged CX3 complex as the interactome had previously only been studied with the individual mouse proteins. Using novel co-expression constructs, small-scale pulldowns, and liquid-chromatography/tandem mass spectrometry (LC-MS/MS) will help in identifying new interaction partners, allowing future structural and biochemical studies that would enrich our understanding of this complex during HRR.

Methods:

Purification of Maltose Binding Protein (MBP)

A purified T7 promoter vector for the expression of His-MBP was transformed into Rosetta 2 *E. coli* cells, and single colonies were grown overnight in 25 ml starter cultures at 37°C. The starter culture was used to inoculate 1 L of Terrific Broth media and grown at 37°C to an OD600 of 0.8, followed by induction with 0.5 mM IPTG for overnight expression at 16°C. The cells were pelleted and resuspended with a buffer composed of 400 mM NaCl, 25 mM HEPES pH 8, 2.5 mM ATP/MgCl2, 1 mM DTT, and 1 mM Na3VO4. Resuspended cells were sonicated at an amplitude of 25% for 30 seconds (8-10x), and the protein was then purified from the cell lysate through batch chromatography using dextrin beads. Elution of the protein was performed using the previous buffer but with 10 mM maltose. MBP protein was further purified with size-exclusion chromatography and then concentrated to 22.73 mg/mL.

Purification of MBP-CX3

Sf9 insect cell pellets that had overexpressed the His-MBP tagged CX3 complex were available in the Williams lab. These cells were re-suspended using the previous buffer. The re-suspended cells were sonicated with an amplitude of 25% for 30 seconds (8-10x) and protein was purified using nickel nitrilotriacetic acid beads (Ni-NTA) using batch chromatography. Elution of the protein complex required the same buffer but with 250 mM imidazole. The extracted MBP-CX3 complex was further purified with size-exclusion chromatography and then concentrated to 12.02 mg/mL.

Pull-downs for Control and Experimental Proteomic Analysis

100 µl of dextrin bead slurry was added to three 1.7 mL microcentrifuge tubes, which were used for the two negative controls (Beads only and MBP only) and the CX3 only experimental

tube. The 20% ethanol was removed and the dextrin beads were equilibrated with 40 column volumes of binding buffer (50 mM NaCl, 25 mM HEPES pH 8, 2.5 mM ATP/$MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4, 0.5 mM EDTA, and 0.5 mM PMSF). HeLa cell pellets were re-suspended with binding buffer and sonicated with an amplitude of 15% for 20 seconds. 1 mL of lysed cells were added to each tube and mixed with their respective bait proteins. The microcentrifuge tubes were incubated overnight in the 4°C room, where the binding fraction was removed the following morning. The beads were washed with binding buffer and sent to the Southern Alberta Mass Spectrometry Center for an on-bead trypsin digestion and LC-MS/MS. Using statistical analysis provided by Scaffold 4 Proteomic software, the proteins were identified with a ≥ 99.9% confidence in a *Homo sapiens* data base containing 99,739 protein sequences. Process was repeated with increases in salt concentration (150 mM and 500 mM) to determine transient or stable protein-protein interactions.

Results:

Following optimization of the binding buffer, it was found that having a salt concentration of 50 mM resulted in the pulldown of most proteins from HeLa cell lysates with the CX3 complex. To increase the specificity of pull-down, washes with increasing salt concentrations were performed in separate tubes, and the identified proteins were analyzed based on specific criteria and the highest level of spectra confidence. The criteria involved in the identification of potential protein-protein interactions include the following: spectral counts have to be present in CX3 pulldowns with 500 mM NaCl binding buffer washes, no spectral counts should be present for that protein in both negative controls after a 500 mM NaCl binding buffer wash, and the confidence of protein identification should be ≥ 99.9%. The CRAPome database, which curates a list of proteins that are common contaminants in such pull-down analyses, further verifies

whether the interactions were novel or an artefact of non-specific protein-protein interactions. After analyses of the data, 84 proteins were found to be potential interaction partners, although they had low spectral counts.

Conclusions:

Even though progress was made in the finding potential interaction partners for the CX3 complex, the overall goal of this project was not reached. Many of the proteins identified based on the criteria suggest that the CX3 complex may be involved in RNA metabolism due to spectral counts found only in the CX3 pulldowns after 500 mM salt washes. Interestingly, the CX3 complex may assist in the dissolution of R-loops which are known to cause DSBs and would require further analysis to validate the mechanism[2]. ATPase SWSAP1, cell division cycle and apoptosis regulator protein 1, and trans-Golgi network integral membrane protein 2 were proteins found in the results and are known protein-protein interactors with either RAD51C or XRCC3, indicating that the pulldown method was adequate for this project.

Many DNA damage repair proteins (DNA-Pkcs, NONO, PARP1, PC4 SRSF1, XRCC5, and XRCC6) found in the pull-downs were ruled out because of their presence in the control as well as CX3 pull-downs, and in general the results displayed high quantities of sugar-binding proteins that generated background. This may be due to the composition of the dextrin beads used to pull-down MBP, with the DNA repair and sugar binding proteins binding to the beads instead of the CX3 complex. When performing future pull-downs, Ni-NTA beads should be used instead of dextrin beads, as this may decrease the overall background and determine whether the DNA repair proteins are specific and novel interaction partners with the CX3 complex. If the 84 proteins that were only identified in CX3 pull-downs are also found in pull-downs with CX3 using Ni-NTA bead, this would provide additional confidence that they are CX3 interaction

partners. Further optimization of the pull-down protocol will allow for the identification of novel protein-protein interactions that would then be validated through co-immunoprecipitation and western blots, and eventually used for biochemical and structural studies

Experience and Knowledge Gained:

This summer research project was a fulfilling experience as I was able to build off and elevate my basic knowledge in the field of biochemistry and structural biology. The newfound knowledge encouraged me to dig further into the topics, but also helped me think more critically and make meaningful connections from my project to DNA repair pathways. My hands-on skills and aseptic technique have significantly improved, which was crucial to minimizing experimental errors and for my development in pursuing graduate studies. Being part of a lab gave me an opportunity to develop my skills in time management and troubleshooting problems that I faced. An important lesson I learnt from this experience was that research requires a considerable amount of patience and perseverance because of obstacles and mistakes that can occur in a lab environment.

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References

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