Deciphering The Interacting Regions And Functions Of The RAD51D-Nono Protein Complex During DNA Damage Response

Latarsha Porcher
University of South Carolina

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DECIPHERING THE INTERACTING REGIONS AND FUNCTIONS OF THE RAD51D-NONO PROTEIN COMPLEX DURING DNA DAMAGE RESPONSE

by

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DEDICATION

I would like to dedicate my graduate work to God, first and foremost, followed by family, dearest friends, and to those that are fighting cancer or know someone who is. It is my hope that the work accomplished will help provide some progression in the fight against cancer.
ACKNOWLEDGEMENTS

To my wonderful family, especially my mom and grandmother, thank you so much for believing in me. Thank you for being there when I was having a tough day and giving me your continual support in pursuing my dreams. I would like to thank my lab-mate, Nicole Reilly, for being there as a friend and fellow scientist. Thank you for allowing me to bounce ideas off you, discuss concepts I did not understand, and listening to my silly stories about Ginger. To my committee members, Dr. Wyatt and Dr. Turner, I can not thank you enough for taking time out of your busy schedules to serve on my committee. Thank you for all your help as mentors that aided my growth as a scientist. To my amazing mentor, Dr. Pittman, thank you so much for allowing me to conduct research in your lab. You have my deepest gratitude for helping me to grow as a scientist and a person.
Genomic instability is one of the major components represented in the “Hallmarks of Cancer.” DNA interstrand crosslinks and double-stranded breaks are two of the most severe causes of genomic instability. Homologous recombination (HR) plays a major role in resolving both types of DNA lesions, requiring a homologous template and the RAD51 paralog complex of proteins. One member of this complex is the RAD51D ovarian cancer susceptibility gene product. To better elucidate RAD51D modifications and functions, several protein interaction screens were performed, and one of the novel proteins identified was NONO (a.k.a. p54nrb). Decreased expression of either RAD51D or NONO conferred increased chromosomal instability and cellular sensitivity to DNA interstrand crosslinking agents. To further characterize RAD51D-NONO interaction, I used the yeast two hybrid approach. Interestingly, the yeast two hybrid data not only identified regions of RAD51D necessary for the interaction but indicated that the RAD51D Walker Box A motif and a specific lysine residue within RAD51D decreased the strength of the interaction with NONO. Taken together, the results from this study suggest specific regions or post-translational modifications regulate binding between these two proteins. Another important feature of the NONO protein is that it is a component of a sub-nuclear structure known as the paraspeckle. The second part of this thesis work investigated whether there was a correlation between paraspeckle numbers and levels of DNA damage resulting from etoposide, mitomycin C, or cisplatin. The results suggest that there is a very modest, yet significant, decrease in the numbers of
paraspeckles after double-stranded DNA break induction following etoposide treatment. Additional data are presented suggesting that the absence of RAD51D confers a decrease in paraspeckle number. In conclusion, these studies revealed that the interaction between RAD51D and NONO may be very tightly regulated, perhaps during the DNA damage response, and that there may be a correlation between DNA damage, the paraspeckle structure and/or numbers, and individual paraspeckle components.
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CHAPTER 1: INTRODUCTION

1.1 Genomic Instability

Cancer is a disease characterized by tumor growth and metastatic dissemination. There are six hallmarks that help explain the development of human tumors: sustaining proliferative signaling, evading tumor suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death (Figure 1.1).

![Hallmarks of Cancer](image)

Figure 1.1 Hallmarks of Cancer. This illustration shows the six hallmarks proposed by Hanahan and Weinberg. (Adapted from Hanahan and Weinberg, 2011).
Underlying all of these hallmarks is genomic instability or alterations in DNA that enables acquisition of these characteristics that allows cancer cells to dominate in normal tissue (Hanahan and Weinberg 2011). The alterations in DNA that contribute to genomic instability may be due to several sources: apurinic site, deamination, pyrimidine dimer, mismatches, double-strand breaks, interstrand crosslinks, bulky adducts. There are five repair pathways that combat these DNA alterations and maintain cell homeostasis (Table 1.1).

Table 1.1 DNA Damage and their Corresponding Repair Pathways (Adapted from Dexheimer 2013)

<table>
<thead>
<tr>
<th>DNA Damage Source</th>
<th>DNA Damage Type</th>
<th>DNA Repair Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive Oxygen Species</td>
<td>Oxidation (8-oxoguanine)</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>X-rays</td>
<td>Single Strand Break</td>
<td></td>
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<tr>
<td>Replication Errors</td>
<td>Mismatches</td>
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<td></td>
<td>Insertions</td>
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<tr>
<td></td>
<td>Deletions</td>
<td></td>
</tr>
<tr>
<td>UV light</td>
<td>Bulky Adducts</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td></td>
<td>Intrastrand Crosslinks</td>
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<tr>
<td>X-rays</td>
<td>Double Strand Breaks</td>
<td>Non-homologous End Joining</td>
</tr>
<tr>
<td>Ionizing Radiation</td>
<td>Interstrand Crosslinks</td>
<td>Homologous Recombination</td>
</tr>
</tbody>
</table>

1.2 Interstrand Crosslink and Homologous Recombination

Interstrand crosslinks are one of the most severe types of DNA damage. Interstrand crosslinks can cause stalled replication forks and ineffective repair can lead to disease. Interstrand crosslinks repair involves several pathways including nucleotide excision repair, translesion synthesis, and homologous recombination (Andreassen and Ren 2009). The interstrand crosslink is unhooked by incision that involves the
nucleotide excision repair pathway resulting in a double-strand break. The gap in the incised strand is filled by translesion synthesis. Translesion synthesis bypasses the lesion and utilizes error-prone DNA polymerases. The double-stranded break on the template strand is then restored by homologous recombination (Figure 1.2, Andreassen and Ren 2009).

Figure 1.2. Interstrand Crosslink Repair. Interstrand crosslinks involve nucleotide excision repair, translesion synthesis, and homologous recombination pathway. The above schematic shows the steps involved in interstrand crosslink repair. (Adapted from Nicole Reilly)

Homologous recombination is a double-stranded break repair pathway that uses a homologous template and the RAD51 complex to repair the damage (Heyer et al. 2010). Homologous recombination is divided into three stages: presynapsis, synapsis, and postsynapsis. In the presynapsis stage, end resection occurs by the MRN complex to leave single-stranded overhangs that are bound by RPA. During synapsis, the RAD51 complex performs a homology search and DNA strand invasion. In the postsynapsis stage, the double-stranded break is resolved through three possible pathways that may
leave crossovers or noncrossovers (Heyer et al. 2010). The RAD51 complex is composed of several paralogs one of which is RAD51D.

1.3 RAD51D

RAD51D is paralog of RAD51 and component of the RAD51 complex used in homologous recombination for the homology search and strand invasion steps (Figure 1.3). It is ubiquitously expressed in all tissues and has alternative transcripts (Pittman et al. 1998, Kawabata and Saeki, 1999). It is composed of two Walker Box motifs and helix-turn-helix motif. The Walker Box motifs are ATP domains that aid in its interaction with RAD51C and decreased sensitivity to crosslinking agents (Gruver et al. 2005). RAD51D is an essential protein in the homologous recombination process. Deletion of RAD51D caused embryonic lethality, reduced RAD51 foci formation, and increased chromosomal aberrations (Smiraldo et al. 2005). It is also necessary for telomere maintenance (Tarsounas et al. 2004). RAD51D has a variety of known functions but not all have been fully elucidated. To identify novel function of RAD51D in DNA repair, a proteomics study was undertaken to reveal novel interaction partners (Rajesh et al. 2009). One novel interaction protein revealed was NONO.

![Figure 1.3. RAD51D. Schematic representation of RAD51D and its motifs. RAD51D has a linker region and two Walker Box motifs.](image-url)
1.4 NONO

NONO is a nuclear localized protein that is a member of the *Drosophila* Behavior Human Splicing (DBHS) protein family (Figure 1.4). It was originally identified by its *Drosophila* homolog (Yang *et al.* 1993). It is also a paralog of SFPQ (Splicing Factor, Proline and Glutamine) protein of the DBHS family and was also found to interact with RAD51D (Rajesh *et al.* 2009). NONO has been implicated in gene regulation through binding of target gene promoters, sequestration of gene activators, binding of RNA transcript and machinery (Knott *et al.* 2016). It is an essential component of a nuclear organelle called the paraspeckle. The paraspeckle retains mRNAs with hyperedited adenosines-to-inosines. Upon cellular stress induction, mRNAs are released from the paraspeckle and into the cytoplasm to be translated. NONO has also been implicated in neuronal function and circadian rhythm (Knott *et al.* 2016). NONO was also found to localize to sites of DNA damage and participate in DSB break through association with Ku70, Matrin3, and PARP (Knott *et al.* 2016). The role of NONO in double-stranded DNA break repair has been primarily seen in non-homologous end joining (Knott *et al.* 2016).

![NONO and domains](image)

**Figure 1.4.** NONO. The above picture is a schematic representation of NONO and the domains within it. At the N-terminus, there is proline and glutamine rich region. It is also composed of two RNA recognition motifs, NonA/paraspeckle domain, and a coiled-coil region. The coiled-coil region is necessary for protein-protein interaction.
1.5 RAD51D and NONO Studies

Our proteomics study identified a possible homologous recombination function for NONO through its interaction with RAD51D (Rajesh et al. 2009). In order to learn more about this interaction, the yeast two hybrid assay was used. The yeast two hybrid assay was used to identify the domains of RAD51D that were involved in its interaction with NONO. In the yeast two hybrid, interaction between NONO and RAD51D would activate reporter genes that would allow the yeast cells to grow on selective dropout medium plates. Through this assay, it was identified that the middle and carboxy terminus of RAD51D interact with NONO. The Walker Box A mutants showed enhanced interaction with NONO. The interaction studies suggested multiple domains of RAD51D interacted with NONO. Lysine mutants did not affect RAD51D’s interaction with NONO. NONO was also an essential component of the paraspeckle. Our studies also investigated if this interaction affected paraspeckle function upon interstrand crosslink and double-stranded DNA break induction. Our results found a decrease in the amount of paraspeckles upon double-stranded DNA break induction and RAD51D deletion. These results suggest that paraspeckles may have a role in DSB repair and RAD51D may be necessary for paraspeckle formation.
CHAPTER 2: INTERACTION PROFILES OF RAD51D AND NONO BY YEAST TWO HYBRID ANALYSIS

2.1 Abstract

Interstrand crosslinks and double-stranded DNA breaks are the most severe type of DNA damage. Ineffective repair of these DNA lesions can lead to mutations or cell death contributing to diseases such as cancer. One repair pathway used to resolve these DNA lesions is homologous recombination. Homologous recombination uses the RAD51 complex and a homologous template to resolve double-stranded DNA breaks. RAD51D is an essential component of the RAD51 complex and the homologous recombination pathway. Disruption of RAD51D function leads to ineffective DNA repair and consequently, decreased genomic stability. Although RAD51D has been implicated in homologous recombination in the RAD51 complex, novel function are still waiting to be identified. To identify novel functions of RAD51D, novel protein interactions need to be found. A proteomics study done by the Pittman laboratory identified the RAD51D and NONO interaction. Yeast two hybrid studies were conducted to further characterize the RAD51D and NONO interaction. Yeast two hybrid studies showed that NONO interacts with the middle and carboxy terminus of RAD51D. The Walker Box A mutants showed stronger interaction with NONO suggesting a possible regulatory mechanism surrounding the interaction with NONO. Yeast two hybrid results also showed that the lysines were not necessary for its interaction with NONO. The interaction analysis of RAD51D and
NONO suggests that Walker Box A motif possibly inhibits RAD51D’s interaction with NONO; while the alternative splice variants show multiple points of interaction that enhance or weaken the interaction depending upon the domains exposed.

2.2 Introduction

Genomic instability is one of the major contributing factors to cancer. One of the factors contributing to genomic instability is DNA damage (Hanahan and Weinberg 2011). Interstrand crosslinks are one of the most severe types of DNA damage. Part of interstrand crosslinks repair involves generation of a double-stranded DNA break (Andreassen and Ren 2009). There are two main pathways that correct double-stranded DNA breaks. The first pathway is error-prone pathway called non-homologous end-joining that just ligates the broken DNA strands back together. It’s an error-prone pathway because the sequence where the double-strand break was is lost. The second pathway used to repair double-stranded DNA breaks is homologous recombination. Homologous recombination is an error-free DNA repair pathway using the RAD51 paralogs and a homologous template usually from a sister chromatid (Heyer et al. 2010).

One of the RAD51 paralogs involved in homologous recombination is called RAD51D. It participates in a complex with the other RAD51 paralogs: RAD51B, RAD51C, XRCC2, and XRCC3 to search for the homologous template and correct the double-stranded DNA break (Heyer et al. 2010). Previous work has shown that disruption of RAD51D function leads to embryonic lethality in mice. The embryonic lethality is most likely due to chromosomal aberrations. Disruption of RAD51D function also leads to reduced RAD51 foci and enhanced sensitivity to DNA damaging agents such as mitomycin C (Smiraldo et al. 2005). Previous research has shown the necessity of
RAD51D in the homologous recombination pathway but there is much that remains unknown about it. To further elucidate RAD51D’s function, a proteomics study was conducted to identify novel interaction partners (Rajesh et al. 2009). A novel interaction candidate discovered was NONO (Figure 2.1).

Figure 2.1. *Drosophila* Behavior Human Splicing Protein Family. Shown above are schematic representations NONO and related proteins. Within this family, the proteins have 2 RNA recognition motifs, NonA/Paraspeckle domain (NOPS), and a coiled-coil domain in common.

NONO is a member of the Drosophila Behavior Human Splicing protein family. It is composed of two RNA recognition motifs, nuclear localization signal, coiled-coiled domain, and a NonA/paraspeckle domain (Figure 2.1). These domains play a role in NONO’s nucleic acid interaction and processing and protein interaction. NONO has been implicated in non-homologous end joining, paraspeckle formation, and gene regulation via nucleic acid processing (Knott et al. 2016). Disruption of NONO has shown enhanced sensitivity to interstrand crosslink agents such as mitomycin C and cisplatin (Unpublished data). To decipher the function of this interaction, the yeast two hybrid
system was used to identify the domains of RAD51D involved in its interaction with NONO.

In the yeast two hybrid system, NONO was placed in a vector with the Gal4 DNA binding domain and RAD51D and its mutants were placed in a vector with the Gal4 activation domain. The observance of yeast growth on selective dropout medium plates suggests interaction of RAD51D or its mutants with NONO. In this system, the interaction of RAD51D or its mutants with NONO will bring together the Gal4 domains and activate its promoter causing expression of reporter genes for adenine, histidine, leucine, and tryptophan allowing the yeast to grow on selective dropout medium plates.

In the yeast two hybrid analysis, growth was observed between NONO and RAD51D and the following RAD51D mutants: 77-329, 234-329, Δ8, Δ7B,Δ7/8, and Δ5 (Figure 2.4b). Growth was also observed with all the RAD51D Walker Box A mutants and the lysine mutants. These results suggest that there are multiple points of RAD51D that interact with NONO.

2.3 Materials and Methods

Yeast vectors and Plasmids

To examine the interaction between Rad51d and Nono in the yeast two hybrid system, plasmids were made from the vector backbones: pGADT7 and pGBKT7 (Figure 2.2). In each of the vectors, there is a two micron origin of replication. The two micron origin of replication allows for the promotion of DNA replication in yeast cells. Without the two micron origin of replication, these vectors would not be able to replicate in yeast cells. It also enables 20-50 copies of any plasmid and ability to monitor overproduction of a particular gene product. The RAD51 paralogs Rad51c, Rad51d, Xrcc2 were cloned into
the pGADT7 vector using restriction enzymes *EcoRI* and *BamHI*. The *Rad51d* truncation mutants 4-77, 77-329, 234-329 were a gift from Dr. Joanna Albala. The *Rad51d* Walker Box A mutants were created by Dr. Aaron Gruver through site-directed mutagenesis through a *Rad51d* cDNA cloned into *EcoRI* and *BamHI* cut sites of pUC19 (Gruver *et al.* 2005). The *Rad51d* alternative splice variants: Δ8, Δ7B, Δ7/8, Δ3, Δ5, and +intron3 were generated as previously described (Gruver *et al.* 2009). (Figure 2.4)

![Diagram of yeast two hybrid vectors](image)

**Figure 2.2.** Yeast Two Hybrid Vectors (A) pGADT7 is a yeast expression vector used for the RAD51 paralogs. This vector has the Gal4 activation domain. It contains an ampicillin antibiotic gene that allows it to be taken up by bacteria and a leucine gene that allows the plasmid to be taken up by yeast. (B) pGBK7 is another yeast expression vector used for the NONO protein in these studies. It has the Gal4 DNA-binding domain that activates reporter genes when in contact with the Gal4 activation domain. It contains a kanamycin antibiotic resistance gene for bacteria and tryptophan gene for yeast. (Clontech 2009, Snapgene Software)

**Bacterial Strain and genotype**

The bacterial strain used for the plasmids in these studies was DH5α. The genotype for DH5α is as follows: F-φ80, lacZΔM15 (*lacZYA-argF*), U169 recA1, endA1, *hsdR17*(rK-, mK+), *phoA*, *supE44*, *λ-thi*-1, *gyrA96*, *relA1*. 
**Bacterial Transformation**

The pGADT7 and pGBKT7 plasmids were transformed into DH5-alpha cells using the heat shock procedure. In this protocol, bacterial cells and less than 100 nanograms of each plasmid were placed on ice for 30 minutes. After a 30 minute incubation, the cells were shocked by being placed in a 42°C water bath for 42 seconds enabling the plasmids to enter the bacterial cells. Following the heat shock, the transformation mixture was placed on ice for 2 minutes. Transformation mixture was pelleted down and then resuspended in 1 ml of LB broth and incubated with shaking at 37°C for one hour. After the one hour incubation, one fifth of the mixture was plated on the appropriate selection plate.

**Yeast Two Hybrid Analysis**

_Yeast strains and genotypes_

AH109 is the yeast strain that was used in all interaction studies. It’s genotype is as follows: \textit{MATa, trp1-901, leu2-3, 112, ura3-52,his3-200, gal4Δ, gal80Δ, LYS2::GAL1\textsubscript{UAS}-GAL1\textsubscript{TATA}-HIS3, MEL1, GAL2\textsubscript{UAS}-GAL2\textsubscript{TATA}-ADE2,URA3::MEL1\textsubscript{UAS}-MEL1\textsubscript{TATA}-lacZ} (Clontech 2009).

_Yeast transformation and selection of colonies_

pGADT7: \textit{Rad51d, Rad51c, and Xrcc2} and pGBKT7:\textit{Nono} were transformed into AH109 yeast strain using ZymoResearch Frozen EZ Yeast Transformation II kit per manufacturer’s instructions. One microgram of each plasmid was placed into the yeast cells. The transformation mixture was incubated at 30 °C for forty-five minutes while shaking at 230 RPM. The transformation mixture was then plated on selective dropout medium lacking leucine and tryptophan. Leucine and tryptophan are markers in the
pGADT7 and GBKT7 vectors that enable yeast cells to take up plasmids containing the Rad51 paralogs or NONO. After the transformation mixtures were plated, they were placed in a 30°C incubator for four days. After four days, the amount of colonies were recorded. Medium or large-sized colonies were selected for interaction analysis.

**Replica Plating and Y2H Analysis**

To study the interaction between the *Rad51d* truncation mutants and splice variants with *Nono*, three colonies were picked and then placed into a 96-well plate with synthetic dropout medium lacking leucine and tryptophan. For each positive or negative control in these interaction studies, one colony was picked and placed into the 96-well plate. The colonies were then grown at 30°C for 24 hrs shaking at 230 RPM. After the 24 hour incubation, a 48 patch replicator (Boekel) was used to stamp the patches on two types of synthetic dropout medium plates with an ethanol sterilization in between each stamp. These plates were either lacking both leucine and tryptophan or lacking leucine, tryptophan, adenine, and histidine. The interaction observations were based on the synthetic dropout medium plates lacking leucine, tryptophan, adenine, and histidine. After the stamping was completed, the plates were placed in a 30°C incubator until growth was observed. When yeast growth was observed on the synthetic dropout medium plates lacking leucine, tryptophan, adenine, and histidine, it suggested that the proteins interacted (Figure 2.3).

**o-nitrophenyl-β-galactopyranosidase Assay**

To quantify the protein interaction observed on the synthetic dropout plates, liquid beta-galactosidase assays were performed using *o*-nitrophenyl-β-galactopyranosidase as a substrate. Beta-galactosidase was an enzyme activated by the
reporter gene lacZ. LacZ was activated upon protein interaction in yeast two hybrid. In this colorimetric assay, ONPG (ortho-nitrophenyl beta galactosidase) was a substrate of

Figure 2.3. Yeast Two Hybrid Analysis Procedure. The RAD51 and NONO plasmids are transformed into AH109 and plated. After the yeast have grown, colonies are picked and grown in 96 well liquid culture overnight. A 48 pin replicator was used to stamp the yeast cells from the 96 well plate onto selective dropout medium lacking leucine or tryptophan (control) or leucine, tryptophan, adenine, histidine (experimental).

beta-galactosidase. When hydrolysis of beta-galactosidase occurred, it cleaved ONPG and left ONP, which caused a yellow color. The yellow color change was measured by absorbance at 420nm. Using the following formula \(1,000 \times \frac{OD_{420}}{(time \times V \times OD_{600})}\), where \(t=\) time in minutes, \(V=0.1\) concentration factor of 15, enzyme units were calculated and corresponded to the amount of beta-galactosidase enzyme activity in the
Colonies were picked for each interaction and placed in synthetic dropout medium lacking leucine and tryptophan and grown overnight at 30°C shaking at 230 RPM. After the overnight culture was grown, a new culture was made in YPD and grown to an OD$_{600}$ of 0.5-0.8. The yeast cells were then pelleted down and washed in Z-buffer. After several washes, the yeast cells were lysed open by a freeze and thaw cycle of liquid nitrogen and 37°C water. ONPG was then added to the cell, placed in a 30°C incubator until a yellow color developed, and a timer started. Once the yellow color developed, 1M sodium carbonate was added to inactivate the reaction and the time for color development was recorded. An absorbance for the yellow color change was taken at OD$_{420}$. The following formula was then used to calculate beta-galactosidase units for each interaction: 1,000* OD$_{420}$/ (time*V* OD$_{600}$). Statistical significance was determined by a two-tailed t-test where p<0.05 was determined to be statistically significant.

2.4 Results

*Characterization of RAD51D truncation and alternative splice variants with NONO*

The novel interaction of RAD51D and NONO was initially identified in a proteomic study (Rajesh et al. 2009). To confirm this interaction and determine what domains of RAD51D were involved in its interaction with NONO, the yeast two hybrid system was used (Figure 2.4). In the yeast two hybrid, activation of reporter genes due to protein interaction enables the yeast to grow on selective dropout medium plates. The control plate is a synthetic dropout lacking leucine and tryptophan. For each control, there is one patch and they are in the left and right most column. For each experimental patch examining interaction between 51D and NONO, there are 3 patches. In this analysis, a positive control of the well-studied interaction between 51D and 51C and
negative controls of NONO or 51D alone was shown. As expected, robust growth was shown for the top left patch on the experimental plate that represents the 51D and 51C interaction. Growth was observed for all 3 patches corresponding to the 51D and NONO A.

<table>
<thead>
<tr>
<th></th>
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<th>51C/NONO</th>
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<td>51D/51C</td>
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Control  Experiment
Figure 2.4. Interaction Analysis between RAD51D and NONO. (A) The NONO and RAD51D expression constructs were co-transformed into AH109 haploid yeast. The data suggest interaction at the RAD51D middle and carboxy-terminus. (B) Representation of RAD51D alternative splice variants and truncation mutants and interaction results. Key: +: Growth=control, +/-: Growth similar to the control, -/+: Growth<Control, -: No Growth. (C) The ONPG colorimetric assay was used for quantification. “*” indicates statistical significance (p<0.05) compared to control group.

sections. Growth was also seen with the 77-329 and 234-329 truncation mutants. Interaction was observed for the alternative splice variants: Δ8, Δ7B, Δ7/8, and Δ5. There were more yeast colonies observed for the 234-329 truncation mutant and alternative
splice variants (Figure 2.4A and B). Quantitation was attempted with a liquid beta galactosidase assay to determine if interaction was stronger with certain regions of RAD51D. Δ5 and Δ8 had statistically significant higher beta-galactosidase activity in comparison to 51D/NONO (Figure 2.4C).

**Increased Interaction between RAD51D Walker Box A ATP Binding and Hydrolysis mutants with NONO**

RAD51D has two Walker Box Motifs labeled A and B. These motifs bind and hydrolyze ATP (Figure 2.5). These motifs play a role in RAD51D’s function and interaction with RAD51C (Gruver *et al.* 2005). The RAD51D Walker Box A mutants were placed into the AH109 yeast cells with NONO to determine if this motif may affect its interaction with NONO (Figure 2.5). These mutants ΔG112K113, G112A, K113R, and K113A inhibit ATP binding and hydrolysis function. The mutant S111T serves as a positive control because it is a similar amino acid. The positive control on this experimental plate was the well-studied interaction of 51D and XRCC2. The negative controls are 51D or NONO alone. Growth was seen in the top left patch corresponding to 51D and XRCC2. No growth was observed in the patch below 51D and XRCC2 corresponding to 51D and GBKT7. Growth was seen for the all interaction patches corresponding to the RAD51D Walker Box A mutants with NONO. Larger colony size was observed for the RAD51D walker box A mutants with NONO in comparison to the 51D and NONO patch (third patch in the leftmost column) except the S111T conserved mutation. The S111T conserved exhibited similar low yeast growth in comparison to the 51D/NONO patch. The similar growth pattern was expected because the S111T was a conserved mutation that does not disrupt the Walker Box A function.
Figure 2.5. Interaction Analysis between RAD51D Walker Box A mutants and NONO. (A) A schematic representation of RAD51D displaying the Walker Box domains and a table showing the different mutations and their effects on Walker Box A function. (B) The RAD51D Walker Box A mutants and NONO were transformed into haploid AH109 yeast. Yeast growth is seen with all of the Walker Box A mutants suggesting Walker Box A’s function was not necessary for RAD51D’s interaction with NONO. Key: +: Growth=control, +/-: Growth similar to the control, -/+: Growth<Control, -: No Growth
**RAD51D K298R Lysine Mutants Enhances Interaction with NONO**

Post-translation modifications, such as ubiquitination, play a role in protein interaction. Ubiquitination describes a post-translational modification that adds a ubiquitin molecule to lysine a protein. Ubiquitination of a protein can alter its function in a variety of ways, such as signaling downstream factors or signaling the cell that the ubiquitinated protein is ready for degradation (Strieter and Korasick 2011). Previous research has shown that RAD51D is ubiquinated by RNF138 (Yard et al. 2016). Lysines are the amino acid targeted by ubiquitin molecules. The goal of these studies was to determine if the lysine mutants affect its interaction with NONO. In these studies, the positive control was 51D with XRCC2. Growth was observed for the 51D and XRCC2 patch seen in the upper left hand corner on the experimental plate in both parts A and B. As expected, small colony growth was observed for the 51D and NONO patch seen in the third row of the left column. The lysine mutants are seen in the four middle columns for rows 1-4 in both parts A and B show yeast growth. K298R showed larger colony growth in comparison to 51D and NONO patch (Figure 2.6).

A.
B.

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<tr>
<th>51D/X2</th>
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<td>GAD/NONO</td>
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C.

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<th>RAD51D Lysine Mutants</th>
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<td>K24R</td>
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<td>K26R</td>
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<td>K42R</td>
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<td>K48R</td>
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<td>K298R</td>
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<td>K327R</td>
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Figure 2.6. RAD51D Lysine Mutants and NONO Interaction Analysis. (A)Lysines in the later half of RAD51D were mutated to arginines and cotransformed into AH109 yeast cells with NONO. Growth was observed for all later half RAD51D lysine mutants with NONO. (B) Lysines in the beginning of RAD51D were also mutated to arginines. Growth was observed in all four patches for each lysine mutants. NOTE: For part B: The second patch seen on the fifth row in both the control and experimental plate was due to accidentally getting yeast in well. (C) A table summarizing the results of RAD51D lysine mutants interaction with NONO. Key: +: Growth=control, +/-: Growth similar to the control, -/+: Growth<Control, -: No Growth

2.5 Discussion

DNA damage is one of the main contributors to genomic instability that can lead to diseases such as cancer. The most severe type of DNA damage is double-stranded breaks that can be repaired by homologous recombination. The homologous recombination pathway uses a template to repair the double-stranded break that’s usually from a sister chromatids. One of the major players in the homologous recombination pathway is the RAD51 complex. It is composed of proteins from the RAD51 family: RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3 (Heyer et al. 2010). Deficiency in RAD51D has been shown to lead to embryonic lethality, chromosomal aberrations, and enhanced sensitivity to DNA damaging agents such as mitomycin C (Smiraldo et al. 2005). However, there is still much that remains unknown about the role of RAD51D in the homologous recombination pathway. To determine more of RAD51D’s function, a proteomics study was conducted to determine novel interactions. One of the novel interactions revealed was NONO (Rajesh et al. 2009). To further elucidate the role of this interaction, the yeast two hybrid system was used to identify the domains of RAD51D involved in this interaction.
The first studies used RAD51D truncation mutants: 4-77, 77-329, and 234-329 and the alternative splice variants: \( \Delta 8, \Delta 7B, \Delta 7/8, \Delta 5, \Delta 3, \Delta 7B \). These studies revealed interaction with the 234-329 truncation mutant and the following alternative splice variants: \( \Delta 8, \Delta 7B, \Delta 7/8, \text{and} \Delta 5 \). Quantitation of these interactions showed that \( \Delta 5 \) and \( \Delta 7/8 \) displayed statistically significant stronger interaction than 51D and NONO. The truncation mutant 234-329 and alternative splice variants: \( \Delta 8 \) and \( \Delta 7B \) showed more yeast growth than 51D and NONO but these differences were not displayed in the beta-galactosidase assay. The truncation mutants and alternative splice variants that displayed interaction with NONO contain the middle and carboxy terminus of RAD51D. It suggests that there may be multiple point of interaction between RAD51D and NONO. However, within the splice variants and truncation mutants, there are regions missing as well. In the splice variants: \( \Delta 8, \Delta 7B, \text{and} \Delta 7/8 \), parts of the carboxy terminus are missing; whereas in \( \Delta 5 \), part of the middle terminus near Walker Box motif A is missing. The stronger yeast growth observed in these interactions in comparison to 51D/NONO suggests there may be an inhibitory mechanism to this interaction. When full-length RAD51D interacts with NONO, not all parts are able to strongly bind with NONO, but when regions are missing it may cause a conformational change that more strongly exposes parts of RAD51D enabling them to bind to NONO.

To further elucidate the function of this interaction, RAD51D Walker Box A mutants were also placed in the yeast two hybrid system to determine if ATP binding and hydrolysis was necessary for its interaction with NONO. All of the Walker Box A mutants disrupt ATP binding and hydrolysis except S111T mutation. The S111T mutant is a conserved mutation that retains the Walker Box A function. The Walker Box A
mutant have shown enhanced sensitivity to DNA damaging agents and affected RAD51D’s interaction with NONO. The results showed more yeast growth with all the mutants that disrupted function in comparison to the 51D/NONO. These results suggest that ATP binding and hydrolysis function may inhibit its interaction with NONO. The Walker Box A motif may require conformational changes upon ATP binding and hydrolysis that could close off certain domains of RAD51D. It could be part of the inhibitory mechanism previously mentioned.

The last set of studies analyzed the necessity of the RAD51D lysines in its interaction with NONO. Lysines are subject to post-translational modifications such as ubiquitination. Ubquitination can activate a protein's function, signal downstream factors, or mark a protein for degradation. Of all the lysine mutants, only K298R displayed more yeast growth compared to 51D/NONO. Interestingly, K298R failed to restore RAD51D function in complementation studies. The results suggest K298R may have different functions in RAD51D (Unpublished data). The larger colony size for K298R suggests that K298 may have a role in inhibiting its interaction with NONO.

The yeast two hybrid analysis suggests that there may be an inhibitory mechanism that plays a role with RAD51D’s interaction with NONO. The splice variants that interacted with NONO had part of the carboxy terminus and middle part of RAD51D missing. The Walker Box A mutants and lysine mutants also displayed more yeast growth in comparison to 51D/NONO. The increased yeast growth in comparison to 51D/NONO suggest those regions cause a weak interaction with NONO. When those regions are absent, there is a stronger interaction with NONO.
Future directions to garner more information about 51D and NONO interaction would be to truncate NONO and analyze what domains interact with the middle and carboxy terminus of RAD51D. Figure 7 shows an X-ray crystal structure of NONO. Exposed to the surface are the coiled-coil, NonA/paraspeckle (NOPS), and RNA recognition motif 1. Their locations suggest that these domains are most likely to be involved in the interaction with RAD51D. The NOPS domain has been implicated in mediating dimerization of NONO with its interaction partners (Knott et al. 2016). RNA recognition motif 2 faces inside the dimer and is most likely not involved in the interaction due to lack of exposure. The coiled-coil domain of NONO has been shown to play a role in homo and heterodimerization (Knott et al. 2016). The NONO truncation yeast two hybrid analysis will possibly yield that the domains involved are either RRM1, NOPS, or the coiled-coil domain.
CHAPTER 3: ANALYSIS OF INTERSTRAND CROSSLINKS AND DOUBLE-STRANDED DNA BREAKS ON PARASPECKLE QUANTITY

3.1 Abstract
If repaired inefficiently, double-stranded DNA breaks (DSBs) are a major source of chromosome rearrangements. One of the primary pathways used to resolve DSBs is homologous recombination that utilizes the RAD51 protein complex and a homologous DNA template, which is thought to primarily be from the sister chromatid. RAD51D is the fourth member of the RAD51 complex and an essential component of this repair pathway. However, the function(s) of RAD51D have not been elucidated. To discover novel RAD51D functions, new interaction partners, such as NONO, were identified from a proteomics screen. NONO is a member of the Drosophila Behavior Human Splicing (DBHS) family that has been implicated in gene regulation and DNA repair. NONO is also an essential component of a nuclear organelle known as the paraspeckle, whose function during cellular metabolism is also unclear. My studies investigated the possible role for paraspeckles in DNA repair by scoring changes in their quantity in Hela and mouse embryonic fibroblast cells during the repair of DSBs or interstrand DNA crosslinks. These results suggest that there is no change in the paraspeckle quantity under cellular stress resulting from interstrand crosslink damage caused by mitomycin C or cisplatin. However, a small decrease was noted in the presence of DSBs resulting from treatment with etoposide. My results also demonstrated a decrease in the number of
paraspeckles in the absence of RAD51D. In conclusion, these results suggest that paraspeckles may have a role during repair of double stranded breaks.

3.2 Introduction

One of the main causes of diseases such as cancer is genomic instability due to defective DNA repair pathways (Abbas et al. 2013). The most severe type of DNA damage is double-stranded DNA breaks (DSBs). To repair DSBs, there are two main pathways involved: non-homologous end joining and homologous recombination. The homologous recombination pathway relies on the RAD51 complex and a homologous template that usually comes from a sister chromatid (Li and Xu 2016). One of the RAD51 proteins, RAD51D, plays an essential role in this complex and in the pathway. The full functionality of RAD51D has not been fully elucidated. To determine functions of this protein and its role in the pathway, novel interaction partners were identified and analyzed by a proteomics screen and yeast 2 hybrid approaches. Two of the most novel interacting partners discovered via the proteomic analysis were NONO and SFPQ (Rajesh et al 2009).

NONO and SFPQ are members of the Drosophila Behavior Human Splicing (DBHS) protein family. They have been implicated in nucleic acid processing and binding, transcriptional regulation, DNA repair, and other cellular processes (Knott et al. 2016). In nucleic acid processing, SFPQ and NONO have been shown to bind to the spliceosome, DNA and RNA, and to play a role in stabilizing transcripts (Knott et al. 2016). For DNA repair, NONO and SFPQ have been primarily implicated in non-homologous end joining through its association with Ku70 and increasing its DNA ligation ability (Bladen et al. 2005). NONO and SFPQ were also shown to bind to
another double-stranded break repair protein called Matrin3. In the absence of Matrin3, NONO along with its paralog, SFPQ had delayed localization to sites of DNA damage (Salton et al. 2014). In the absence of SFPQ and NONO, cells have also shown enhanced sensitivity to DNA damaging agents and chromosomal aberrations (Rajesh et al. 2011).

NONO and SFPQ were also identified as essential components of a nuclear organelle called the paraspeckle. The paraspeckle is located in the interchromatin space near nuclear speckles. It is composed of a long noncoding RNA named NEAT1 (nuclear enriched transcript 1) and several essential proteins such as PSPC1, NONO, and SFPQ (Yamazaki & Hirose 2015). Essential paraspeckles components, such as SFPQ and NONO, are necessary to its formation. Within the body of a paraspeckle, there are RNAs containing long poly A chains that can be edited to inosines during cellular stress events. These RNAs have their poly A ends changed to inosines and are then released to be translated into a protein product (Yamazaki, Hirose 2015). Another currently known function of paraspeckles involves their ability to sequester proteins from their target promoters either causing transcriptional repression or activation. An example lies with SFPQ that when bound to the promoter of IL-8 represses its expression. In times of cellular stress, such as during viral infections, SFPQ is sequestered within the paraspeckle allowing for increased expression of IL-8 (Imamura et al. 2014).

Since NONO and SFPQ, along with several other proteins localized to the paraspeckle, have been implicated in DNA repair, the purpose of my studies was to analyze if there was a role for paraspeckles during DNA damage response. Paraspeckle quantity was analyzed in the presence of the DNA damaging agents mitomycin C, cisplatin, and etoposide. The results suggest that interstrand crosslink damage displayed
no effect on the number of paraspeckles per cell. However, DSBs resulting from etoposide treatment, caused a very small, yet statistically significant, decrease in the number of paraspeckles per cell. These results suggest that the effect on paraspeckle quantity may differ depending upon the type of DNA damage encountered by a mammalian cell.

3.3 Materials and Methods

Plasmids

To analyze paraspeckles exogenously, GFP plasmids were transfected into HeLa or mouse embryonic fibroblast cell lines. The following plasmids shown in figure 3.1 were used to perform these experiments: pEGFP-N1, pEGFP-SFPQ and pEGFP-NONO. NONO and SFPQ were cloned into pEGFP at the XmaI and SmaI restriction enzyme sites.
Figure 3.1. pEGFP, pEGFP-SFPQ, and pEGFP-NONO plasmid maps. Pictured above are schematics of the pEGFP plasmids used to transfect HeLa cells. The GFP gene is fused to the NONO or SFPQ gene. Each plasmid contains a kanamycin antibiotic resistance gene. Plasmid maps shown here were generated using SnapGene (GSL Biotech)
Bacterial genotype and Transformation

The bacterial strain used for the plasmids in these studies was DH5α. The genotype for DH5α is as follows: F-ϕ80, lacZΔM15 (lacZYA-argF), U169 recA1, endA1, hsdR17(rK-, mK+), phoA, supE44, λ-thi-1, gyrA96, relA1.

The pEGFP-N1, pEGFP-SFPQ, and pEGFP-NONO plasmids were transformed into DH5-alpha cells using the heat shock procedure. In this protocol, bacterial cells and less than 100 nanograms of each plasmid were placed on ice for 30 minutes. After a 30 minute incubation, the cells were shocked by being placed in a 42°C water bath for 42 seconds enabling the plasmids to enter the bacterial cells. Following the heat shock, the transformation mixtures were placed on ice for 2 minutes. The cells were pelleted, resuspended in 1 ml of LB broth, and incubated with shaking at 37°C for one hour. After the one hour incubation, one fifth of the mixture was plated on the appropriate antibiotic selection plate at 50 µg/ml for kanamycin. The colonies were then grown overnight into liquid culture. After overnight incubation, DNA was isolated from bacterial cells using Bio-Rad Quantum Midiprep Plasmid Kit (7326120). The plasmids were then placed into mammalian cells by transfection.

Mammalian Cell Line Transfection

To analyze paraspeckle quantity using the pEGFP plasmids, Mirus TransX- LT1 transfection reagent was used. Each plasmid was individually transfected into a 6-well plate. Each well contained 300,000 HeLa cells and 3 µg of each plasmid was used. The plasmids, transfection reagent, and OPTI-MEM medium were warmed to room temperature. Afterwards, 250 microliters of OPTI-MEM was placed into a microcentrifuge tube, followed by 3 microliters of transfection reagent to 1 ug of DNA.
After this mixture was created, a 30 minute incubation followed. The transfection mixture was then added to the cells in a dropwise manner and placed at 37°C with 5% CO₂ (Thermo Scientific).

**DNA Damage Treatment**

The following DNA damage agents were used to perform these experiments: mitomycin C, cisplatin, and etoposide. For each drug treatment, a low concentration was chosen that would not affect cell survival and a higher concentration was chosen that would result in approximately 50% cell death. Mitomycin C was resuspended in water at a concentration of 2mg/ml. From this stock, the following concentrations were used to treat HeLa cells: 150, 250, and 550 ng/mL. Cisplatin was resuspended in PBS at a concentration of 5 mM. The following concentrations were used to treat HeLa cells: 1 and 10 uM. Etoposide was resuspended in DMSO at a concentration of 42 mM. The following concentrations were used for treatment of HeLa cells: 5 and 50 uM. Mitomycin C treatment was done to transfected and non-transfected cells. Cisplatin and etoposide were only used to treat non-transfected cells. In transfected cells, mitomycin C treatment was completed 12 hours after the transfection. Mitomycin C, cisplatin and etoposide treatment in non-transfected cells were completed 24 hours after the cells were plated.

**Cell Fixation and Immunofluorescence**

For transfected and non-transfected HeLa cells, after drug treatment was completed, fixation occurred in 4% paraformaldehyde for 20 minutes. After fixation, the cells were washed twice with 1X PBS. Permeabilization occurred with 0.1% Triton X-100 for 5 minutes at room temperature. The cells are again washed in 1X PBS twice. For transfected cells, Prolong Diamond Antifade Mountant with DAPI (Life Technologies)
was placed on slides and the coverslips were mounted. The slides are then placed in the dark overnight and analyzed on the microscope afterwards. For untransfected cells, after permeabilization, blocking occurs in 1% BSA in PBS for 30 minutes to 1 hour. After the blocking, a primary antibody to SFPQ (mouse, ab11825) or NONO (goat, ab50411) (Abcam) at 1:1000 in 1% BSA in PBS was incubated with the cells overnight at 4°C. The following day, the cells were washed twice for 5 minutes each in 1X PBS. A secondary AlexaFluor 594 donkey anti-goat (A11058), AlexaFluor 488 donkey anti-goat (A11055), Oregon green anti-mouse 488 (06380), or Texas red anti-mouse antibody (16930, Molecular Probes) was then added to the cells at a 1:1000 dilution and incubated for 1 hour shaking in the dark. After the 1 hour incubation, the cells were washed 5 times for 15 minutes each. After the washes, a single drop of Prolong Diamond Antifade Mountant was placed on slides. The coverslips were then placed on top of the slides and stored in the dark overnight to cure. Paraspeckles were then counted using a Nikon Eclipse E600 at 100X magnification.

**Paraspeckle Scoring**

For paraspeckle scoring, 30-50 cells were counted for each treatment condition. For transfected and non-transfected HeLa cells, distinct large foci within the cells were scored as positive. Foci counts were placed in an Excel spreadsheet. Beeswarm plots were made using GraphPad (GraphPad Software Inc.) to plot the data.

**Statistical Significance**

A two-tailed t-test was used to analyze significance in paraspeckle quantity between untreated and treated conditions. The two-tailed t-test was conducted in Excel. P<0.05 was considered statistically significant.
3.4 Results

*Paraspeckle Numbers following Mitomycin C Treatment*

NONO and SFPQ have been implicated in DNA repair and shown to be essential components of the paraspeckle. The purpose of these studies was to determine if there was a connection between interstrand crosslinks, a type of DNA damage, and paraspeckle quantity. To complete the analysis, HeLa cells were either transfected with pEGFP plasmids or antibodies were used against SFPQ and NONO. For each treatment condition, approximately 30-50 cells were scored. In transfected cells, the treated and untreated conditions maintained about 2-5 paraspeckles per cell (Figure 3.2A). There was no statistically significant difference in cells treated with mitomycin C versus untreated. In untransfected cells, the number of paraspeckles were approximately 3 per cell (Figure 3.2B). Over the 24 hour drug treatment, the paraspeckle number remained unchanged.

A.
B.

Figure 3.2. Mitomycin C Treatment Effect on Paraspeckle Quantity. A. Hela cells were transfected with pEGFP-NONO and counted for paraspeckle foci. For the untreated and treated conditions, the paraspeckle number ranged from 1-3 per cell. To the right are representative images of transfected HeLa cells with paraspeckles. B. Hela cells were stained with the anti-NONO antibody and scored for paraspeckles. In the right image are representative pictures of paraspeckles observed using anti-NONO antibody.

*Paraspeckle Numbers following Cisplatin Treatment*

Cisplatin is a drug that causes DNA interstrand crosslinks. The absence of SFPQ and NONO caused cells to have enhanced sensitivity to interstrand crosslink drugs (unpublished data). In three independent trials, non-transfected HeLa cells were subjected to cisplatin at 1 and 10 µM for 24 hours. After 24 hours and cell fixation and immunofluorescence staining with anti-SFPQ antibody, 30-50 cells were scored. In the untreated and treated conditions, there were approximately 1-3 paraspeckles per cell. The number of paraspeckles per cell from untreated to treated did not significantly change (Figure 3.3).
Figure 3.3. Effect of Cisplatin on Paraspeckle Quantity. Left: Bee-swarm plot made using Graphpad analyzing the effect on paraspeckles under cisplatin treatment. The HeLa cells were treated with cisplatin for 24 hours at two different doses: 1 and 10uM. In the untreated and treated conditions, there were about 1-3 paraspeckles per cell. Right: Representative images of paraspeckles in Hela cells under untreated and treated conditions.

Paraspeckle Numbers following Etoposide Treatment

Etoposide is a topoisomerase II inhibitor that causes double-stranded DNA breaks through prevention of the linkage between DNA and the topoisomerase. SFPQ and NONO have been implicated in double-stranded DNA break repair. In these studies, the purpose was to analyze, using SFPQ as a paraspeckle marker by immunofluorescence, if etoposide affected paraspeckle quantity. In three independent experiments, HeLa cells were treated with etoposide at 5 and 50 µM for 24 hours. In the untreated cells, they averaged about 3-5 paraspeckles per cell (Figure 3.4). At 5 uM, there were approximately
1-3 paraspeckles per cell. At 50 µM, there were on average 0-2 paraspeckles per cell.

The results suggest that DSBs induced by Etoposide treatment significantly decreased the number, of paraspeckles per cell (P<0.05).

![Graph showing paraspeckle concentration](image)

**Figure 3.4. Etoposide Decreases Paraspeckle Quantity**

Left: Bee-swarm plot made in Graphpad displaying results of paraspeckle scoring. HeLa cells were treated with cisplatin at two doses for 24 hours. Paraspeckle number per cell decreased from 3-5 in the untreated conditions to 1-3 at 5 and 50 µM. Right: Representative images of HeLa cells stained with anti-SFPQ antibody.

*Deletion of Rad51d Decreases Paraspeckle Quantity*

Through a proteomics analysis and my yeast two hybrid studies, an interaction was identified between RAD51D and NONO. Because this interaction is likely necessary for repairing DNA damage, I wanted to determine if that might extend to NONO’s role in paraspeckle formation. In these studies, RAD51D proficient and deficient mouse embryonic fibroblasts were transfected with pEGFP-SFPQ and pEGFP-NONO and
scored for the presence of paraspeckles (Figure 3.5). In these studies, scoring was accomplished by analyzing cells that contained greater than three paraspeckles. In the *Rad51d*-deficient MEFs, there were fewer cells that contained more than three paraspeckles in comparison to the *Rad51d*-proficient MEFs.

![Graph](image)

**Figure 3.5.** Loss of RAD51D causes a reduction in paraspeckles. pEGFP-SFPQ and pEGFP-NONO were transfected into mouse embryonic fibroblasts (MEFs) that were RAD51D proficient or deficient. Around 50 cells were scored for each cell on whether they had greater than 3 paraspeckles per cell. In the *Rad51d/-* MEFs for both pEGFP-SFPQ and pEGFP-NONO, there were less cells with greater than 3 paraspeckles. All MEF cell lines were *Trp53* deficient.

### 3.5 Discussion

RAD51D is a RAD51 paralog that participates in a complex in homologous recombination to search for the homologous template that is used to resolve double-
stranded DNA breaks. Without RAD51D, homologous recombination is impaired. For example, mutations in the Rad51d gene confers disposition towards ovarian cancer and ineffective treatment (Loveday et al. 2011). Loss of RAD51D can lead to chromosomal aberrations and enhanced sensitivity to DNA damage (Smiraldo et al. 2005). In order to fully identify the functions of RAD51D, novel interaction partners must be found.

Through a proteomic study, it was found that RAD51D interacted with NONO (Rajesh et al. 2009). Loss of RAD51D and NONO led to chromosomal aberrations and increased sensitivity to DNA damage such as interstrand crosslinks or DNA double-stranded breaks. The increased sensitivity to interstrand crosslinks or DNA double-stranded breaks could be due to delayed recruitment of downstream DNA factors, weakened association with site of DNA damage, ineffective homology search or strand invasion. It suggests that this interaction is necessary for the repair of interstrand crosslinks and double-stranded DNA breaks.

NONO is a member of Drosphila Behavior Human Family that has been implicated in several cellular processes. NONO, in its interaction with its paralog SFPQ, has been found to interact with splicesome components such us U5 snRNA, stabilize mRNA transcripts, and other steps in nucleic acid processing (Yamazaki & Hirose 2015). NONO has also been implicated in DNA repair through its interaction with non-homologous end joining protein Ku70, and other DNA repair proteins such as Matrin3 and PARP (Yamazaki & Hirose 2015). Loss of NONO impairs DNA repair and can lead to genomic instability. In NONO-deficient mouse embryonic fibroblasts, there was no effect upon gamma-H2AX and 53BP1 foci resolution suggesting a stage downstream (Li et al. 2014).
NONO is also an essential component of a nuclear organelle called the paraspeckle. Paraspeckles are composed of proteins and a long noncoding RNA called NEAT1. The paraspeckle retains hyperedited adenosine-to-inosine mRNAs until times of cellular stress. Paraspeckles are also known to sequester proteins from their target promoters. For example, under viral infection, SFPQ is sequestered in the paraspeckle away from the IL-8 promoter to allow its expression (Imamura et al. 2014). Various paraspeckle proteins, such as NONO, SFPQ, HNRNPK, FUS have been implicated in DNA damage response (Salton et al. 2010, Mastrocola et al. 2013, Moumen et al. 2005). The various paraspeckle proteins implicated in DNA damage response suggest there may be a correlation between DNA damage and paraspeckle function.

Since the interaction between RAD51D and NONO appears to be necessary for DNA repair, I wanted to determine if that interaction extended to the paraspeckle function of NONO. In these studies, we looked at a paraspeckle quantity in the presence and absence of RAD51D. The results demonstrate that more cells in Rad51d-deficient MEFs were absent of paraspeckles in comparison to Rad51d-proficient. They suggested that RAD51D may have a role in paraspeckle formation. To further analyze the possibility of paraspeckles in DNA repair, HeLa cells were treated with interstrand crosslink and double-stranded DNA break causing drugs. Our results showed that interstrand crosslink damage does not affect paraspeckle quantity but that paraspeckles are decreased in the presence of double-stranded DNA breaks. These results suggest that paraspeckles may have a role in double-stranded DNA break repair and the interaction between RAD51D and NONO may play a role in that function.
A decrease in paraspeckle quantity may indicate that NONO or other DNA repair proteins in the paraspeckle are being released to fulfill their function in the resolution of double-stranded DNA breaks. It is also possible that the interaction between RAD51D and NONO may enable release of mRNA transcripts during times of stress that possibly play a role in the resolution of double-stranded DNA breaks. To analyze this possibility, fluorescent tags could be placed on RAD51D, NONO, and a known paraspeckle mRNA and live-cell imaging could be used to track the release of the mRNA from the paraspeckle when analyzing RAD51D and NONO interaction. Further work needs to be done to identify how the interaction of RAD51D and NONO may affect paraspeckle function in DNA double-stranded breaks.

Future studies will use RAD51D and NONO knockout cell lines and analyze the actions of downstream HR proteins. It is possible that deletion of this interaction could cause delayed or no DSB repair. To analyze the effects of this interaction, foci scoring of downstream DSB repair factors such as BRCA1 at different time points could be completed to confirm if disruption of this interaction causes a delay. This interaction may also serve as an activation signal through a post-translational modification. Methylation of NONO by CARM1 affects its interaction with RNA (Hu et al. 2015). In order to determine if RAD51D and NONO serve as an activation signal in DSB repair, yeast two hybrid analysis with site-directed mutagenesis will be performed targeting amino acids that are phosphorylated, ubiquitinated, or methylated. If this interaction serves as an activation signal, fluorescent microscopy will be used to determine if plays in release of mRNA transcripts from the paraspeckle.
REFERENCES


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