Implementing the Bimolecular Fluorescence Complementation assay to study protein interactions in the cell cycle checkpoint response

by

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Abstract

The genomic integrity of a cell is constantly being pressured by both intrinsic and extrinsic forces. Cell cycle checkpoints exist to protect the cells by arresting cell cycle progression in response to DNA damage or replication stress. It has been shown that the interaction between the checkpoint proteins Rad9A and TopBP1 is a crucial upstream event required for the ATR-dependent checkpoint response to DNA damage, which can be activated throughout different points in the cell cycle. The Bimolecular Fluorescence Complementation (BiFC) technique has recently emerged as a simple and effective tool for analyzing protein-protein interactions in live cell cultures. By fusing complementary fragments of fluorescent proteins to proteins of interest, one can visualize protein-protein interactions through the formation of a mature fluorophore from these fragments. In the current work, the BiFC assay system was employed to study the interaction between TopBP1 and Rad9A; the human homologue of fission yeast Rad9. BiFC vectors expressing TopBP1, Rad9A, and the Rad9A-S387 mutant were constructed and optimized for transfection in HeLa cells. It was shown that the BiFC fusion protein of Rad9A lacked phosphorylation on its constitutive S387 site, although it retained its upstream damage dependent S272 phosphorylation after IR treatment. BiFC signals could be detected in cells containing the BiFC fusion proteins of Rad9A and TopBP1 using confocal microscopy and flow cytometry techniques. However, the signals could not be distinguished from that of the negative control samples. Our results suggest a possibility that our BiFC fusion proteins of interest interact in a non-specific manner, although further characterization is required to confirm this. The BiFC assay employed in this project must be further optimized to effectively study the interaction between Rad9A and
TopBP1, as well as other checkpoint proteins. However, this study has given us great insight into the implementation of this new BiFC technique for studying protein interactions in the context of cell cycle proteins, and the knowledge gained from this study will be invaluable for future work.
Acknowledgements

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<tr>
<td>9-1-1</td>
<td>Rad9A-Rad1-Hus1A</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53 Binding Protein 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>ATRIP</td>
<td>ATR interacting protein</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer associated gene 1</td>
</tr>
<tr>
<td>BRCT</td>
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<tr>
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<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
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<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
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<tr>
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<td>Fluorescence/Förster resonance energy transfer</td>
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<tr>
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<td>Hydroxyurea</td>
</tr>
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<td>Immunoglobulin G</td>
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<tr>
<td>IgY</td>
<td>Immunoglobulin Y</td>
</tr>
<tr>
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<td>Ionizing radiation</td>
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<td>Mediator of DNA damage checkpoint protein 1</td>
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<td>Normal goat serum</td>
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<tr>
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<td>Phosphate buffered saline</td>
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<td>Proliferating cell nuclear antigen</td>
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<td>Single stranded DNA</td>
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<td>Tris-Acetate-EDTA</td>
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<tr>
<td>TopBP1</td>
<td>Topoisomerase II beta binding protein 1</td>
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Chapter 1: Introduction

1.1 The cell cycle and the DNA damage checkpoint response

1.1.1 Overview of cell cycle checkpoint control

The cells of an organism must be able to accurately duplicate and pass on its genetic material to its daughter cells in order to exhibit proper functioning and survival. However, the genomic stability of a cell is constantly being pressured by extrinsic DNA damaging agents as well as intrinsic stresses caused by regular metabolic processes [1-4]. Eukaryotic cells have developed a complex system of regulatory mechanisms called cell cycle checkpoints to ensure that certain cellular processes are completed before downstream cell cycle events can occur by delaying or arresting cell cycle progression [5-8]. The many proteins and processes involved in the checkpoint response often overlap and function interdependently with the mechanisms of DNA damage repair, and apoptosis [9]. The cell cycle checkpoints regulate entry of cells into S-phase, progression through S-phase, and entry into and exit out of mitosis in response to DNA damage [8]. Due to the importance of cell cycle checkpoints in maintaining genetic stability, defects in this system can contribute to diseases such as cancer.

The accumulation of genetic mutations in cells causes the activation of proto-oncogenes and the inhibition of tumour suppressor genes that can lead to cancer formation [10]. Defects in the mechanisms of DNA repair and cell cycle checkpoints may allow cells to continue dividing in the presence of DNA damage. One of the most commonly mutated genes in human cancer is p53, which has a number of roles involved in DNA repair, apoptosis and checkpoint control [11, 12]. Other checkpoint proteins
implicated in various cancers include Chk1, Chk2, BRCA1, and ATM [10, 13-15]. Recently, checkpoint proteins have also been increasingly studied as targets for therapeutic cancer drugs. Although the loss of checkpoints can lead to uncontrolled cell proliferation, it can also lead to cell death, which can be desirable for the treatment of cancer [16]. Many cancer cells have intact G2/M checkpoints, while they have defects in the G1/S checkpoint [16]. Thus, the targeting of G2/M checkpoint proteins such as Chk1 have been of keen interest in recent years for the study of cancer therapeutics [16]. Further insight into the mechanisms and specific protein interactions of the various checkpoint signaling pathways in the checkpoint response to DNA damage will be valuable for the study and treatment of human cancers.

1.1.2 Checkpoint proteins

The proteins involved in the DNA damage checkpoints are generally grouped into four categories; sensors, transducers, mediators, and effectors [17]. However, these are loosely defined categories for conceptual purposes and many proteins can be thought of as belonging to more than one group. Also, although each DNA damage checkpoint pathway is unique, they may share many of the same proteins in their signaling cascade.

1.1.3 Checkpoint sensors

Sensors are the proteins/protein-complexes that recognize the sites of DNA damage or replication stress and initiate the checkpoint response. One important sensor complex in the DNA damage checkpoint response is made up of the Rad17-RFC (Replication Factor-C) complex and the 9-1-1 complex. The related ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia telangiectasia and Rad3 related) kinases also act as sensors, responding to different types of DNA damage [18, 19]. Although these
sensors all respond to DNA damage, they are thought to be loaded to sites of damage independently of one another [18].

The 9-1-1 complex is made up of the Rad9A, Rad1, and Hus1A proteins that interact to form a heterotrimeric ring structure [19, 20]. Structurally, the 9-1-1 complex resembles the homotrimeric Proliferating Cell Nuclear Antigen (PCNA) complex [21, 22]. PCNA is a sliding clamp processivity factor for DNA polymerase δ that is involved in DNA replication and repair in eukaryotic cells [23, 24]. In response to DNA damage, areas of ssDNA are produced that are subsequently coated by replication protein A (RPA) [25]. Rad17 forms a complex with four of the small RFC subunits to form the Rad17-RFC clamp loader structure, which binds to the ssDNA through its interaction with RPA [25]. In much the same way that RFC loads PCNA onto DNA during replication, Rad17-RFC loads the 9-1-1 complex at the site of DNA damage [26]. Although the 9-1-1 complex has been primarily associated with the checkpoint response, emerging evidence has also linked the complex with a role in the closely associated DNA repair pathway [5, 18, 27].

The human Rad9A gene was first identified based on sequence homology to the rad9+ gene of fission yeast [28]. A unique feature of Rad9A is that it contains a ~120 amino acid C-terminal tail region that is not required for 9-1-1 complex formation [29]. This heavily modified tail region is made up of many constitutive, cell cycle dependent, and damage dependent phosphorylation sites [19, 30-32]. It is believed that the phosphorylated C-terminal tail of Rad9A may act as a loading platform for many checkpoint proteins, so as to bring them to sites of DNA damage. Recently, a related protein called Rad9B has been identified, which is highly expressed in human testes and
expressed in lower amounts in skeletal muscle [33]. Rad9B can form a unique 9-1-1 complex with Rad1 and both Hus1A/Hus1B. It is speculated that these unique complexes may exhibit distinct cellular functions that are yet unidentified [33].

As mentioned previously, the ATM and ATR kinases make up the other major group of sensor proteins, although they can also be classified as transducers. ATM and ATR are part of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of serine/threonine kinases [19, 34, 35]. ATM and ATR are activated and brought to sites of damage in response to different types of DNA damage or replication stress. ATM is thought to be primarily activated in response to DNA double strand breaks (DSB), whereas ATR is thought to localize to sites of RPA coated ssDNA and activated in a Rad17-RFC/9-1-1 complex dependent manner, [18, 36, 37].

Further complicating matters, there is much overlap in the downstream targets of the signaling pathways initiated by both ATM and ATR and recent evidence has shown that both kinases can be activated by multiple agents of DNA damage [19]. This is believed to be because one specific type of DNA damage may lead to the eventual formation of a different type of lesion. Such can be the case with DSBs, which can be processed into ssDNA to recruit various proteins required for the ATR-dependent checkpoint pathway and DNA damage repair [38]. However, much is still unknown about the exact mechanisms and the intertwined relationships of the different checkpoint signaling pathways. Upon sensing the DNA lesions and the activation of the checkpoint response, the checkpoint signal is passed down to downstream checkpoint proteins called mediators, transducers, and effectors.
1.1.4 Checkpoint mediators, transducers, and effectors

The mediator group of checkpoint proteins is recruited to sites of DNA damage by the sensors and may interact with both ATM/ATR and downstream transducers to relay specific checkpoint signals. Mediators include Topoisomerase Binding Protein 1 (TopBP1), Claspin, Mediator of DNA Damage Checkpoint Protein 1 (MDC1), and p53 Binding Protein 1 (53BP1) [39-42]. In the DNA damage checkpoint response, the main transducers are the Chk1 and Chk2 kinases [43, 44]. Although there is overlap between the pathways, generally the damage signal sensed by ATR is transduced by Chk1, while that of ATM is transduced by Chk2 [45, 46]. These transducer kinases ultimately relay the damage signal to proteins such as the Cdc25 phosphatases and p53 that directly target the effector proteins responsible for cell cycle transition, which are primarily cyclin-dependent kinases (Cdks) [47].

1.1.5 Checkpoint activation by DNA damage

Exposure to different types of genotoxic agents, and even regular metabolic processes such as replication can lead to the formation of DNA structural abnormalities or intermediates. These include stalled replication forks, double strand breaks (DSBs), pyrimidine dimers, and other structural changes of DNA that activate the checkpoint response [48-50]. As noted earlier, DSBs are associated primarily with the activation of ATM and is recognized as one of the most dangerous forms of DNA damage [48]. Ionizing radiation (IR) sources can easily induce many types of DNA lesions, including DSBs and is used commonly in the laboratory for this purpose. IR is able to ionize molecules with which it collides, causing damage directly to DNA or indirectly through the production of reactive oxygen species [51]. Another common experimental
compound for checkpoint activation is hydroxyurea (HU). HU works by blocking DNA replication through the inhibition of the enzyme ribonucleotide reductase (RNR), which is responsible for the conversion of ribonucleotides into deoxyribonucleotides [52]. Nucleotide depletion leads to stalled replication forks, creating stretches of ssDNA that primarily activates the ATR-mediated checkpoint response [48, 53].

1.1.6 The eukaryotic cell cycle

In order for eukaryotic cells to divide properly, a number of complex events must take place for its genome to be correctly replicated, divided, and distributed into two new daughter cells. These complex events collectively make up the eukaryotic cell cycle. The cell cycle is made up of four distinct phases that include the Gap1 (G1), Synthesis (S), Gap2 (G2), and Mitosis (M) phases. During S phase, DNA replication commences and is completed, resulting in twice the amount of DNA in the cell [54-56]. The M phase consists of nuclear and cytoplasmic division resulting in two identical daughter cells. The G1 and G2 phases occur before S and M respectively. During these phases, the cell gets ready for the next phase of the cell cycle by synthesizing the necessary factors required for cell cycle progression [54-56]. Quiescent cells that are not dividing are referred as being in the Gap0 phase. The progression between the phases of the cell cycle is controlled by the key regulatory proteins known as cyclins and Cdk's [54-56].

Cdk's are serine/threonine kinases whose kinase activity is activated upon interaction with the regulatory cyclin proteins to form specific cyclin-Cdk complexes. This process is regulated by the synthesis and degradation of specific cyclins in a phase-specific manner during the different phases of the cell cycle [56]. In mammalian cells, there are a number of different Cdk's and cyclins that interact in specific combinations.
This leads to the phosphorylation of downstream substrate targets that control specific mechanisms of the cell cycle progression. Progression out of G0 and through G1 until the late G1 restriction point is controlled by the D-cyclins, which interact with Cdk4 and Cdk6 [57-59]. Cdk2 is then responsible for promoting entry and progression into the S phase by interacting with cyclin E and cyclin A [60, 61]. Finally, mitosis occurs by the cyclin B dependent activation of Cdc2 (Cdk1) [62, 63]. As mentioned previously, these cyclin-Cdk complexes and subsequently, cell cycle progression is regulated by cell cycle checkpoints that responds to DNA damage. The specific DNA damage checkpoints including the G1/S, intra-S, and G2/M checkpoints will be discussed below.

1.1.7 The G1/S checkpoint

In the presence of DNA damage in the G1 phase, the G1/S checkpoint is activated, preventing the initiation of replication. There appears to be two distinct signaling pathways involved in the G1/S checkpoint; the first rapid response to initiate the G1/S cell cycle arrest, and the second prolonged response to maintain it [64]. In the first pathway, depending on the type of damage, either ATM or ATR is activated leading to Chk1/Chk2 phosphorylation. Chk1/Chk2 can then inhibit the Cdc25A phosphatase by phosphorylating it, leading to nuclear exclusion and ubiquitin mediated proteolytic degradation [65]. The decrease in Cdc25A levels results in the accumulation of the inactive form of Cdk2-cyclinA/E, preventing the transition into the S phase by preventing Cdc45 dependent initiation of replication [66, 67].

The second G1/S checkpoint response is also activated in response to DNA damage, but may take several hours after damage to become fully functional as it affects the transcriptional machinery [64]. In this p53 dependent pathway, ATM/ATR
phosphorylates p53 directly and indirectly through Chk2/Chk1, preventing its nuclear export and degradation [68-70]. The accumulation of p53 allows for the increased activation of target genes, such as p21 [71]. p21 then binds and inhibits the Cdk2-CyclinE and Cdk2-CyclinD complexes required for S-phase progression leading to the maintenance of the G1/S checkpoint arrest [64]. It has also been shown recently that Cdk4 activity is inhibited by p21 following DNA damage [72]. Cdk2-CyclinE dependent phosphorylation of Cdc45 is required for the initiation of DNA replication, while Cdk4-CyclinD dependent phosphorylation of retinoblastoma protein (Rb) is required for the transcription of a number of S-phase genes required for progression into S-phase [64, 73, 74]. The p53 protein also regulates the expression of a number of genes involved in apoptosis and p53 mediated apoptosis is another regulatory mechanism used to rid the cell population of damaged DNA [75].

1.1.8 The intra-S checkpoint

In order for eukaryotic DNA to be replicated correctly during the S-phase, both the firing of multiple origins of replication and replication fork progression must be carefully maintained [76]. DNA lesions can impede the replication fork and result in replication fork collapse [3, 77]. Thus, in the presence of unrepaired DNA damage from the G1 phase or new DNA damage inflicted on the cell during S-phase, the intra-S phase checkpoint is activated. The intra-S checkpoint regulates the rate of replication elongation and inhibits the firing of all origins of replication yet to be replicated [76]. Checkpoint deficient cells fail to inhibit DNA replication in response to DNA damaging agents [78]. This failure in response to IR damage is known as radioresistant DNA synthesis (RDS). Similarly to the G1/S checkpoint, the intra-S checkpoint response to DNA damage can
lead to the ATM/ATR mediated degradation of Cdc25A, leading to delayed S-phase progression [79]. However, there are also many other checkpoint proteins believed to be involved in the S-phase checkpoint response, which are intertwined with the DNA repair pathway such as MDC1, and 53BP1. These proteins are recruited to sites of damage after IR treatment and in the case of MDC1, its downregulation leads to a RDS phenotype [80, 81].

1.1.9 The G2/M checkpoint

The G2/M checkpoint ensures that cells with damaged DNA do not enter mitosis [82]. Similarly to the other checkpoints, different types of DNA damage during G2 can activate the ATR-Chk1 or ATM-Chk2 arms of the checkpoint response [83]. When the ATR-Chk1 pathway is activated by DNA damage, the ATM-Chk2 pathway is later activated to maintain the checkpoint response and vice versa [17, 84, 85]. Ultimately, both pathways prevent mitotic entry by regulating the Cdc2-CyclinB complex. Chk2/Chk1 phosphorylation leads to Cdc25A sequestering by the 14-3-3 complex and ubiquitin mediated degradation, leaving Cdc2 in its inactive form [86, 87]. Wee1, an inhibitor of Cdc2 is also upregulated leading to cell cycle arrest at G2/M [88, 89]. The checkpoint proteins and their role in the DNA damage checkpoint response throughout the different phases of the cell cycle are summarized in Figure 1.
Figure 1: The cycle checkpoint response to DNA damage.

In response to DNA damage at specific points in the cell cycle, different checkpoint signaling pathways are activated. DSBs predominantly activate the ATM mediated response, while stalled replication forks leading to ssDNA predominantly activate ATR. The sensor protein complexes such as Rad17-RFC, the 9-1-1 complex, ATM, and ATR sense the DNA damage. The damage signal is then relayed onto mediator proteins such as TopBP1, MDC1, Claspin, and 53BP1, which help activate the Chk1 and Chk2 transducer kinases. The transducer kinases ultimately help relay the damage signal to the cyclin-Cdk effector complexes, which directly regulates cell cycle progression. In the G1/S checkpoint, p53 accumulation and Cdc25A degradation leads to cell cycle arrest. p53 is also involved in the apoptosis response to DNA damage. The intra-S checkpoint response also leads to Cdc25A degradation and subsequent inhibition of DNA synthesis and origin firing. In the G2/M checkpoint, both Cdc25A degradation and Wee1 upregulation leads to cell cycle arrest.
The diagram represents a detailed pathway of cellular mechanisms involving sensors, mediators, transducers, effectors, and checkpoints of the cell cycle. The sensors include proteins like Rad17, RFC, and ATR. The mediators such as TopBP1, MDC1, S3BP1, and Claspin transmit signals to the transducers, which include Chk1 and Chk2 proteins. These transducers regulate the cell cycle checkpoints at G1, S, and G2 phases. The effectors such as p53 and p21 play crucial roles in controlling cell proliferation and apoptosis. The ubiquitin (Ub) modification is also depicted as a key regulatory mechanism in this pathway.
1.1.10 TopBP1 and the ATR-dependent checkpoint response

As previously discussed, DNA damage or replication stress caused by both exogenous and endogenous factors can lead to the activation of the ATR kinase through exposure to RPA covered ssDNA [37]. The 9-1-1 complex is loaded onto these sites by Rad17-RFC, while ATR is loaded in an independent manner through its association with ATR interacting protein (ATRIP) [18]. Direct interaction between ATRIP and RPA was shown to load the ATR kinase onto DNA [37]. Recently, it has been shown that the mediator protein TopBP1 is required for ATR-dependent activation of Chk1 in the DNA damage checkpoint response [90]. TopBP1 is a multi-BRCA1 C-terminal (BRCT) domain containing protein that is required for DNA replication [39]. TopBP1 is brought to sites of DNA damage or replication stress by interacting with the C-terminal tail of Rad9A [32, 90, 91]. This interaction occurs between the constitutive Serine 387 phosphorylation site of Rad9A and the BRCT1 and BRCT2 domains of TopBP1 [90]. Following this interaction, TopBP1 is able to interact with and activate the ATR/ATRIP complex via its activation domain located between BRCT domains 6 and 7 [90]. Although TopBP1 is required for ATR-dependent activation of Chk1, the mediator protein Claspin is also required [92]. Claspin functions downstream of TopBP1 in the ATR-dependent checkpoint response and directly interacts with Chk1 to activate it [92]. Collectively, TopBP1 can be looked upon as the general activator of ATR, while Claspin is a specific downstream activator of ATR dependent Chk1 activation [92]. Thus, the Rad9A-TopBP1 interaction is very important, as it is one of the most upstream components of the ATR-mediated DNA damage checkpoint response.
Figure 2: The ATR-dependent DNA damage checkpoint response.

At sites of DNA damage and replication stress, stretches of ssDNA appear and is subsequently bound by RPA. The Rad17-RFC clamp loader interacts with RPA and then loads the 9-1-1 complex onto DNA. The ATR-ATRIP complex is also loaded onto DNA through its interaction with RPA in a Rad17-RFC/9-1-1 independent manner. TopBP1 is brought to sites of DNA damage through the interaction between its BRCT1 and BRCT2 domains with the Serine 387 phosphorylation site (Red P) of Rad9A. This allows TopBP1 to then interact and activate ATR with its activation domain (AD). This interaction is crucial for the downstream activation of Chk1, which also requires Claspin. Some of the identified phosphorylation sites of Rad9A are listed. Green denotes constitutive phosphorylation sites, red denotes damage dependent sites, and blue denotes cell cycle dependent sites. Black denotes unique phosphorylation sites determined by the Karnitz group [31].
hRad9A – Phosphorylation Sites
S272 – damage induced
S277 - constitutive
T292 - mitotic
S328
S336
S341
T355
S375
S380
S387 – interacts with TopBP1
1.2 Protein Interaction Studies

1.2.1 Methods of studying protein interactions

The study of macromolecular interactions is an area of great interest in cellular biology and many different assay systems have been implemented in the past to study these relationships. These include such methods as two-hybrid screening, co-immunoprecipitation, and various co-purification techniques [93-96]. However, it is often desirable to visualize direct protein interactions in the context of live cells using microscopy techniques. Such methods have the advantage of studying protein interactions in their live cellular environment, as well as being able to visualize the subcellular localizations of such an interaction. To date, the most popular method to visualize protein interactions in live cells has been the Fluorescence/Förster Resonance Energy Transfer (FRET) technique [97-99].

When visualizing protein interactions by FRET, proteins of interest are fused to a donor or acceptor fluorophore. The principle of the technique is based on the idea that when proteins of interest are within close enough proximity (usually less than 10nm), the excited donor fluorophore is able to transfer its energy to the acceptor fluorophore, whose emission fluorescence can be measured using various confocal microscopy techniques [97-99]. The related technique called Bioluminescence Resonance Energy Transfer (BRET) works in a similar way, except that it uses a bioluminescent enzyme such as luciferase as the donor molecule [100].

An assay system called Bimolecular Fluorescence Complementation (BiFC) has recently gained popularity as an alternative tool for visualizing protein interactions in live cells. First described by Hu et al. using the bZIP regions of the proteins c-Fos and c-Jun
(bFos, bJun), the BiFC technique involves the use of non-fluorescent protein fragments derived from whole fluorescent proteins [101]. These fragments are carefully designed so as to be able to interact and form a fully functional fluorophore when they come into close enough proximity [102, 103]. Furthermore, these complementary protein fragments can be fused onto proteins of interest, whereupon specific interaction between the proteins of interest will bring the fragments into close enough proximity to form a mature fluorophore [102, 103]. These protein interactions can then be detected in live cells using standard techniques of confocal microscopy and flow cytometry [102-104]. The principle behind such a technique has evolved from prior protein interaction studies that made use of protein complementation between fragments of enzymes such as β-galactosidase and dihydrofolate reductase [105, 106]. The BiFC technique has now been successfully used to study many different types of molecules including nuclear proteins, signaling proteins, and peptides in a wide variety of cell types [107-109]. It has also been used in a wide variety of applications such as in the study of subcellular localization of protein interactions, macromolecular complex formation, and interactions involving post-translational modifications [101, 110, 111].

The biggest advantage of BiFC over past techniques is that the source of fluorescence is limited to those mature fluorophores that are formed under conditions of protein interaction [102, 103]. Thus, it eliminates the need for complex data processing and complications that may arise with the use of extrinsic fluorophores or multiple interacting fluorophores [101]. This can theoretically lead to increased signal sensitivity and greater signal/background noise ratio [102, 103]. Also, due to the large combinations of different fluorescent protein fragments that are complementary, one can use BiFC to
study multiple protein interactions simultaneously in the same cell [112]. However, it is also worth noting that fluorophore formation can often take a long time (>30 minutes), which may limit the study of certain dynamic or induced interactions in the cell [113]. Based on current studies in vitro, the BiFC complex is also thought to be irreversible; a consequence that may perhaps be exploited, such as in the trapping of transient protein interactions, but this may also limit the assay system in some ways [113]. However, studies have put doubt in this hypothesis as the disassociation of BiFC complexes has been shown under certain conditions with fragments of EGFP [111].

1.2.3 Complementary fluorescent protein fragments

As mentioned previously, many different combinations of fluorescent protein fragments can interact to form a complementary fluorophore, many of which have been successfully implemented with the BiFC technique [112]. In the original study by Hu et al., various C- and N-terminal fragments of EYFP were tested for their complementation and fluorescence intensities [101]. They determined that the EYFP fragment containing residues 1-154 and the EYFP fragment containing residues 155-238 produced the highest signal [101]. However, recent studies have shown that better combinations of fluorescent protein fragments exist for BiFC analysis. In 2006, Shyu et al. showed that the BiFC combinations between the C-terminal region of ECFP (CC155 – residues 155-238) and the N-terminal residues 1-173 of either Cerulean or Venus (CrN173, and VN173 respectively) yielded much improved results [113]. Cerulean and Venus are fluorescent proteins derived from ECFP and EYFP that are engineered to improve such properties as fluorescence intensity or rate of maturation [114, 115]. These two new protein fragment combinations for BiFC were shown to improve upon previous combinations by requiring
less plasmid and incubation time, leading to reduced non-specific interactions [113].
Also, the new combination got rid of the need for a pre-incubation step of cells at a lower
temperature for fluorophore maturation [113]. Thus, this new combination of
complementary fluorescent protein fragments has made the BiFC assay simpler and has
increased both the signal and specificity of the assay [113].
Figure 3: The mechanism of Bimolecular Fluorescence Complementation.

The BiFC technique is based on the complementation of two non-fluorescent protein fragments derived from a whole fluorescent protein (denoted as fragments N and C). These fragments can be fused onto proteins of interest indicated here as protein A and protein B as fusion tags. If proteins A and B are able to interact, this allows the BiFC tags to come into close enough proximity to interact and allows for the maturation of complete fluorophore. These interactions can be directly visualized using standard methods of confocal microscopy.
1.3 Thesis Project

1.3.1 Introduction and hypothesis

The study of the cell cycle checkpoints and their role in the maintenance of genomic integrity has been an area of great interest in recent years, especially for its involvement in tumorigenesis and cancer therapeutics as previously described. Greater research into the field has revealed cell cycle checkpoints to be a very complex web of signaling pathways that are intertwined with both DNA replication and DNA repair. With the focus of the Rad9A protein in our lab and the ongoing elucidation of its increasingly complex C-terminal phosphorylation sites, new and improved methods to study its interactions have been an area of interest. Thus, it has been hypothesized that the BiFC assay can be used to study cell cycle checkpoint protein interactions in live cells. This will allow for an alternative and improved method of studying the Rad9A interaction with other checkpoint proteins, such as TopBP1.

1.3.2 Aims and objectives

The general objective of the project was to optimize the BiFC assay to determine if it is a viable assay to study Rad9A interactions under various conditions, such as DNA damage. Due to the significance of the Rad9A-TopBP1 interaction in the ATR-dependent checkpoint response, it was chosen as the specific interaction of study. If the implementation of the BiFC technique to study this interaction was successful, then it could subsequently be applied to study the interactions of other checkpoint proteins.

Specifically, this project entailed the need to construct BiFC plasmid constructs that can synthesize Rad9A and TopBP1 fusion proteins with complementary fluorescent protein derived protein fragments on its C-terminal end. Co-transfection of the BiFC
constructs and the detection of the protein interactions then had to be optimized using confocal microscopy and flow cytometry techniques. These interactions were studied under both normal and DNA damaging conditions. Lastly, due to the importance of the Rad9A C-terminal tail phosphorylation sites, the effects of the BiFC fusion tag on the post-translational modifications of Rad9A were determined. If the optimization of the BiFC assay in the context of cell cycle checkpoint proteins was successful, it was hoped that such a system could be coupled to a high-throughput protein interaction assay. For example, coupling the BiFC assay with flow cytometry could yield a simple and effective drug screen assay to determine the effects of cancer drugs on specific protein interactions involved in the cell cycle checkpoint pathways.
Chapter 2 – Materials & Methods

2.1 Construction of the BiFC plasmid vectors

2.1.1 Cloning vectors

The BiFC control and cloning vectors were provided by Dr. Cheng-Deng Hu (Purdue University) (refer to Figure 4 for pictorial representation). The plasmids were created from pHA-CMV (Clontech Laboratories Inc., Mountain View, CA) or pFLAG-CMV2 (Sigma-Aldrich, Oakville, Ontario) expression vectors containing their respective cDNA sequences (bJun, bFos, bFos-ΔZIP) or multiple cloning region. Refer to reference [101] for further details. Table 1 shows the detailed components of the BiFC constructs used in this project and the shorthand nomenclature that will be used to refer to the constructs from henceforward. The BiFC constructs and their expressed proteins will be referred to as follows; bFos*, bJun*, bFos-Mut*, empty CC155*, empty CrN173*, Rad9A*, Rad9A-Mut* and TopBP1*.

2.1.2 Polymerase Chain Reaction (PCR)

All PCR experiments were performed using the Eppendorf Mastercycler epgradient S cycler (Eppendorf, Mississauga, ON). PCR was performed using existing Rad9A (pyDF31) [30], Rad9A-S387A (pyDF31) [32], and TopBP1 (pBluescript II SK+) (Kazusa DNA Research Institute, Kisarazu, Japan) vectors as the template DNA to obtain PCR products containing the sequences of Rad9A, Rad9A-S387A, and TopBP1 with new restriction sites. The Rad9A and Rad9A-S387A PCR products were created to contain SalI/BglII restriction sites, while the TopBP1 PCR product was created to contain NotI/ClaI restriction sites (on the 5’ and 3’ ends, respectively). Primers were designed
using the online Primer3Plus program and synthesized through an external service (Operon, Huntsville, Alabama). The PCR products were made to be in frame with the start codon of the N-terminal HA/FLAG tag regions when they are ultimately inserted into their respective destination BiFC expression vectors. The stop codons were also removed so as to allow for the fusion of the C-terminal BiFC tags to the proteins of interest in the final BiFC expression vectors. The Rad9A-S387A sequence differs from that of Rad9A by the serine to alanine mutation on residue 387 caused by the codon change from AGT to GCT. Refer to Table 2 for the sequences of the PCR primers used in this experiment and their respective restriction sites.

The Platinum Pfx DNA polymerase system (Invitrogen, Burlington, Ontario) was used to obtain the Rad9A and Rad9A-S387A PCR products. The PCR reaction mixture contained 1ng of the template DNAs described above, deoxynucleotide triphosphates (dNTPs; 300μM final concentration of each), magnesium sulfate (MgSO₄; 3mM final concentration), forward and reverse primers (10μM final concentration of each), Pfx polymerase (1U), and 1X Pfx amplification buffer. Thermal cycling conditions were as follows: 2 min at 94°C, followed by 30 cycles of 94°C for 15s and 72°C for 75s, with a final extension at 68°C for 7 minutes.

The Finnzymes Phusion high-fidelity DNA polymerase system (New England Biolabs/NEB, Pickering ON) was used to obtain the TopBP1 PCR product. The PCR reaction mixture contained 5ng of the template TopBP1 DNA, deoxynucleotide triphosphates (dNTPs; 200μM final concentration of each), forward and reverse primers (10μM final concentration of each), Phusion polymerase (1U), and 1X Phusion HF buffer (containing a final concentration of 1.5mM MgCl₂). Thermal cycling conditions were as
follows: 30s at 98°C, followed by 30 cycles of 98°C for 10s and 68°C for 80s, with a final extension at 72°C for 10 minutes.

2.1.3 Purification and extraction of PCR products

The samples containing the PCR products of Rad9A, Rad9A-S387A, and TopBP1 were separated on a 1% agarose gel using 1X Tris-Acetate-EDTA (TAE) buffer and stained with crystal violet dye (1% solution in TAE) for 20 min. The gel was destained for 10 min using distilled water. The PCR products were excised from the gel with a scalpel and extracted using the QIAquick Gel Extraction kit (Qiagen, Mississauga, ON).

2.1.4 Restriction enzyme digestion and phenol-chloroform extraction

The PCR product inserts and their target BiFC expression vectors were restriction digested. The Rad9A PCR product, the Rad9A-S387 PCR product, and the empty CC155* vector was digested using 30U of SalI and BglII (NEB), while the TopBP1 PCR product and the empty CrN173* vector was digested using 30U of NotI and ClalI (NEB) for 2 hours at 37°C (mixtures contained 1X BSA, 1X NEB Buffer 3).

The DNA products were purified by phenol-chloroform extraction after restriction digest. DNA samples were diluted with water to 300 μL and mixed with an equal volume of phenol-chloroform. Samples were centrifuged at 16 000 x g in a tabletop centrifuge and the upper aqueous phase containing the DNA was saved. This was performed a total of three times. Samples were then mixed with an equal volume of chloroform to remove trace phenol. Samples were centrifuged again and the aqueous phase was saved. This was performed a total of two times. Samples were mixed with three times total volume of ethanol/5M NaOAc, incubated at -80°C for at least 30 minutes, and spun at 4°C for 30 minutes. Samples were then decanted, 300 μL of 70% ethanol was added, and samples
centrifuged at 4°C for 5 minutes. Samples were decanted, dried, and the DNA resuspended using the Qiagen elution buffer (Qiagen).

2.1.5 Phophatase treatment and ligation

The restriction digested BiFC expression vectors (empty CC155* and empty CrN173*) were treated with 10U of calf intestinal phosphatase (CIP) (NEB) for 30 minutes at 37°C and purified by phenol-chloroform extraction to prevent vector religation. The purified PCR insert products of Rad9A, Rad9A-S387A, and TopBP1 were then ligated to their corresponding linearized, CIP treated and purified BiFC vectors. Ligation reactions took place in 10μL of total volume containing 1μL of T4 DNA ligase (NEB), 1X T4 DNA ligase buffer (NEB). Molar ratios (ng insert: ng vector) of 5:1, 3:1, and 1:1 of insert to vector (~300ng vector) DNA was used. Ligation reactions were carried out at RT for 30 minutes.

2.1.6 Bacterial transformation by electroporation and colony selection

The ligated BiFC expression vectors were transformed into ElectroTen-Blue Electroporation Competent Cells (VWR International, Mississauga, ON) using the method of electroporation. 1μL of the ligation mix was added to 40μL of ElectroTen-Blue and gently mixed. The transformation mixture was then added to pre-chilled 2mm gap electroporation cuvettes (Molecular BioProducts, San Diego, CA) and tapped to eventually distribute the mixture in the bottom of the chamber. The transformation mixture was subjected to electroporation using the Bio-Rad MicroPulser (Bio-Rad). 1mL of SOC media was then immediately added to the cuvette and mixed. The transformation mixture was then incubated at 37°C for ~20 minutes on a rotating nutator. 200μL of the transformation mixture was added to Luria Bertani (LB) agar plates containing ampicilin
(Amp; 40μg/mL) and spread evenly until it was sufficiently absorbed into the agar. The plates were incubated at 37°C overnight (O/N; ~16-18 hours) Individual bacterial colonies were picked and grown O/N as starter colonies in 5mL of LB media containing a final concentration of 100μg/mL of Amp.

2.1.7 Analytical digest, storage, and sequencing of BiFC constructs

The O/N starter cultures were purified for their plasmid DNA using the QIAprep Spin Miniprep Kit (QIAGEN). The plasmid DNA was then subjected to restriction digest using either SalI/BglII (Rad9A*, Rad9A-Mut*) (NEB) or NotI/ClaI (TopBP1*) (NEB) as before and run on a 1% agarose gel containing ethidium bromide (EtBr; 0.5 μg/mL) and visualized to determine if the newly ligated BiFC constructs contained the correct insert sequences. Select colonies with the appropriately transformed BiFC constructs were grown in 100 mL of LB media (with 100μg/mL of Amp) O/N and 1mL stored in equal volume of 100% glycerol at -80°C. The remaining volume of the cell culture was purified for their respective BiFC plasmid DNA using a QIAGEN Plasmid Maxi Kit (QIAGEN). Lastly, the sequences of the newly subcloned BiFC constructs containing the inserted PCR products were confirmed through the external sequencing services of The Centre for Applied Genomics (TCAG, Toronto, ON).

2.2 Cell culture

The HeLa CCL-2 cell line was used for all the cell culture experiments (ATCC, Burlington, Ontario). All HeLa cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (Invitrogen) (complete DMEM), 100 U/ mL of Penicillin, and 100 ug/ mL of Streptomycin (Invitrogen). Cells were cultured at 37°C in a 5% CO₂ atmosphere using
10cm, 60mm, and 6 well dishes (Sarstedt, Montreal, Quebec). Cells were routinely split at approximately 80-100% confluency at ratios of 1:5 and 1:10 using 2X 0.5% Trypsin-EDTA (Invitrogen).

2.3 Transfection and harvesting cells

The transfection agent, Lipofectamine 2000 (Invitrogen) was used to transfect cells at ~80-90% confluency. 24 hours prior to transfection, the culture media was removed from the cell culture dishes and replaced with complete DMEM without antibiotics. A DNA (μg): lipofectamine 2000 (μL) ratio of 1:2.5 was used to transfect the cells, using Opti-MEM I Reduced Serum Medium (Invitrogen) as the complex media. Control cultures without transfection were mock transfected in the absence of DNA. Transfected cells were cultured at 37°C in a 5% CO₂ atmosphere for ~42-48 hours.

Cells were then washed 2X with warm 1X phosphate buffered saline (PBS), tryspinized, centrifuged at 200 x g, washed, and then lysed using NETN lysis buffer (20mM Tris-Cl pH 8.0, 0.5% Nonidet P-40, 125mM NaCl, 1mM EDTA, 1.6mg/mL β-glycerol phosphate, 1 tablet complete mini protease inhibitor cocktail/10mL buffer (Roche, Laval, Quebec)). The cells were incubated on ice with lysis buffer for 30 minutes with occasional mixing and centrifuged at 16 000 x g to remove insoluble material. An equal volume of 3X sample buffer (SB; 30% glycerol, 6% SDS, 15% β-mercaptoethanol) was then added to the cell lysate.

2.4 Ionizing radiation

DNA damage induced by IR was conducted through the exposure of plated HeLa cells to a cesium-137 source using the GammaCell 20 irradiator. The dose rate was between 0.46-0.49 Gy/min, varying between the timeline of each experiment. Cells were
exposed to 10Gy of IR in total 24 hours after transfection and harvested 18 hours after treatment unless otherwise indicated.

2.5 Hydroxyurea treatment

Cells were treated with 5mM hydroxyurea (HU) (Sigma Aldrich) in fresh DMEM culture media as indicated above. Cells were treated with HU 24 hours after transfection for 18 hours and then harvested, unless otherwise indicated.

2.6 SDS-PAGE and immunoblotting

All protein samples were heated at 100°C for 5 minutes and subjected to SDS-PAGE on a 7% polyacrylamide gel, unless otherwise noted. The electrophoresis analysis was run at 80-120V in 1X Laemmli Buffer until the desired separation was achieved. The separated proteins were then transferred to Amersham Hybond-ECL nitrocellulose membranes (GE Healthcare, Baie d’Urfe, Quebec). The polyacrylamide gels, the nitrocellulose membranes, and the filter papers were soaked in cold Bjerrum transfer buffer for a few seconds and assembled onto the transfer apparatus. The protein transfer was run at 20V for 45 min on a TransBlot SD Semi-dry Transfer Cell (Bio-Rad) in all cases, except when analyzing TopBP1. On gels analyzing for TopBP1, the protein transfer was run at 20V O/N at 4°C using the wet transfer Criterion Blotter apparatus (Bio-Rad).

The nitrocellulose membrane blots were then blocked in 1X PBS containing 0.1% Tween (PBS-T) and 5% non-fat milk powder for 1 hour at RT. The blots were then washed two times with PBS-T at RT and incubated in PBS-T containing the appropriate primary antibody O/N at 4°C. After the primary antibody incubation, blots were washed with PBS-T every 10 minutes three times and then incubated with PBS-T containing the
appropriate secondary antibody for 1 hour at RT. Blots were then washed with PBS-T every 5 minutes for at least 30 minutes and the binding of the antibodies were detected using the Supersignal West Pico Chemiluminescent Substrate (Fisher Scientific, Ottawa, ON) for 2 minutes. The blots were then visualized using Kodak Image Station 4000mm PRO (with Kodak MI software) or by exposure on X-ray film and subsequent development of the film. Please refer to Table 3 for specific antibodies and dilutions used in this project.

2.7 Co-immunoprecipitation and CIP experiment

All steps in this protocol were performed on ice, and all incubation steps were performed on a nutator unless otherwise noted and refer to lysates from confluent HeLa cells grown on 60mm dishes. After cells were lysed with NETN lysis buffer as previously described, lysates were incubated with 40 μL of prewashed protein-A-sepharose (CedarLane Laboratories, Burlington, ON) (TopBP1 and HA IP) or PrecipHen goat α-chicken-IgY-agarose (Aves Labs, Tigard, OR) (Rad9A and Rad9A-S387A IP) for at least 1 hour to preclear the lysates. Following the preclear step and removal of α-protein-A-sepharose/ α-IgY-agarose, lysates were incubated with 5μg of polyclonal antibody directed against the target protein or epitope (TopBP1, HA, Rad9A) O/N at 4°C. Following this, samples were incubated with 40 μL of protein-A-sepharose/ IgY-agarose to pull down the immune complexes for at least 3 hours at 4°C. Samples were then centrifuged for 30 seconds, supernatant removed, and the complexed agarose/sepharose beads washed with lysis buffer at 4°C. This was repeated a total of five times. After the final wash, supernatant was removed and samples resuspended in 1.5X SB. The samples
were heated at 100°C, centrifuged at 16 000 x g, and the supernatant kept for immunoblotting as described above.

The CIP treated samples were not resuspended in 1.5X SB but instead resuspended in a 40 μL solution containing 0.2X NEB buffer 3 (NEB3) and 1% SDS. Samples were heated at 100°C, centrifuged at 16 000 x g, and the supernatant split into 2 samples. Equal volume of 3X SB was added to the first sample as the non-CIP treated control. The second sample was diluted up to a total volume of 200 μL using a 0.2X NEB3 solution containing 30U of CIP (NEB). The CIP sample was incubated at 37 °C for 30 minutes and the reaction was stopped by adding 100 μL of 3X SB.

2.8 Determination of transfection efficiency by flow cytometry

The EPICS Altra HSS flow cytometer (Beckman Coulter, Mississauga, Ontario) was used for all flow cytometry experiments and data analysis was performed by the Queen’s Cancer Research Institute (QCRI) cytometry and imaging service (QCRI, Kingston, ON). The data was analyzed using the program EXPO32 MultiComp software (Beckman Coulter).

The transfection efficiency of co-transfection between Rad9A* and TopBP1* was determined by transfecting HeLa cells using 0.2μg Rad9A* and 3.8μg TopBP1* with lipofectamine 2000 as previously described using a 60mm dish. A mock transfected control sample was also used. Cells were trypsinized, washed with cold PBS, and resuspended in 200 μL of cold PBS. 70% EtOH (ice cold) was then slowly added and mixed on ice for at least 1 hour to fix the cells.

After fixation, cells were washed, resuspended in cold 1mL PBS/0.5% Triton X-100, and washed again. Cells were then resuspended in 100 μL of PBS/0.5% Tween 20/
1% Normal Goat Serum (NGS) containing 1μg of primary antibody (Rad9 or TopBP1) and incubated at RT for 1 hour with occasional mixing. After this incubation, cells were washed, resuspended in 100 μL of PBS/0.5% Tween 20/1% NGS containing 0.1μg of secondary antibody and incubated at RT for 30 minutes with constant mixing in the dark. Finally, cells were washed, resuspended in PBS, and aliquoted into 5mL flow tubes for flow cytometry analysis. The secondary antibodies used in this project were Alexa Fluor 488 (Invitrogen) and Pe-Cy5 (Santa Cruz Biotechnology, Santa Cruz, CA). Transfection efficiency was determined by gating sample fluorescence above the endogenous level as determined by non-transfected control samples.

2.9 Flow cytometry analysis of BiFC interactions

HeLa cells were grown and transfected as indicated previously, in the presence or absence of DNA damage. DNA damage was applied to damaged samples by exposure to 10 Gy of IR or 5mM of HU as described previously. HeLa cells were trypsinized, washed with PBS, and aliquoted into flow tubes for analysis. The samples containing the BiFC control vectors (bJun*, bFos* bFos-Mut*) were analyzed using an excitation laser at 514 nm and emission detection at 529 nm. The samples containing the BiFC proteins of interest (empty CC155*, empty CrN173*, Rad9A*, Rad9A-Mut*, TopBP1*) were analyzed using an excitation laser at 457 and emission detection at 488nm (+/- 20nm range).

2.10 Visualization of BiFC interactions using confocal microscopy

The Leica TCS SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) with 63X and 100X oil immersion optics was used for all confocal microscope experiments for live visualization of BiFC interactions in HeLa cells. HeLa cells were
grown on disposable Bioptechs Delta T dishes (Carl Zeiss, Toronto, ON), but otherwise all other cell culture and transfection protocols were followed as previously described. Image capturing was performed with the help of the QCRI cytometry and imaging service. The images were captured using the Image Pro Plus Basic Leica Operating Software program (Leica Microsystems). Laser lines at 458nm and 514nm for excitation of cerulean/cyan constructs and venus/yellow control constructs were provided respectively. Detection ranges were set to eliminate crosstalk between fluorophores at 480+/-20 and 530+/-20 nm.
Figure 4: BiFC Plasmid Constructs.

The BiFC plasmid constructs used in this project are shown. The sequences coding for Rad9A, TopBP1, and the Rad9A mutant containing a Serine to Alanine mutation on residue 387 (RadA-S387A) were cloned into their respective BiFC plasmid constructs as indicated (Top). The control plasmid constructs containing the interacting bZIP regions of the proteins c-Jun and c-Fos (bJun and bFos), as well as the bFos protein with a deletion in the bZIP interacting region (bFos-mutant) are also shown (Bottom). Each of the BiFC constructs code for a fusion protein with an HA or FLAG tag on the N-terminal end and a BiFC tag on the C-terminal end. CC155 is a fragment of ECFP containing residues 155-238, CrN173 is a fragment of Cerulean containing residues 1-173, VN173 is a fragment of Venus containing residues 1-173, and VC155 is a fragment of Venus containing residues 155-238. MCS denotes the multiple cloning site.
Table 1: BiFC constructs used in this project

<table>
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<tr>
<th>Full Name</th>
<th>N-terminal Tag</th>
<th>C-Terminal Fluorescent Protein Fragment</th>
<th>Insert sequence</th>
<th>Shorthand Nomenclature (both plasmid and expressed protein)</th>
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<tbody>
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<td>HA</td>
<td>VC155</td>
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<tr>
<td>BiFC-bJun-VN173</td>
<td>FLAG</td>
<td>VN173</td>
<td>bJun</td>
<td>bJun*</td>
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<tr>
<td>BiFC-bFosΔZIP-VN173</td>
<td>FLAG</td>
<td>VN173</td>
<td>bFosΔZIP (deletion in the interacting ZIP region)</td>
<td>bFos-Mut*</td>
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<td>CC155</td>
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<td>empty C155*</td>
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<tr>
<td>BiFC-CrN173</td>
<td>FLAG</td>
<td>CrN173</td>
<td>None/empty</td>
<td>empty CrN173*</td>
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<td>CC155</td>
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<tr>
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<td>CC155</td>
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<td>CrN173</td>
<td>TopBP1</td>
<td>TopBP1*</td>
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Table 2: PCR primer sequences and their residing restriction sites

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<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Restriction Site (Restriction Enzyme)</th>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CCCAGATCTGGCCTTCAC</td>
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</tr>
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<td></td>
<td></td>
<td>CCTCAGCTGTCTTC</td>
<td></td>
</tr>
<tr>
<td>Rad9A-S387A</td>
<td>Forward</td>
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<td>G</td>
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<tr>
<td></td>
<td></td>
<td>GCCTGGTCACGGGC</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CCCAGATCTGGCCTTCAC</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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<td>AT</td>
</tr>
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<td></td>
<td></td>
<td>C TAGGTCGTTTGAT</td>
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Table 3: Antibodies for immunoblotting experiments

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<th>Primary Antibody</th>
<th>Dilution Ab:PBS-T (μL)</th>
<th>Source</th>
<th>Secondary Antibody</th>
<th>Dilution Ab:PBS-T (μL)</th>
<th>Source</th>
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</thead>
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<td>α-chicken IgG goat</td>
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<td>Cedarlane Laboratories</td>
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<td>1:1000</td>
<td>BD Biosciences (Mississauga, ON)</td>
<td>α-mouse IgG goat</td>
<td>1:10000</td>
<td>Cedarlane Laboratories</td>
</tr>
<tr>
<td>α-HA</td>
<td>1:500</td>
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<tr>
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<td>Cedarlane Laboratories</td>
<td>α-rabbit IgG goat</td>
<td>1:50000</td>
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<tr>
<td>α-Rad9 P-S272</td>
<td>1:500</td>
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<td>α-rabbit IgG goat</td>
<td>1:50000</td>
<td>Bio-Rad</td>
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Chapter 3 – Results

3.1 Determining if the BiFC constructs contain the proper insert DNA

The BiFC vectors containing Rad9A*, Rad9A-Mut*, and TopBP1* were constructed through the PCR amplification of existing plasmids containing the sequences of Rad9A, Rad9A-S387A, and TopBP1, restriction digest, and insertion into either empty CC155* or empty CrN173*. This was followed by transformation of the plasmids into *Escherichia coli* cells and the subsequent purification of these plasmids. To ensure that the final BiFC constructs contained the appropriate insert sequences, an analytical digest was performed (Figure 5). As expected, in the case of Rad9A*, and Rad9A-Mut*, the sample digested with both *Sal*I and *Bgl*II showed a band of ~1200 bp consistent with that of the insert, while the samples digested individually with *Sal*I or *Bgl*II migrated at ~5300 bp, which is consistent with the size of the linearized plasmid (~1200 bp insert and ~4100 bp vector). This was also true with TopBP1*, as the sample digested with both *Not*I and *Cla*I migrated at ~4600bp, while the samples digested individually with *Not*I or *Cla*I migrated at ~9800bp, consistent with the size of the linearized plasmid (~4600 bp insert and ~ 5200 bp vector). The undigested control samples migrated at a smaller apparent bp size than the linearized plasmid, as it migrated as circular DNA. The sequences of the inserts were subsequently analyzed for any mutations by reviewing the chromatogram data. All the BiFC constructs were confirmed to contain the correct insert sequences without any mutations.
Figure 5: Analytical digest of the Rad9A*, Rad9A-Mut*, and TopBP1* BiFC plasmids. Plasmid samples containing A) Rad9A*, B) Rad9A-Mut*, or C) TopBP1* were digested by the indicated restriction enzymes and separated on a 1% agarose gel containing EtBr. The 1Kb GeneRuler ladder (Fermentas) was also run on each gel. C indicates the mock control samples containing no restriction enzyme.
3.2 Co-transfection of the BiFC constructs

To ensure that the transfected HeLa cells express the proteins of interest and to visualize their expression levels, we co-transfected the BiFC constructs of Rad9A*/TopBP1* and Rad9A-Mut*/TopBP1*. This was done with varying ratios of the DNA plasmids of interest to determine which ratio would be optimal for future experimentation. This was important for the subsequent flow cytometry and confocal microscopy protein interaction studies that required enough protein expression to visualize the interaction, while minimizing the background signal and non-specific interactions that may result with protein expression levels that are too high. With Rad9A*/TopBP1*, the transfection ratios used were 4:0 (Rad9A* μg/ TopBP1 μg), 1:3, 0.5/3.5, 0.2/3.8, and a mock transfected sample in 60mm dishes. The migration and expression of the proteins of interest in these samples were visualized by immunoblot analysis.

As it can be seen in Figure 6, endogenous Rad9A migrated at ~60 kDa, while Rad9A* migrated slightly higher at about 65 kDa. Rad9A* was expressed greatly above the endogenous levels even with very little Rad9A* being used for transfection. Endogenous TopBP1 was seen to migrate at ~180 kDa, while TopBP1* migrated a little bit higher. As opposed to Rad9*, TopBP1* expression levels were quite low, expressing less than endogenous levels in most cases. These results suggest that TopBP1* transfection may be the limiting factor for further BiFC experiments. Based on the findings however, it was decided that a Rad9*/TopBP1* co-transfection ratio of 0.2/3.8 should be used for further experiments. This allowed for expression of TopBP1* at levels close to endogenous expression, while Rad9* was expressed at a lower level than the
other ratios; closer to endogenous levels. The Rad9A-Mut* expressed at a similar level to Rad9A* during co-transfection with TopBP1*, as they are identical in sequence except for the AGT to GCT mutation on bases 1159-1161.

Although the above results showed the relative expression levels of our proteins of interest across the whole cell population as visualized on an immunoblot, it did not indicate how well the HeLa cells were being transfected on a cell by cell basis. This was important to consider as subsequent protein interaction studies analyzed the protein interactions on a cell by cell basis. To analyze the transfection efficiency, HeLa cells were co-transfected with Rad9A*/TopBP1* at the 0.2/3.8 DNA ratio determined previously and analyzed using flow cytometry analysis. The fluorescence levels corresponding to that of Rad9A* and TopBP1* above the levels of their endogenous protein counterparts were determined. The generated dot plot is shown in Figure 7.

As it can be seen, the majority of the cells did not show fluorescence significantly above endogenous levels. Only 8.6% (9.4%-0.8% on upper right quadrant) of the cell population showed significant fluorescence above endogenous levels at both emission spectra (Alexa 488 and Pe-Cy5) corresponding to Rad9A* and TopBP1*. Also, it was observed that whereas there existed many cells (17.4%) that suggested that they were transfected with only Rad9A*, there were almost no cells (0.2%) that exhibited only TopBP1* transfection. Thus, the results suggest that the low level of co-transfection of Rad9A*/TopBP1* is predominantly caused by the low transfection efficiency of TopBP1*, a relatively large protein when compared with Rad9A*.
Figure 6: Immunoblot showing co-transfection of Rad9A* and TopBP1*.

HeLa cells were co-transfected with different DNA ratios of Rad9*/TopBP1* as indicated. The cell lysates were run on a 7% SDS-PAGE gel and immunoblotted with antibodies directed against A) Rad9 or B) TopBP1. The Prestained Broad Range Protein Marker (NEB) was also run on each gel. C indicates control mock transfected cell lysate samples.
Figure 7: Dot plot showing the co-transfection efficiency of Rad9A* and TopBP1* using flow cytometry. HeLa cells were either A) mock transfected or B) co-transfected with Rad9A*/TopBP1*. These cells were permeabilized and probed with antibodies directed against Rad9 or TopBP1, which were then tagged by secondary antibodies conjugated with either Alexa 488 (Rad9A*) (x-axis) or PE-Cy5 (TopBP1*) (y-axis). The emission fluorescence levels were gated above the endogenous levels observed in the control (A) to determine the transfection efficiency of the transfected Rad9A* and TopBP1* plasmids in the HeLa cells (B). The top right quadrant indicates cells successfully co-transfected with both BiFC constructs.
Control - Mock Transfection

Rad9A*/TopBP1*
Co-Transfection

y-axis: TopBP1^1*, Pe-Cy5^2*

x-axis: Rad9 1^*, Alexa 488 2^*

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3.3 Co-immunoprecipitation experiments show that Rad9A* and TopBP1* interact

To ensure that Rad9A* and TopBP1* behave like their endogenous counterparts and are able to interact with one another; a co-immunoprecipitation experiment was carried out (Figure 8) using cell lysates from co-transfected HeLa cells. This was achieved by immunoprecipitating Rad9A* with antibodies directed against the HA tag and immunoblotting for TopBP1. Although FLAG antibodies were not able to immunoprecipitate TopBP1* effectively, TopBP1* immunoprecipitation was achieved using an antibody directed against TopBP1 and immunoblotting for Rad9A. The results showed that TopBP1* interacts and co-immunoprecipitates with Rad9A* as expected. In the opposite direction, Rad9A* also co-immunoprecipitated when using antibodies targeting TopBP1, but this result did not distinguish the Rad9A* interaction between endogenous TopBP1 or transiently expressed TopBP1*. Although positive controls transfected with only Rad9A* or TopBP1* was not implemented for this co-immunoprecipitation experiment, past studies by members of our lab have demonstrated with the necessary controls that Rad9A and TopBP1 co-immunoprecipitate, while the Rad9A-S387A mutant does not. Together, these results suggested that Rad9A* and TopBP1* interact.
Figure 8: Co-immunoprecipitation of Rad9A* and TopBP1*.

HeLa cells were either mock transfected (indicated by C; control) or co-transfected with Rad9A*/TopBP1*. Co-immunoprecipitation was carried out by either A) HA directed Rad9A* pull-down or B) TopBP1 directed TopBP1* pull-down. Samples were subsequently separated on a 7% SDS-PAGE gel and immunoblotted using antibodies directed against A) TopBP1 or B) Rad9.
A

Rad9A⁺/TopBP1⁺
Co-Transfection

HA Immunoprecipitation, TopBP1 Immunoblot

B

Rad9A⁺/TopBP1⁺
Co-Transfection

TopBP1 Immunoprecipitation, Rad9 Immunoblot
3.4 Confocal microscopy and flow cytometry analysis of BiFC protein interactions

With the knowledge that Rad9A* and TopBP1* can interact, confocal microscopy experiments were carried out to visualize protein interactions via the formation of BiFC fluorescent signals. Live HeLa cells were transfected with bJun*/bFos*, bJun*/bFos-Mut*, or Rad9A*/TopBP1* in the presence or absence of DNA damage. Cells were subject to DNA damage either by exposure to 10Gy of IR with 18 hours recovery or 5mM of HU for 18 hours. bJun* and bFos* are BiFC fusion proteins containing the known interacting partners composed of the bZIP domains of the proteins c-Jun and c-Fos [101] and used as the positive control. bFos-Mut* contains a mutation on the interacting bZIP region required for interaction with bJun*, thus acting as the negative control. The results can be seen in Figure 9A.

As expected, the bJun*/bFos* cells exhibited bright nucleolar yellow signals caused by the complementation of the Venus fluorescent protein fragments. On the other hand, the negative control bJun*/bFos-Mut* cells showed significantly decreased signals. Cells co-transfected with Rad9A*/TopBP1* showed cells containing relatively bright punctuate signals in the nucleus of the cells. This was consistent with what we may have expected, as Rad9A/TopBP1 interacts in the nucleus and past co-localization studies showed a similar pattern of punctate nuclear signals [91]. However, Rad9A*/TopBP1* cells damaged with IR or HU did not seem to exhibit increased signal or a change in the fluorescence pattern compared to the undamaged cells. As an alternative tool to determine if fluorescence did indeed remain unchanged after DNA damage, flow cytometry was used to compare the relative fluorescence level of IR damaged and undamaged Rad9A*/TopBP1* cells (Figure 9B). In the flow cytometry experiment, the
damaged cells did not show a significant increase in fluorescence signal over undamaged cells, although they both showed significant signal above the mock transfected control. In both damaged and undamaged samples, approximately 10% of cells showed fluorescence above control levels (data not shown). The relative fluorescence levels of cells transfected with the bJun*/bFos* and bJun*/bFos-Mut* controls were also determined to show that specifically interacting BiFC fusion proteins can yield significantly higher BiFC signal than the negative control (Figure 9C). The relative fluorescence level of the positive control was seen to be more than two times that of the negative control.
Figure 9: Detecting protein interactions using confocal microscopy and flow cytometry. HeLa cells were co-transfected with the indicated BiFC plasmids and visualized using confocal microscopy (A). These included the positive control (i), negative control (ii), as well as undamaged (iii) and damaged (iv, v) Rad9A*/TopBP1* samples. A laser line at 514 nm was used for the excitation of the controls (a, b) and detection was carried out at 530 +/-20 nm. For the Rad9A*/TopBP1* samples, a laser line at 458nm was used and detection was carried out at 480 +/-20 nm. B) Separate flow cytometry experiments were also carried out in parallel to determine if relative fluorescence levels differed between undamaged and damaged Rad9A*/TopBP1* samples. Flow cytometry profiles indicate the % of cells showing fluorescence above the gated level (indicated as a line). Histograms indicate the relative median fluorescence of these cells compared to the median fluorescence of all mock transfected control “c” cells.

C) This flow cytometry experiment was also performed with the positive and negative controls containing bJun*/bFos* or bJun*/bFos-Mut* to show that the positive control exhibits significantly higher BiFC signals than that of the negative control.
3.5 The BiFC fusion tag on the C-terminal tail of Rad9A* may be preventing the proper phosphorylation of Rad9A*

The C-terminal tail of Rad9A contains many different constitutive, cell cycle, and damage dependent phosphorylation sites that are important for the checkpoint response, including the S387 phosphorylation site that is required for TopBP1 interaction [20, 64]. Thus, it is important to determine if these phosphorylation sites are intact after the addition of a fluorescent protein tag on its C-terminal end. Endogenous Rad9A has a predicted molecular mass of ~43 kDa, but migrates on a SDS-PAGE gel with an apparent molecular mass of ~60 kDa; a difference of ~17 kDa. This is mainly caused by the phosphorylation of the protein at Ser277, Ser328, Ser336, and Thr355 [32]. We would expect that Rad9A lacking one or more of these phosphorylation sites to migrate closer to 43 kDa than 60 kDa. Thus, by comparing the migration of endogenous Rad9A with the migration of Rad9A* on a SDS-PAGE gel; in the presence or absence of CIP (to remove phosphate groups), one may be able to determine if the BiFC tag is affecting the phosphorylation sites of Rad9A*.

Cell lysates from untransfected or Rad9A* transfected HeLa cells were run on a SDS-PAGE gel that were previously CIP treated or untreated. The samples were then immunoblotted with antibodies directed against Rad9 (Figure 10). The results show that endogenous Rad9A migrated at ~46 kDa in the presence of CIP and ~60 kDa in its absence. The Rad9A* protein has a predicted molecular mass of ~57 kDa and the protein migrated as expected after CIP treatment. However, untreated Rad9A* migrated only slightly higher at ~65 kDa. The difference in migration of CIP treated and untreated samples of endogenous Rad9A was ~14 kDa while that of Rad9A* was smaller at ~8
kDa, as determined by visual analysis of the immunoblot. Based on this result, it is possible that one or more phosphorylation sites on Rad9A* are being negatively affected by its C-terminal BiFC tag, thus contributing to the smaller difference in migration between CIP treated and untreated samples compared to endogenous samples. However, it is also possible that Rad9A* migration is not analogous to that of endogenous Rad9A as it is a different protein with possibly different migration properties due to the BiFC tag and the different location of the phosphorylation sites (they would be more central to the protein due to the presence of the BiFC tag on the most C-terminal region of the protein). Thus, further experimentation was performed to confirm the phosphorylation status of Rad9A*.

For this approach, specific phosphorylation sites of Rad9A* were analyzed by immunoblotting with phospho-specific antibodies. Due to its great importance in TopBP1 binding, the S387 phosphorylation site was analyzed. The damage dependent S272 phosphorylation site was also analyzed to see if the fusion tag was affecting more upstream phosphorylation sites. The results are shown in Figure 11. The S272 phosphorylation was retained in Rad9A* after IR damage, showing that at least some phosphorylation sites are unaffected by the BiFC tag. However, the S387 constitutive phosphorylation appeared to be lost in Rad9A*, showing similar banding patterns to mock transfected, and Rad9A-Mut* transfected samples. These results suggest that perhaps the C-terminal BiFC tag is somehow blocking the phosphorylation of the most downstream phosphorylation sites on the Rad9A* C-terminal tail.
Figure 10: Determining the SDS-PAGE migration pattern of Rad9A*

HeLa cells were either mock transfected (C) or transfected with Rad9A* and their lysates either treated with CIP or left untreated. The lysates were then separated on a 7% SDS-PAGE gel and Rad9 immunobloting was performed. CIP untreated endogenous Rad9A migrated at ~60 kDa, while the CIP treated sample migrated at ~ 46 kDa. CIP untreated Rad9A* migrated at ~65 kDa, while the CIP treated sample migrated at ~ 57 kDa.
<table>
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**Rad9 Immunoblot**

C (Endogenous Rad9A)  Rad9A*
Figure 11: Determining the phosphorylation status of Rad9A* at S272 and S387. HeLa cells were either mock transfected (C) or transfected with the specific Rad9A plasmids as indicated and immunoblotted with phoso-specific antibodies directed against A) P-S272 or B) P-S387. For the DNA damaged Rad9A* sample, cells were treated with 10 Gy of IR 24 hours after transfection and allowed to recover for 18 hours prior to lysis.
3.6 The association between Rad9A*/TopBP1* may be caused by non-specific interactions – determination by co-immunoprecipitation

With the knowledge that the important S387 phosphorylation is absent in Rad9A* and that the BiFC tag is possibly affecting the phosphorylation status of Rad9A*, a number of specific control experiments were performed. The goal of these experiments were to determine if the previous results suggestive of an interaction between Rad9A* and TopBP1* were due to specific interactions, or if the interactions may have been caused by non-specific interactions.

This was first determined by performing a co-immunoprecipitation experiment in cell lysates previously co-transfected with TopBP1* and Rad9A-Mut* (Figures 12A and 12B). As Rad9A-Mut* lacks the S387 phosphorylation required for TopBP1 binding, we may expect TopBP1* to not co-immunoprecipitate with Rad9A-Mut*. However, the results showed that Rad9A-Mut* and TopBP1* co-immunoprecipitated, thus existing in the same protein complex through some sort of interaction. This was seen in either case using an antibody directed against the HA tag of Rad9A-Mut* or an antibody directed against TopBP1 to co-immunoprecipitate the complex. These results support the idea that Rad9A* lacks a viable S387 phosphate group and that both the Rad9A* and Rad9A-Mut* are interacting with TopBP1* in the same way, via a non-specific interaction. There is also the alternative possibility that these proteins are being co-immunoprecipitated together indirectly in a shared multi-protein complex, without direct, specific interaction. The co-immunoprecipitation samples of cells co-transfected with Rad9A*/TopBP1* were also run in parallel as comparison. In both cases (with mutant or non-mutant Rad9A), the
co-immunoprecipitation immunoblot yielded similar protein levels for both Rad9A*/Rad9A-mut* and TopBP1*.

If Rad9A* is indeed interacting with TopBP1* non-specifically, it was hypothesized that perhaps Rad9A* is interacting with the BiFC tag region of TopBP1* and vice versa. It is also possible that the BiFC tags of Rad9A* and TopBP1* are themselves interacting. To test this hypothesis, HeLA cells were co-transfected with either Rad9A*/empty CrN173*, TopBP1*/empty CC155*, or empty CC155*/empty CrN173* and co-immunoprecipitation experiments were performed immunoprecipitating each protein of interest and immunoblotting for the second protein of interest in each of the scenarios. Co-immunoprecipitation of the co-transfected proteins was not achieved in most of the cases. However, the results showed that Rad9A* co-immunoprecipitated with empty CrN173* as seen in Figure 12C. Since Rad9A* is able to interact with the empty CrN173*, this supports the idea that Rad9A* may be interacting with TopBP1* in a non-specific manner via an interaction mediated by the BiFC tag region of TopBP1*. 
Figure 12: Rad9A*/TopBP1* interaction may be caused by non-specific interactions. A, B) HeLa cells were either mock transfected or co-transfected with Rad9A-Mut*/TopBP1* and co-immunoprecipitation was performed with antibodies directed against HA or TopBP1, and subsequently immublotted for Rad9 or TopBP1. Co-immunoprecipitation samples co-transfected with Rad9A*/TopBP1* were also run in parallel for comparison. C) HeLa cells were also co-transfected with Rad9A*/empty CrN173* and co-immunoprecipitation was performed with antibodies directed against Rad9 and immunoblotted for the FLAG tag. Cell lysates from samples transfected with empty CrN173 are also shown for comparison.
A

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HA Immunoprecipitation, TopBP1 Immunoblot

B

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TopBP1 Immunoprecipitation, Rad9 Immunoblot

C

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empty CrN173*

- empty CrN173*

FLAG Immunoblot
3.7 The association between Rad9A*/TopBP1* may be caused by non-specific interactions – determination by confocal microscopy and flow cytometry

Although the results of the co-immunoprecipitation experiments showed some evidence for the possibility of Rad9A* and TopBP1* interaction through a non-specific interaction between Rad9A* and the BiFC fragment tag of TopBP1* (and possibly vice versa), further control experiments were performed to confirm these findings using confocal microscopy and flow cytometry. HeLa cells were co-transfected with empty CC155*/empty CrN173*, Rad9A*/empty CrN173*, TopBP1*/empty CC155*, and Rad9A-Mut*/TopBP1* and visualized using a confocal microscope (Figure 13A). Although no fluorescent signal was observed with the empty CC155*/empty CrN173* and Rad9A*/empty CrN173* transfected cells, they were seen in the cells transfected with TopBP1*/empty CC155* and Rad9A-Mut*/TopBP1*. As in previous confocal microscopy experiments, small punctate fluorescent signals were localized to the nucleus. These fluorescence patterns did not differ from those seen previously with Rad9A*/TopBP1*. Along with the co-immunoprecipitation studies, this may further suggest a possibility that Rad9A* and TopBP1* are interacting non-specifically. However, there is also the possibility that the similar fluorescence patterns seen with the Rad9A*/TopBP1* sample and the negative controls may be due to a high background fluorescence signal. If this was the case, specific interaction signals may not be easily detected through visual analysis of the cells, as they would be masked. Thus, further analysis of the BiFC signals was performed using flow cytometry to quantitatively determine if their relative fluorescence levels differ.
The overall fluorescence levels of the Rad9A*/TopBP1* and Rad9A-Mut*/TopBP1* transfected HeLa cells were compared using flow cytometry as seen in Figure 13B. Although both the Rad9A*/TopBP1* and Rad9A-Mut*/TopBP1* cells showed significant fluorescence levels above the mock transfected control, their fluorescence levels were not significantly different from one another. Several other negative controls were also tested including empty CC155*/empty CrN173*, Rad9A*/empty CrN173*, and TopBP1*/empty CC155*, but these controls too showed similar or even greater signal than the aforementioned samples. All three samples co-transfected with TopBP1 showed a smaller number of cells that exhibited fluorescence above the mock control levels (1.9% with empty CC155*, 6.4% with Rad9A*, 2% with Rad9A-Mut*). As with the confocal microscopy experiments, the similar signal between the negative controls and the Rad9A*/TopBP1* sample suggests the following possibilities; 1) The BiFC fusion proteins may be interacting in a similar, non-specific manner resulting in similar fluorescence levels. 2) Even without direct non-specific interaction between the BiFC fusion proteins, significant background fluorescence signals may exist, possibly even masking existing specific interactions of the BiFC constructs. Collectively, the results of this project suggest that the BiFC assay system may need to be further adjusted for future studies in order to confirm its viability as a means to studying the interaction between Rad9A and TopBP1. However, some of the results also suggest a strong possibility that the C-terminal phosphorylation status of Rad9A is being negatively influenced by the C-terminal BiFC tag.
Figure 13: Determining if Rad9A*/TopBP1* interactions are specific by confocal microscopy and flow cytometry.

A) HeLa cells were co-transfected with Rad9A-Mut*/TopBP1* or empty CC155*/TopBP1* and visualized using confocal microscopy as in previous experiments. In the case of the CC155*/TopBP1* cells, a red nuclear stain was also used simultaneously. Small punctate, nuclear signals were seen in both samples. B) Flow cytometry was also used to determine the relative fluorescence levels of HeLa cells transfected with TopBP1* and either Rad9A* or Rad9A-Mut*, as well as the negative controls indicated. Both Rad9A*/TopBP1* and Rad9A-Mut*/TopBP1* exhibited fluorescence above the mock transfected control, but fluorescence levels did not differ significantly between the two samples. Their fluorescence levels also did not differ significantly from that of the negative controls. Flow cytometry profiles indicate the % of cells showing fluorescence above the gated level (indicated as a line). Histograms indicate the relative median fluorescence of these cells compared to the median fluorescence of all mock transfected control “c” cells.
Chapter 4 – Discussion

4.1 Co-transfection of BiFC constructs

After the successful construction of the BiFC constructs containing the proteins of interest, one of the first aims of the study was to determine the co-transfection efficiency of Rad9A*/TopBP1*. The results have shown that TopBP1* is the limiting factor during co-transfection as only 0.2% of total cells contained only TopBP1*, while 8.6% of total cells contained both TopBP1* and Rad9A*. On the other hand, 17.4% of cells contained only Rad9A*. The difficulty of transfecting TopBP1* was highlighted by the fact that several different transfection reagents and transfection conditions had to be analyzed before TopBP1* transfection was even detected. This low transfection efficiency of TopBP1* may have hampered our BiFC assay as only 8.6% of cells showed co-transfection, limiting the number of cells that we can visualize for BiFC analysis. On the other hand, Rad9A* transfection was seen in a greater number of cells, and immunoblot analysis showed very high transient expression of the protein even with very little plasmid DNA used during transfection. This too may have caused some problems for our BiFC analysis, as overexpressed Rad9A* protein may have contributed to increased background fluorescence signals, and non-specific interactions during the interaction studies.

For future consideration, these concerns may need to be addressed to increase BiFC sensitivity for specific interactions and to optimize the number of cells that carry the BiFC fusion proteins. One such approach may be to establish stable cell lines expressing both Rad9A*/TopBP1*. Such a stable cell line may express our proteins of interest at levels closer to endogenous levels depending on the integration site on the host
chromosome, while at the same time allowing for the detection of BiFC signals in the whole cell population [116, 117]. This approach does not guarantee that the proteins will express at an optimal level, but the production of stable cell colonies with the integrated genes of interest and subsequent analysis of their protein expression levels may allow us to screen for the cell population with expression closest to endogenous levels. An alternative method of transfection for future consideration may also be electroporation, as it has been shown to be highly efficient even in transfecting large DNA into mammalian cells [118, 119]. Although such an approach may not satisfy the need for lower transient expression of Rad9A*, more cells may be able to obtain copies of the TopBP1* plasmid, leading to greater signals caused by specific interaction between TopBP1 and Rad9A.

4.2 Is the C-terminal BiFC fragment tag affecting the phosphorylation status of Rad9A*?

Due to the close proximity of the BiFC tag to the heavily phosphorylated C-terminal tail of Rad9A, there was some concern that it would somehow block the proper phosphorylation of residues at certain sites on Rad9A. The CIP experiment revealed that the Rad9A* migration pattern was different to that of endogenous Rad9A. This may have been due to the loss of certain C-terminal phosphorylations, namely the phosphorylation at sites of Ser277, Ser328, Ser336, and Thr355 [32] responsible for a migratory shift on SDS-PAGE gels. However, with Rad9A* being a different protein product than endogenous Rad9A, the difference in migration patterns may have been due to differing electrophoretic mobility properties rather than a loss of phosphorylation and no concrete conclusions could be made.
Further experiments with phospho-specific antibodies showed that Rad9A* is phosphorylated at S272 after IR damage as expected. However, the constitutive phosphorylation on the S387 site of Rad9A required for TopBP1 interaction was missing. This indicated that if indeed the BiFC fragment tag is affecting the phosphorylation pattern of Rad9A*, its negative effects may only contribute up to a certain point on the C-terminal tail. If this steric hindrance of phosphorylation is real, determining the phosphorylation status of sites upstream of S387 may indicate at what point the BiFC tag is no longer an issue on Rad9A phosphorylation. It is also possible that even though the results of the immunoblot indicated that Rad9A* is not phosphorylated at S387, perhaps a very small fraction of the population is being phosphorylated, but is not being picked up by the immunoblot. One possible future experiment to test this theory may be to analyze the effects of overexpressed Rad9A* on G2/M arrest. A past study by St. Onge et al. [32] has shown that in HeLA cells overexpressed with the Rad9A-S387A mutant protein, cells exhibited prolonged G2/M arrest after IR. This prolonged G2/M arrest is a phenotype seen in S-phase checkpoint defective cells damaged with IR and is thought to be a compensatory mechanism giving cells extra time to fix their replicated damaged DNA [32, 84]. Thus, by transfecting cells with Rad9A* and analyzing for G2/M arrest after IR, one may be able to find functional evidence for Rad9A* lacking its S387 phosphorylation.

4.3 Is Rad9A* and TopBP1* interacting non-specifically?

Co-immunoprecipitation experiments have shown that both Rad9A* and Rad9A-Mut* interact with TopBP1*. This was unexpected, especially with the finding that Rad9A* does not likely possess S387 phosphorylation, as direct interaction between
Rad9A and TopBP1 requires the S387 phosphorylation of Rad9A. Thus, it was possible that the co-transfected BiFC fusion proteins were directly interacting through a non-specific interaction. Evidence for this was supported by the co-immunoprecipitation of empty CrN173* with Rad9A*. It is unlikely that the BiFC tags of Rad9A* and TopBP1* themselves were interacting as empty CC155* and empty CrN173* did not interact.

However, in light of a past finding in our lab by Blair Besley, an alternative hypothesis also exists where the Rad9A* and TopBP1* may have been indirectly interacting in a shared protein complex. In this study, Rad9A was shown to exist in three distinct complexes by gel filtration chromatography corresponding to complex sizes of 100 kDa (corresponding to the 9-1-1 complex), 500 kDa, and 670 kDa. Since, Rad9A is known to interact with many different proteins; it is possible that the two larger complexes consist of the 9-1-1 complex and its interactions with other checkpoint proteins [19, 120-121]. Interestingly however, their relative abundance did not change throughout different points of the cell cycle, nor did new Rad9A containing complexes appear. Since Rad9A contains the mitotic hyperphosphorylation site T292, we may have expected that Rad9A containing complexes of varying sizes may appear throughout different points of the cell cycle as different interaction partners interact with Rad9A. However, this was not the case. Thus, it has been hypothesized that perhaps the 9-1-1 complex may exist in a higher order trimeric form corresponding to the large complexes that were eluted by gel fractionation. If this were to be the case, a lack of phosphorylation at the S387 site of Rad9A would not impede interaction between Rad9A and TopBP1, as the 9-1-1 complex containing the mutant Rad9A protein would be associated with endogenous 9-1-1 complexes containing wild type Rad9A, allowing for TopBP1
interaction. However, further characterization of the gel filtration samples would need to be carried out to confirm this hypothesis.

Confocal microscopy and flow cytometry results also showed that not only Rad9*/TopBP1* co-transfected cells exhibited BiFC signals, but so did many different negative control sample, including Rad9A-Mut*/TopBP1* cells. Again, these results suggested the possibility that Rad9A* and TopBP1* are not directly interacting, but are associating in a non-specific manner leading to similar fluorescence signals of Rad9A*/TopBP1* as the negative controls. However, there is also the possibility that the background fluorescence signals created by the BiFC tags were sufficiently high as to possibly mask any specific interaction signals that may exist. This factor may have been compounded by the relatively high expression of Rad9A*. The difference in percentage of cells showing fluorescence above the mock transfected levels were also of concern. All cells containing TopBP1* showed a lower number of cells showing fluorescence above the mock control levels, whereas both the empty CC155*/empty CrN173* and Rad9A*/empty CrN173* showed much fluorescence in a much greater percentage of cells. Although relative fluorescence was determined from only those cells showing fluorescence above the mock control levels, a smaller sample size in the TopBP1* containing cells was not ideal. This could be due to the low transfection efficiency of TopBP1* as described previously. Alternatively, this may be caused by decreased survivability of cells transfected with TopBP1* if the fusion protein is having a toxic dominant negative effect on the checkpoint pathway, preventing cell cycle arrest. Thus, it cannot be concluded that specific interactions signals do not exist until co-transfected
BiFC proteins can be expressed closer to endogenous levels to reduce the background signal and more consistent transfection efficiency can be obtained across all samples.

Also, the control bFos/bJun based vectors used in this project was not ideal, as the present control system employs the complementary fragments of the Venus fluorescent protein, while our proteins of interest use the Cerulean based vectors. Alternatively, examining our protein interactions in the same Venus based expression vectors as the bFos*/bJun* controls would be preferred, as comparisons can be problematic when switching between different wavelengths for detecting BiFC signals. We must also acknowledge that the strength of interaction between the control bFos/bJun proteins may be substantially different than that of Rad9A/TopBP1, so a direct comparison of BiFC signals may not be ideal. Thus, a positive control consisting of a whole fluorescent protein such as Venus, fused to Rad9A would make for a better control in the future.

4.4 The cell cycle checkpoint response to DNA damage

We may intuitively expect BiFC fluorescence signals to increase in response to DNA damage due to the recruitment of TopBP1 to Rad9A at sites of damage. However, recent evidence has shown that this may not be the case, as the Rad9A-TopBP1 interaction may be present throughout unperturbed DNA replication. Studies have shown that ATR is enriched in the chromatin fraction during replication [122], TopBP1 is upregulated and localized to chromatin during S-phase [39, 123], and Rad17 is constitutively chromatin associated and phosphorylated during normal replication [124, 125]. ATR and Chk1 were also shown to regulate origin firing in the absence of DNA damage [126, 127]. Thus, many checkpoint proteins, including those involved in the ATR-dependent pathway of the checkpoint response are activated during normal
replication, likely due to the resemblance of certain replication intermediates (ie causing ssDNA) to that of DNA damage, such as the stalling of replication forks at regions of DNA that is difficult to replicate [128]. This is important to the current study as we did not observe increased signal in our Rad9A*/TopBP1* cells after damage by IR or HU. Although our results regarding the specific interaction of Rad9A and TopBP1 are inconclusive, we may still expect to see little change in fluorescence after DNA damage, assuming that we are able to obtain specific interactions between our BiFC constructs. Rad9A and TopBP1 may be interacting constantly throughout replication, so the introduction of new damage sites may not cause a significant shift in BiFC signal. However, due to the role of the ATR-dependent checkpoint response at multiple points of the cell cycle, differences in damage induced fluorescence may be possibly observed in synchronous population of cells after DNA damage; particularly at the G2/M checkpoint.

4.5 Can the Rad9A* BiFC construct be modified for success in the future?

The results of this project suggest that the C-terminal BiFC tag of Rad9A* is affecting its S387A phosphorylation site, possibly making it a poor candidate for the BiFC based interaction studies. However, several alternative methods of implementation are worth considering for the future. The first would be to determine if the BiFC fragment tag could be fused onto a different part of the Rad9A protein, namely the N-terminal globular region. The initial concern and justification for avoiding this approach was that the N-terminal region is important for 9-1-1 complex formation and a fluorescent tag may interrupt such complex formation. This would be a significant problem, as the 9-1-1 complex is required for loading of Rad9A at sites of DNA damage [124].
Recently however, the crystal structure of the 9-1-1 complex, including the N-terminal region of Rad9A has been solved [129-131], revealing that perhaps the most N-terminal region of the protein may be a suitable location for a BiFC fragment tag. This is because the most N-terminal residues of Rad9A face outwards in the 9-1-1 complex and are not involved in the interacting surfaces of 9-1-1 complex formation. However, the most N-terminal residues of Rad9A are situated relatively close to the Rad9A-Rad1 interface, which is a potential opening site for 9-1-1 loading onto DNA based on its weaker binding compared to the Rad9A-Hus1A and Rad1-Hus1A interactions. [129]. Alternatively, it may be possible to insert the BiFC tag in the region between the C-terminal tail of Rad9A and its PCNA-like N-terminal region, as the most N-terminal residues of the PCNA-like region also points away from the 9-1-1 complex. However, how such a repositioning of the BiFC tags would affect the 9-1-1 complex structure or function would have to be carefully analyzed thereafter.

Another possible modification of the Rad9A* BiFC construct would be to increase size of the linker region between Rad9A and the C-terminal BiFC tag. This would allow for increased flexibility and movement of the BiFC fragment tag, perhaps allowing for the appropriate kinases to come into close enough proximity to phosphorylate Rad9A* at the appropriate sites. However, such an increase in linker size may also increase the incidence of non-specific interactions as the BiFC fragment tags increased radius of mobility may allow it to be in contact with a larger number of nearby proteins. These modifications are summarized in Figure 14.
Figure 14: Possible modifications of the current BiFC assay for future implementation. The implementation of the BiFC assay to study RadA9A interactions may be improved in the future through several avenues. A) Electroporation or the establishment of stable cell lines expressing the BiFC constructs of interest may achieve improved transfection efficiency and/or protein expression closer to endogenous levels. B) Repositioning of the BiFC fragment tag to different regions of Rad9A; namely the most N-terminal region may possibly solve the issue of C-terminal phosphorylation interference. C) Increasing the linker length between Rad9A and its BiFC fragment tag may also allow for Rad9A to be properly phosphorylated, especially at the S387 site.
4.7 BiFC and the high-throughput drug screen

Although the BiFC assay could not be optimized to effectively study the interactions between Rad9A and TopBP1 in the current study, the hope is that with the knowledge obtained, a working BiFC system can be utilized in the future with many different proteins involved in the checkpoint response. The original long term goal of such a project was to eventually implement the BiFC assay into a high-throughput drug screen. Such a drug screen would allow for the efficient analysis of drugs and their effect on protein interactions in the cell, as indicated by the fluorescence signal strength caused by the complementation of the BiFC tags. Such a drug screen would be highly valuable for its speed, simplicity, and ease of analysis. Recently, flow cytometry and BiFC have been coupled to study weak protein-protein interactions, and inhibitors of protein interactions, showing the merit of such an approach [104, 132]. The availability of multi-colour BiFC constructs [112] would also allow for such a drug screen to simultaneously analyze the effects of particular drugs (or other compounds such as DNA damaging agents) on more than one interacting protein partner.

The BiFC based drug screen would especially be of great value in the context of cell cycle checkpoint proteins, as many components of the checkpoint have been implemented in cancer research as explained previously. Through this drug screen, screening of a large library of drugs or peptides for inhibition of checkpoint protein interactions would be theoretically possible at every level of the checkpoint signaling response. Thus, in conjunction with further insight into protein interactions involved in the checkpoint response, a detailed map of the signaling pathways can be devised,
indicating which drugs affect which protein interactions at specific points in the checkpoint response.

4.8 Conclusions

Recent research in the area of cell cycle checkpoints have shed some light on the specific proteins interactions involved in the complex web of signaling pathways in response to DNA damage. The Rad9A and its heavily modified C-terminal tail has been of particular interest as recent findings have shown that the interaction between Rad9A and TopBP1 is an essential upstream component of the ATR mediated DNA damage response required for Chk1 activation [32, 90]. The objective of this study was to optimize the newly developed BiFC assay system as an alternative way to study the important Rad9A-TopBP1 checkpoint interaction, and eventually utilize the assay system in the context of studying many different components of the checkpoint response. In this study, BiFC constructs containing our proteins of interest were successfully generated and expressed in HeLa cells. Although BiFC fluorescence signals were observed by confocal microscopy and flow cytometry, the results could not distinguish a specific interaction signal between Rad9A and TopBP1 above that seen in negative control samples. The C-terminal BiFC tag on Rad9A was also shown to affect at least one phosphorylation site on the protein; preventing the phosphorylation of the constitutive S387 site, although the damage dependent S272 phosphorylation was intact after IR treatment. These results suggest a possibility that Rad9A and TopBP1 BiFC fusion proteins may be interacting non-specifically, although future adjustments to the BiFC assay system must be made to confirm this. Although the study was not successful in its original objective, the current work has provided invaluable insight into the BiFC
technology that can be used as a building block for future implementation of the technique to study proteins interactions in the checkpoint response.
References


