BIOFLAVONOIDS GENISTEIN AND QUERCETIN TRIGGER ALTERNATIVE END JOINING PATHWAY OF DNA REPAIR

By

Anindita Ghosh

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Approved by:

_____
Dr. Christine Richardson

_____
Dr. David. L Tait

_____
Dr. Didier Dréau

_____
Dr. Andrew Truman

_____
Dr. Juan Vivero-Escoto

ABSTRACT

ANINDITA GHOSH. Bioflavonoids Genistein and Quercetin trigger Alternative End joining Pathway of DNA Repair. (Under the direction of DR. CHRISTINE RICHARDSON)

Faithful repair of chromosomal double-strand breaks (DSBs) is crucial to genome stability and normal cell survival and proliferation. During normal metabolic processes and especially following exposure to DNA damaging agents including irradiation, alkylating agents, and inhibitors of topoisomerase II such as etoposide chromosomal DSBs occur. More recently, researchers have investigated the potential of DSBs from exposure to dietary and environmental compounds, including bioflavonoids. Bioflavonoids are found in fruits, tea, coffee, wine, soy, and cocoa, as well as anthraquinone laxatives, podophyllin resins, flame retardants, quinolone antibiotics, pesticides, and phenolic compounds. Previous studies demonstrated that several bioflavonoids induce DNA DSBs, including cleavage within the *MLL* gene leading to chromosomal translocations. Importantly, the cleavage sites colocalize with cleavage sites induced by chemotherapeutic agents such as etoposide. This research focuses on the mechanisms of DNA damage signaling and the DSB repair pathways preferentially triggered following cell exposure to two specific bioflavonoids, i.e., genistein and quercetin. The hypothesis tested is that genistein and quercetin induce DNA DSBs in the MLL and AF9 genes, promote canonical/specific DNA damage responses and specifically suppress classical nonhomologous end-joining (C-NHEJ) repair but stimulate error-prone alternative end-joining (Alt-EJ) repair events that favor chromosomal translocations. Additionally, the hypothesis that genistein and quercetin mechanistically impact DNA repair pathways by affecting post-translational

modifications of DNA repair proteins is tested. To address these hypotheses, parental embryonic stem cells as well as CRISPR XLF (-) XRCC4 (-) cells were treated with increasing doses of genistein and quercetin and the expression of C-NHEJ protein DNA-PKcs, XLF, XRCC4, and Alt-EJ protein CtIP, Pol θ determined. Additionally, the phosphorylation status of DNA-PKcs was defined. Results indicate that cells treated with genistein and quercetin consistently favor the Alt-EJ DNA repair pathway. Overall these findings indicate genistein and quercetin can initiate DNA damage, influence DSB repair pathway choice and affects post-translational modifications of DNA repair genes. This research significantly broadens our knowledge about the potential for supplemental dietary compounds to induce DNA damage and to influence subsequent DNA repair pathway choice.

DEDICATION

I dedicate my doctoral dissertation to my parents - Dr. Anupam Kumar Das Burman and Mrs. Alpana Bose, to my sister - Antara Das Burman for their continuous love, blessings and inspiration. Without them, this work would not have been possible.

Finally, I am most thankful to my husband - Dr. Angshuman Ghosh, my beloved son and the most precious gift of my life - Abhigyan Ghosh for their constant love and support throughout the period of my doctoral work.

ACKNOWLEDGEMENTS

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CHAPTER 1: Introduction

1.1 DNA double-strand breaks

Genome integrity is critical for cell survival. DNA double-strand breaks (DSBs) are the most deleterious type of DNA damage that may lead to genome instability, cell death, or oncogenic transformation [1].

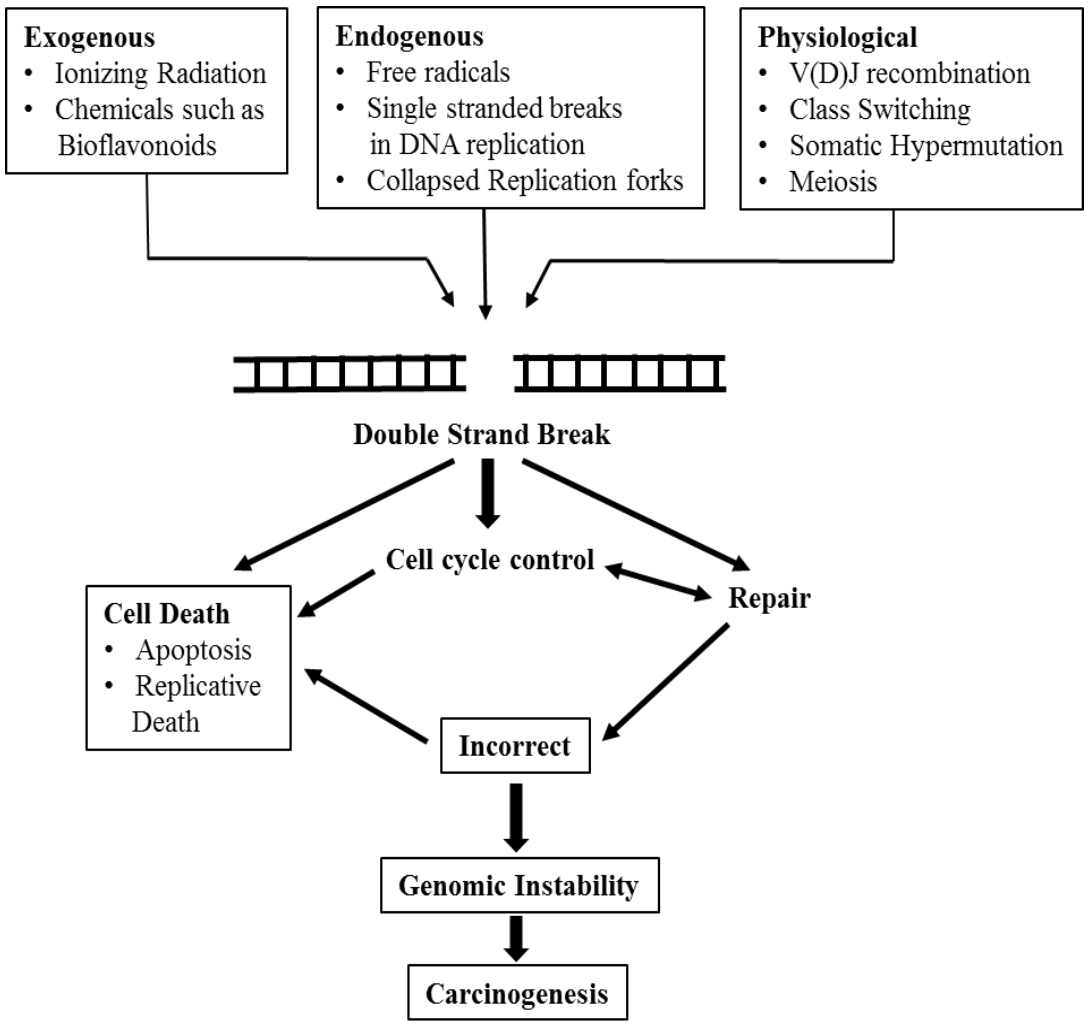


Figure 1.1: Causes, Cellular responses and Consequences of DNA Double-strand breaks

DSBs occur regularly from endogenous processes such as variable (diversity) joining V (D) J rearrangement in lymphoid cells, DNA replication and meiosis and as a result of oxidative radicals. Several external factors including ionizing radiation (IR), reactive oxygen species, topoisomerase II (TOPO II) inhibitors such as the chemotherapeutic agent etoposide and alkylating agents can induce DNA DSBs [2, 3]. It has been estimated that there are 10 DSBs formed daily per cell, or more accurately, 50 DSBs per cell per replication cycle [4]. Any one of these DSBs has the potential to be inaccurately repaired which could result in chromosome rearrangements in the form of deletions, duplications, inversions, or translocations. These types of chromosomal rearrangements could lead to mutagenesis or cancer. Any one of these DSBs has the potential to be inaccurately repaired which could result in chromosome rearrangements in the form of deletions, duplications, inversions, or translocations. These types of chromosomal rearrangements could lead to mutagenesis or cancer.

In higher eukaryotic cells, DNA DSBs in chromatin promptly initiate the phosphorylation of the histone H2A variant, H2AX, at Serine 139 to generate γH2AX [5]. Thus γH2AX is used as an indicator of DNA DSBs. Phosphorylation of Ataxia Telangiectasia Mutated (ATM) is one of the initial events that follow DNA damage. Phospho-ATM (pATM) plays a key role in the activation of DNA damage response and several oncogenic pathways as well as in the maintenance of genomic integrity [6, 7].

1.2 DNA DSB repair and cell cycle

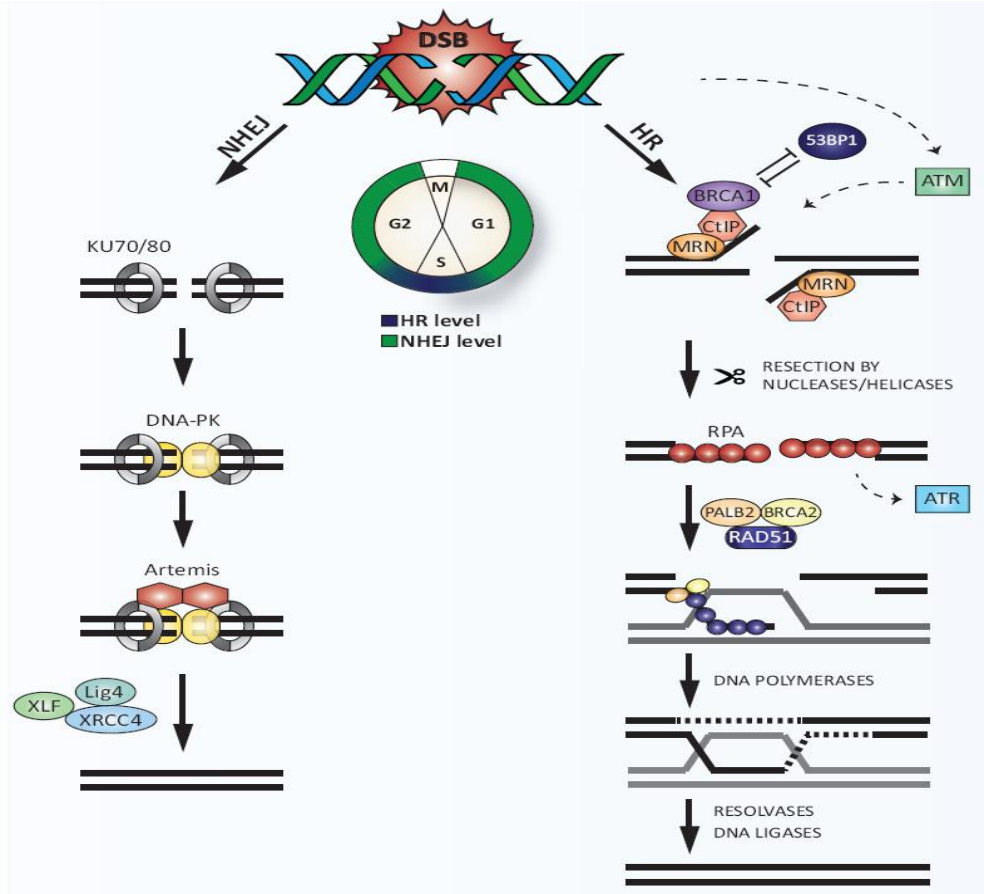


Figure 1. 2: DNA DSB repair pathway and Cell cycle

(Figure Source DOI: https://doi.org/10.4414/smw.2013.13837)

DSBs are predominantly repaired by two distinct pathways: NHEJ or HR. NHEJ operates throughout the cell cycle, but mainly during the G1 and G2 phases, whereas HR peaks in S phase. Rapid association of the Ku70/80 heterodimer to DSBs promotes NHEJ by recruiting DNA-PKcs. DNA ends are processed by the nucleolytic activity of Artemis, followed by religation catalysed by a complex of XLF, Ligase IV (Lig4) and XRCC4 [8]. Alternatively, MRN, which is initially recruited to DSBs in competition with Ku70/80,

initiates DSB resection together with CtIP thereby promoting HR. 53BP1 antagonises BRCA1 in DSB resection. Extensive DSB resection by other nucleases and formation of RPA-coated ssDNA stimulates the activation of ATR. Displacement of RPA by RAD51 is mediated by BRCA2 and PALB2, resulting in the formation of RAD51 nucleoprotein filaments. Subsequent strand invasion into the homologous DNA template and capturing of the second DNA end leads to the formation of a double Holliday junction, which is processed by resolvases. Finally, the DNA is sealed by ligases to accomplish error-free repair of the DSB [9] [10].

1.3 Competing DNA repair pathways

DSBs can be repaired by different and competing pathways depending on the stage of cell cycle and availability of necessary factors that drive the repair pathway [11]. One major pathway for DSB repair is non-homologous end joining (NHEJ) which is active in all phases of the cell cycle but most robust during G0/G1 phase [12]. Another pathway is homologous recombination (HR) which is predominant during S/G2/M cell cycle phases when a sister chromatid with complete homologous template for repair is available in close proximity [13, 14]. The HR pathway is an accurate repair mechanism since it uses the sister chromatid as a template to repair the broken DNA duplex. However, HR can also occur using homologous sequences on an allelic chromosome or heterologous chromosome. Compared to HR, NHEJ is a less accurate repair mechanism since the two termini of the broken DNA molecule are often processed to form compatible ends that are directly ligated and result in small deletions or insertions [15, 16]. NHEJ has two sub pathways--classical nonhomologous end joining (C-NHEJ) and alternative end joining (Alt-EJ).

1.4 Homologous Recombination

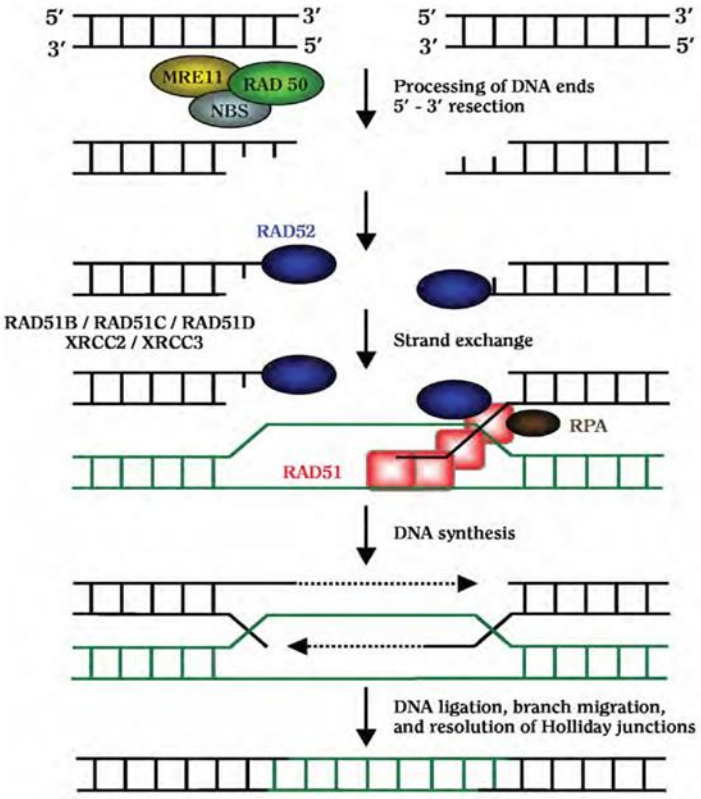


Figure 1.4 : Homologous Recombination pathway

(Figure source https://sites.google.com/site/bi6101dnarepair/homologous-recombination)

The eukaryotic proteins involved in repair of DSBs by HR were identified in *S. cerevisiae* by genetic screening of radiation sensitive and meiotic recombination deficient mutants. The proteins that were identified are known as the RAD52 epistasis group [17]. The mammalian HR proteins include RAD 50, RAD51, RAD 52, RAD54, Mre11, Nbs1, Xrcc2, and Xrcc3[18]. Mre11, RAD50, and Nbs1 form a protein complex in mammalian cells known as the MRN complex [19]. The MRN complex senses DSBs and quickly establishes a protein-DNA complex in order to stabilize and process the free DNA ends. Subsequently,

MRN is responsible for the regulation of exonucleases CtIP and EXO1 and helicase BLM which interact to process the DNA ends in the 5'-3' direction leaving 3' overhanging single stranded DNA ends [20, 21].

DNA resection is followed by the recruitment of other HR proteins including replication protein A (RPA), BRCA1, BRCA2, RAD51, RAD52 and RAD54. RPA binds to the single stranded DNA preventing endonuclease activity and removing any secondary structure. RPA is then replaced by RAD51 which is the main protein involved in HR. RAD51 is a recombinase that catalyzes the homology search and strand exchange with a homologous sequence ensuring accurate repair of the DSB. RAD51 strand exchange activity is inhibited *in vitro* by the addition of RPA to the reaction before or at the same time as RAD51. RAD51 is known to catalyze strand exchange activity. BRCA2 also binds to DNA and physically interacts with RAD51 and is required for the formation of DNA damage induced RAD51 foci. The RAD51 nucleoprotein filament is required for strand invasion, homologous pairing, and D-loop formation. The RAD54 protein, an ATPase and RAD51 dependent helicase, promotes RAD51 mediated strand invasion into duplex template DNA. RAD54 promotes strand invasion and D-loop formation by transiently opening the template DNA duplex, allowing invasion of the RAD51 nucleoprotein filament and binding to homologous sequence.

Homologous pairing of the incoming strand with a template provides a primer for new DNA synthesis to take place and restore information lost at the DSB. In mammalian cells, DNA polymerase η interacts with RAD51 and extends DNA synthesis from the D-loop recombination intermediates. The non-invading end of the DSB is then captured and ligated to the D-loop by DNA ligase-1 forming the hetero-duplexed Holliday Junctions. These

DNA structures are resolved by a resolvase enzyme, possibly by RAD51C in complex with XRCC3.

1.5 Classic non-homologous end-joining

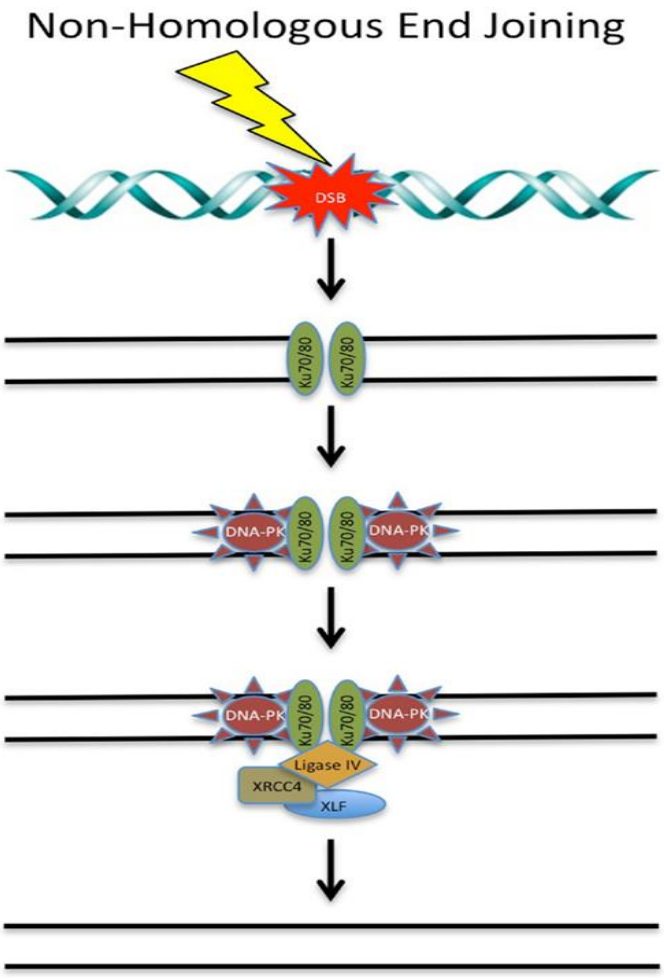


Figure 1.5: Classical Non homologous End Joining (C-NHEJ)

(Figure source DOI: 10.3389/fonc.2014.00086)

In C-NHEJ, repair of DSBs occurs by a ligation reaction which requires ligatable ends and is associated with small insertions or deletions of nucleotides at the junctions. C-NHEJ restores molecular integrity but not sequence information in the DNA [22]. One of the most

abundant cellular proteins Ku has a high affinity for broken DNA ends initiates the process of C-NHEJ. Ku is a heterodimer consisting of Ku 70/80 subunits which recognize and bind to broken DNA ends to protect and recruit the catalytic subunit of the DNA dependent protein kinase (DNA-PKcs), a serine/threonine kinase of the PI3 family of kinases, leading to the formation of a holoenzyme [23, 24]. The serine/threonine kinase function of DNA-PKcs is to bring the broken DNA ends to the close proximity, remodel the break site, and recruit additional NHEJ factors.

Additionally, this kinase component phosphorylates other proteins involved in C-NHEJ [25, 26]. DNA-PKcs undergoes auto-phosphorylation and recruits other kinases such as ATM to activate an endonuclease Artemis for processing of the broken DNA ends. Artemis, in complex with DNA-PKcs, also becomes phosphorylated and exerts endonuclease activity on both the 5′ and the 3′ DNA overhangs. This unique family of nucleases can hydrolyze DNA in various configurations [27, 28]. In the case of IR- induced DNA damage, 20-50% of DSBs require Artemis for repair [29]. However, it is not known if TOPO II inhibitor-induced or bioflavonoid-induced DSBs utilize Artemis or another nuclease. Other nucleases that might be responsible include the MRN complex (MRE11–RAD50–NBS1), CtBP-interacting protein (CtIP; also known as RBBP8) and exonuclease 1 (EXO1). The abundance and localization of these nucleases at DSB sites may determine which are responsible for the greatest amounts of resection at DSBs.

The final stage of C-NHEJ is catalyzed by the ligation complex comprising of DNA ligase IV (LIG4), X-ray repair cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF; also known as Cernunnos) and Paralog-of-XRCC4-and-XLF (PAXX; also known

as XLS/C9orf142) [30-33]. Formation of the ligation complex LIG4/XRCC4/XLF is essential for C-NHEJ [72]. XLF and PAXX interact with XRCC4 and Ku respectively. Studies have shown that XLF stimulates NHEJ of incompatible 3' ends, and PAXX has a role in blunt end ligation.[34]. XRCC4 has the capability to stimulate the enzymatic activity of DNA ligase IV. The N-terminal head domain of both XLF and XRCC4 interact with each other, and the resulting XRCC4/XLF complex forms a sleeve-like structure around a DNA duplex resulting in ligation of broken DNA ends [35-37].

1.6 Alternative end-joining

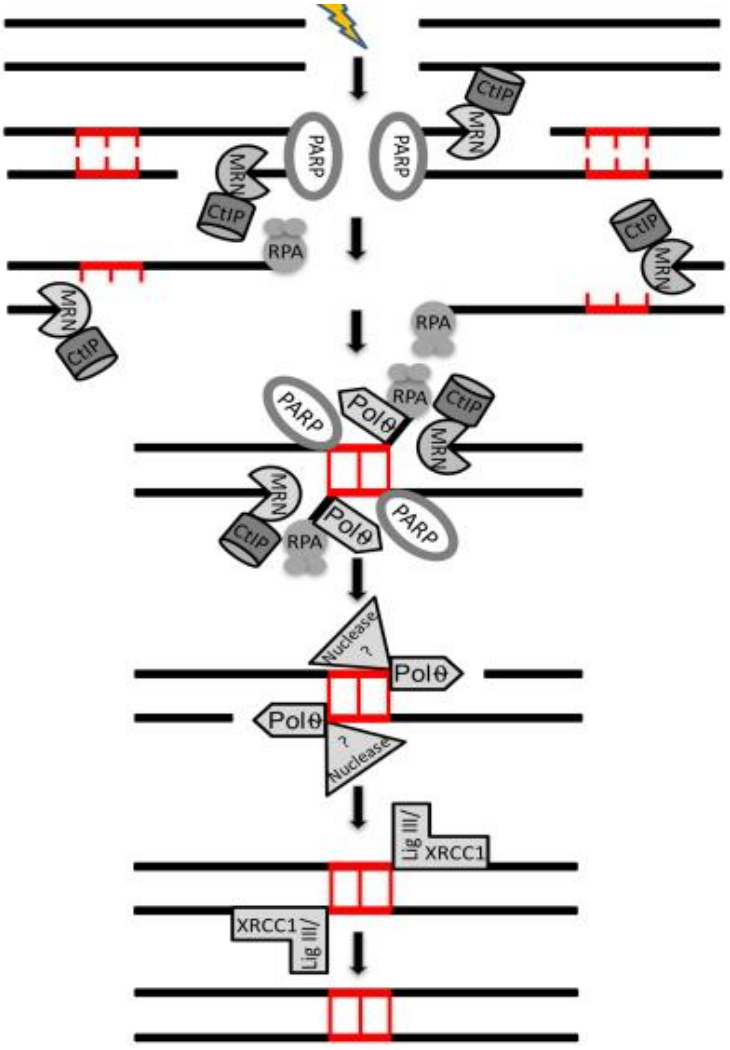


Figure 1.6: Alternative End Joining Pathway (ALT-EJ)

(Figure source DOI: 10.1074/jbc.TM117.000375)

The initiation of the Alt-EJ pathway of DNA DSB begins with Poly ADP ribose polymerase-1 (PARP-1) which has high affinity for ssDNA nicks and blunt DSB ends and influences the activation and recruitment of other repair proteins involved in Alt-EJ [38, 39]. After binding of PARP-1 to the broken DNA end, end resection occurs in two steps to remove small oligonucleotides. The first step involves the MRN complex and the second step requires C-terminal binding protein (CTBP) interacting protein (CtIP) [20, 40]. MRN and CtIP also play roles in promoting HR-mediated DNA repair in association with BRCA-1. Initially CtIP was recognized as a transcriptional co-repressor and a tumor suppressor but it has important role in DNA damage response and end resection. However, it is still unclear how repair pathway preference/choice is determined for annealing the resected ends [41, 42]. During G1 phase Polo-like Kinase 3 phosphorylates CtIP and activates it. During S/G2 phases Ataxia telangiectasia mutated (ATM), Ataxia telangiectasia Related (ATR) and cyclin dependent kinases (CDKs) phosphorylate and activate CtIP [20, 43].

Following end resection the next step in Alt-EJ is annealing of complementary micro-homology DNA sequences and polymerase mediated fill-in synthesis. The exact mechanisms through which intra- or inter-chromosomal complementary base pairing occurs in Alt-EJ requires further elucidation. Recent studies showed that Polymerase theta (Pol θ) encoded by the Pol Q gene facilitates the fill in synthesis in Alt-EJ after the complementary base pairing. The DNA dependent ATPase domain in Pol θ suppresses the RAD51- ssDNA nucleofilament formation due to Pol θ interacting with Rad51 and in this

way HR is inhibited [44, 45].The polymerase domain of Pol θ contains a conserved loop domain essential for extending resected ends. These functions support a critical role of Pol θ in Alt- EJ [42, 46].

The final step of Alt-EJ is ligation of the broken DNA ends. Ligase 3α (LIG 3α) has an effective intermolecular DNA end joining activity and thus plays a role in final ligation step in Alt-EJ [47, 48]. X-Ray Repair and Cross Complementing Factor 1(XRCC1) and LIG3α work in coordination such that XRCC1 acts as a scaffold protein that guides LIG3α to the DNA [49]. PARP-1 interacts with both XRCC1 and LIG3α and gets recruited to the sites of DNA damage. MRN also is known to be associated with LIG3α and XRCC1, especially in context of DNA damage in C-NHEJ deficit conditions [42, 50].

1.7 Post translational modification of DNA repair proteins

Post-translational modifications play a crucial role in coordinating cellular response to DNA damage. Functional interplay between multiple protein modifications, including phosphorylation, ubiquitination, acetylation and sumoylation, combine to propagate the DNA damage signal to elicit cell cycle arrest, DNA repair, apoptosis and senescence [51].

Phosphorylation plays a major role in activating DNA repair proteins [52]. Studies have shown phosphorylation of Ku can determine DNA repair pathway choice in S phase [44]. The NHEJ pathway utilize DNA-PK phosphorylation, whereas the HR pathway mediates repair with phosphorylation of RPA2, BRCA1, and BRCA2. Also, stability of different DNA repair protein depends on phosphorylation [53, 54].

1.8 Repair pathway choice influences repair products and genome stability

Though both end-joining pathways are error-prone, the Alt-EJ pathway of DSB repair poses a particular threat to genomic integrity by promoting joining between heterologous chromosomes leading to chromosomal translocations [55, 56]. Under which circumstances Alt-EJ becomes activated or favored over C-NHEJ requires further investigation [42].

Studies have shown that presence of proteins involved in the C-NHEJ pathway can suppress the activation of Alt-EJ pathway and translocation formation [57]. Conversely absence of proteins from C-NHEJ pathway can promote micro-homology mediated end joining by facilitating Alt-EJ [58-60]. Studies have shown overexpression of Rad 51 protein involved in HR can also lead to Alt-EJ mediated repair of DNA damage [61] but how this overexpression of RAD 51 influences the status of repair protein in end joining pathways have not been elucidated.

Several previous studies implicated the repair pathway choice after IR-induced DNA DSBs [1]. It is clear that IR-induced DNA damage could play role in repair pathway choice by creating variation in the resected ends of dsDNA and modify the functional effect of ATM protein [42, 62]. However, the impact of IR and the cellular response on the functionality of specific DNA repair proteins involved in C-NHEJ and Alt-EJ have not been elucidated.

1.9 Consequences of illegitimate DNA repair events: *MLL* rearrangements and infant leukemia

A chromosomal translocation is a genomic rearrangement that is a reciprocal exchange of genetic material from two heterologous chromosomes that can result in the formation of novel fusion genes and oncogenic transformation, particularly in hematological malignancies and sarcomas, but more recently appreciated in a more diverse array of cancers (Figure 1.9.1) [63-65].

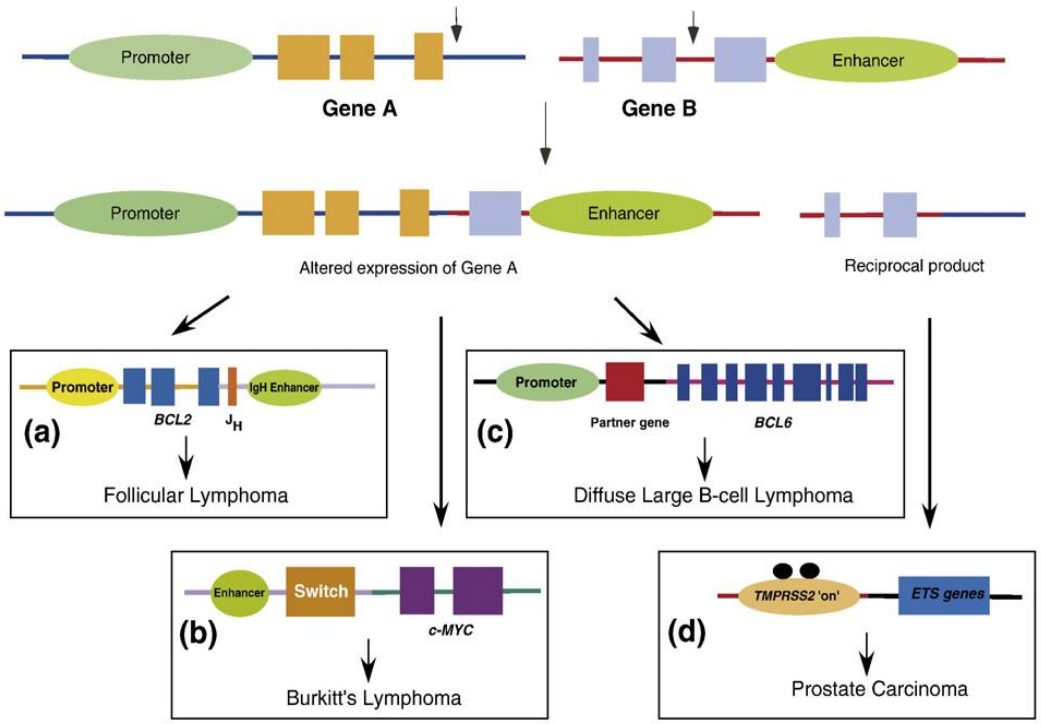


Figure 1.9.1: Chromosomal translocation and hematological malignancy

(Figure source https://doi.org/10.1016/j.bbcan.2008.07.005)

The Mixed Lineage Leukemia (*MLL*) gene is located at chromosomal position 11q23, and frequently rearranged in acute leukemia (Figure 1.9.2). Within the *MLL* gene locus is an 8.2 kb breakpoint cluster region (BCR) where *MLL* translocations arise. *MLL* rearrangements are found in 44-85% of the infants (<1-year-old) with acute lymphocytic leukemia (ALL). These *MLL* rearrangements can arise during intrauterine fetal hematopoiesis [66] suggesting that DSBs occur with high frequency at the BCR either due to gene fragility or due to exposure to chemicals or compounds that promote DSBs in the BCR region itself. [67-71].

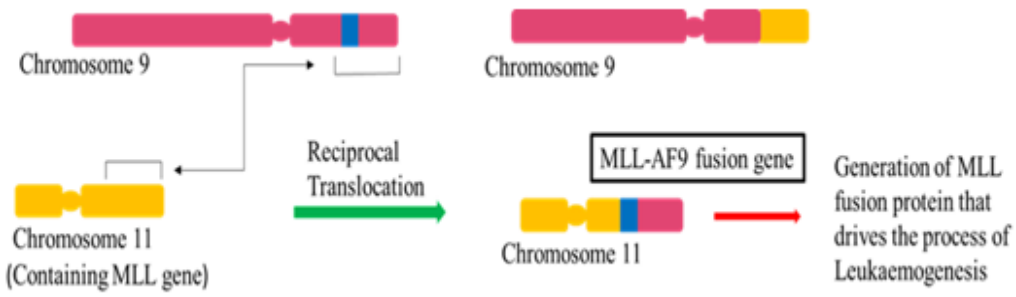


Figure 1.9.2: A Chromosomal translocation t (11; 9) is associated with Mixed Lineage Leukemia

Previous exposure to etoposide, a potent TOPO II inhibitor, is well associated with the development of therapy-related leukemias and *MLL* rearrangements. The similarity of mapped *MLL* rearrangement breakpoints identified in both therapy-related and infant cases suggests a similar mechanism of DSB induction in these two [72].

1.10 Potential Promoters of Genome Rearrangements Observed in Infant Leukemias: Bioflavonoids

Etoposide is characterized by phenolic ring structure, and bioflavonoids contain similar phenolic rings, thus suggesting that exposure of the developing fetus to these compounds through maternal diet can promote DSBs and repair to result in an *MLL* translocation. Bioflavonoid (or flavonoid) is a general name of a class of more than 6500 molecules based upon a 15-carbon skeleton and phenolic ring structures. Two important groups are isoflavones and flavonols (Figure 1.10). Genistein is abundant in soy and is a member of the isoflavones family. Quercetin is present in onions, cherries, apples, broccoli, kale, tomatoes, berries, tea, and red wine and belongs to the family of flavonols [73, 74].

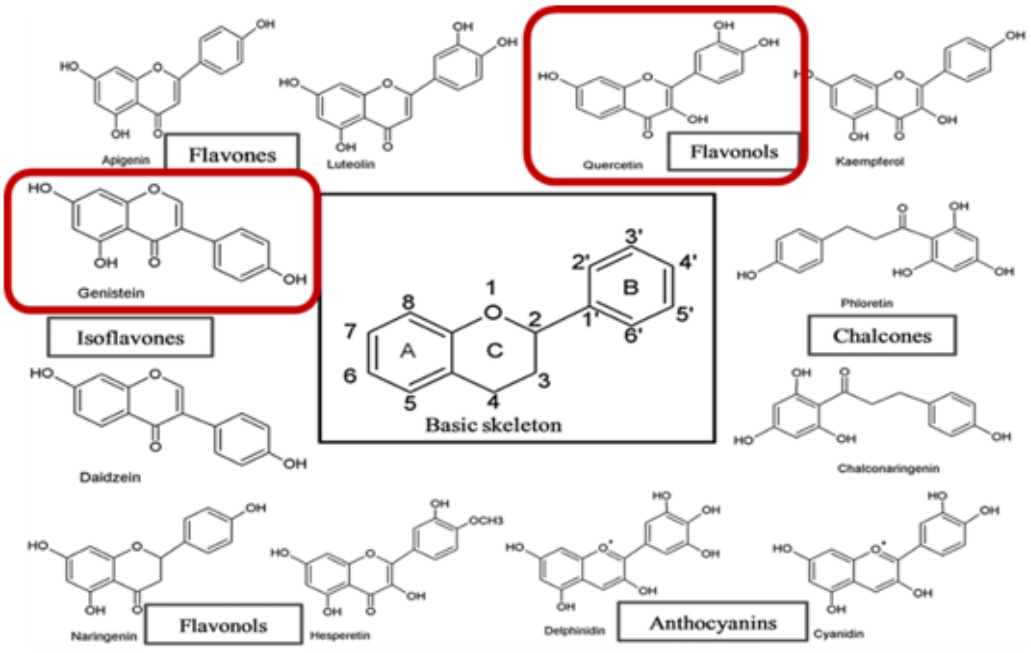


Figure 1.10: Structure of bioflavonoids

It has been shown that bioflavonoids such as genistein and quercetin can induce DNA DSBs in a dose dependent manner including in the *MLL* BCR; in addition, genistein and quercetin exposure can lead to detection of *MLL* BCR translocations [75-77].

However, it is not clear if these bioflavonoids have any direct or indirect effect on DNA repair proteins or DNA repair pathway choice. Genistein is known to have pleiotropic effects including acting as a tyrosine kinase inhibitor [78]. Further investigation is required to determine whether together with other bioflavonoids or genistein alone can also inhibit serine threonine kinases or not, since most of the DNA repair proteins are phosphorylated in the position of serine and threonine which mediates their functionality [79].

1.11. My Dissertation Work

Understanding the crosstalk involved in genistein and quercetin exposure, DNA damage response signaling, and repair pathway choice and the underlying mechanisms of chromosomal translocation in embryonic stem cell is critical. Understanding the integrated activity and role of the proteins involved in C-NHEJ and Alt-EJ pathway, in preventing or promoting translocations, particularly in the context of exposure to genistein and quercetin, is essential to prevent/limit the potential leukemia risk increase of specific isoforms or mutants.

CHAPTER 2: Materials and Methods

2.1 Treatment of MAG cell lines by genistein and quercetin

MAG cells were treated with genistein and quercetin at increasing doses (0, 50,100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. Cells were collected and nuclear protein extracts isolated at 4, 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of γH2AX, pATM, DNA-PKcs, XRCC4, XLF, CtIP and Pol θ were analyzed by densitometry using Quantity One software (Bio-Rad).

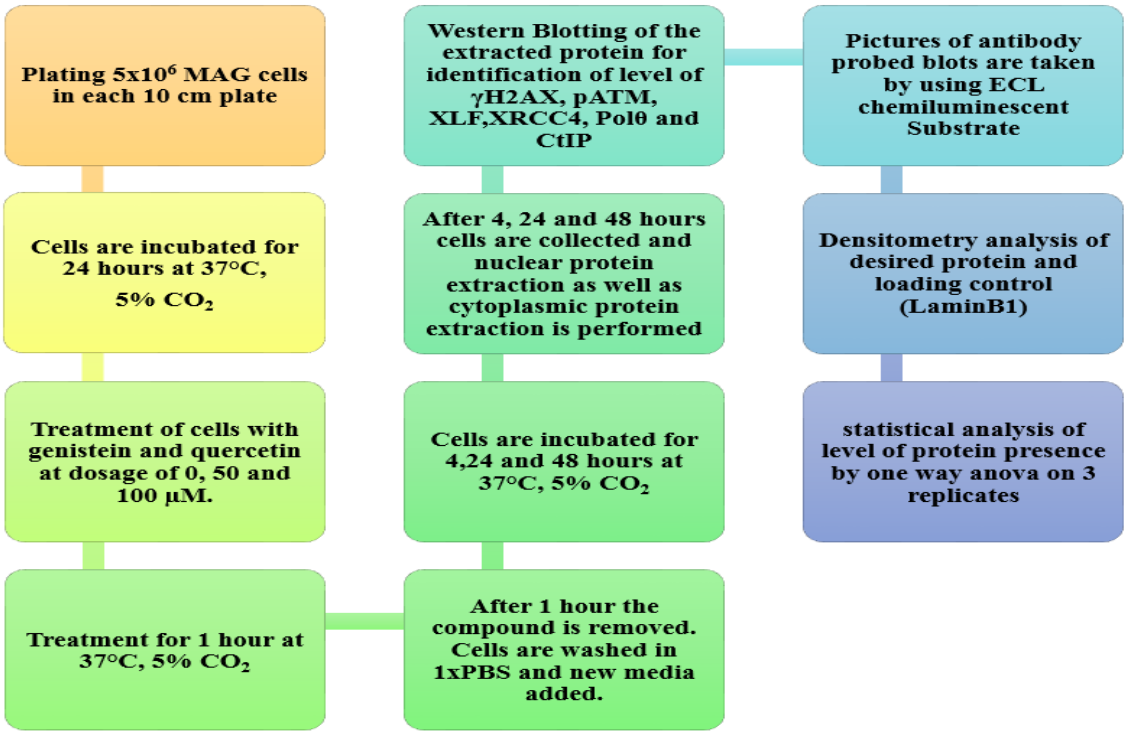


Figure 2.1: Treatment of MAG cell lines by genistein and quercetin at different doses and different time points.

2.2 Generation of CRISPR knockout cell line

Using established protocol [80] we knocked out XLF and XRCC4 protein in MAG cells.

Designing gRNA: Guide RNA (gRNA) for XRCC4 were designed using the website crispr.mit.edu. (For XLF Plasmid with guide RNA were purchased from Genecopia)

Guide RNA for XRCC4 Knockout

5’ CACCGTACCGTGGTTTGCGAGTCGT 3.’

3’ CATGGCACCAAACGCTCAGCACAAA 5’

Cloning: gRNA was generated from purchased nucleotides (from?). pSpCas9 (BB)-2A-GFP (PX458) plasmid was cut with BbSI restriction enzyme followed by XRCC4 gRNA insertion into pSpCas9 (BB)-2A-GFP (PX458) plasmid.

Selection of Correct XRCC4 gRNA inserted Clone: DNA from several clones was isolated using DNA miniprep and clone(s) with XRCC4 gRNA + pSpCas9 (BB)-2A-GFP (PX458) plasmid were identified using polymerase chain reaction (PCR) with the gRNA specific forward primer. Clone(s) was/were further cultured, and the DNA isolated using a maxi prep protocol.

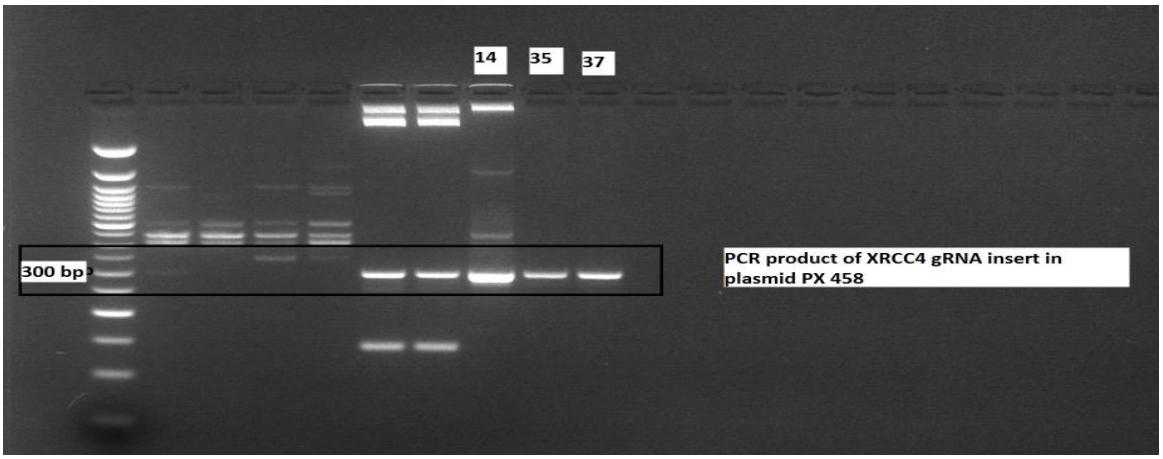


Figure 2.2.1: Agarose Gel electrophoresis of DNA XRCC4 clones

Lipofection: Using Lyovec™ (InvivoGen, San Diego, California) and according to manufacturer's protocol maxipreped DNA of a clone with XRCC4 gRNA was inserted into MAG cells. Control cells were transfected using the purchased XLF gRNA containing clone were into MAG cells using the same protocol.

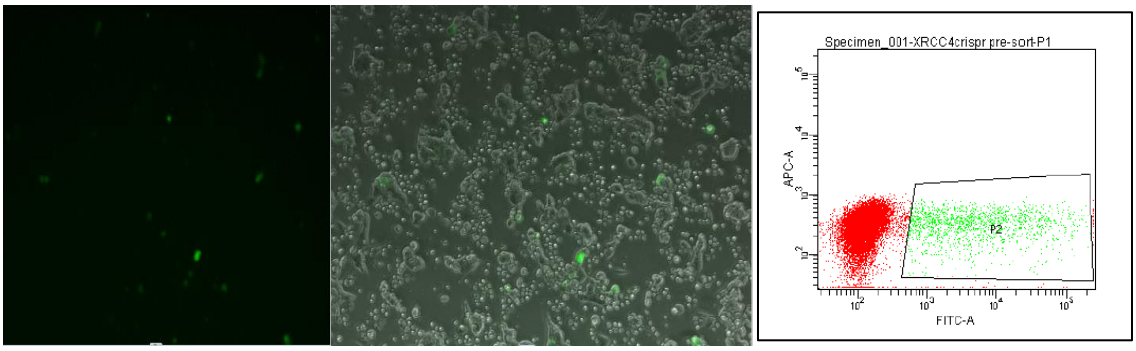


Figure 2.2.2: MAG32A cells transfected with gRNA for XRCC4 PX458 plasmid. (A) Green Fluorescence in transfected cells (Inverted fluorescence microscope, 10X magnification)

(B) Flow-cytometry cell sorting of positively transfected MAG32A + gRNA for XRCC4 PX458 cells.

Flow cytometry: MAG32A cells successfully transfected with pSpCas9 (BB)-2A-GFP (PX458) Plasmid+XRCC4 gRNA and Plasmid with XLF gRNA expressed green fluorescent protein. These green fluorescent cells were cell-sorted using flow cytometry.

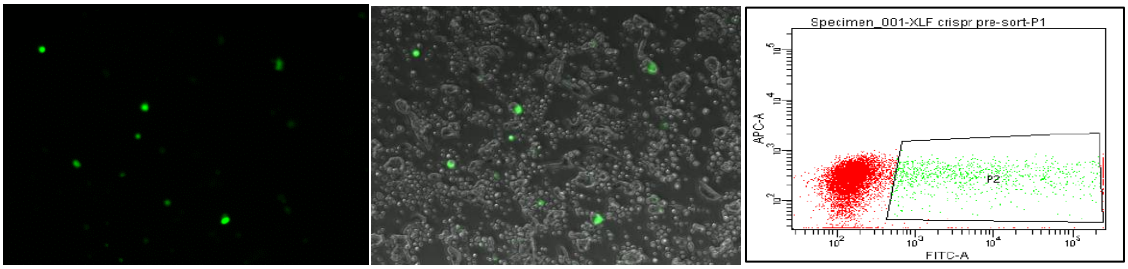


Figure 2.2.3: Cell transfected with gRNA for XLF plasmid. (A) Successfully transfected cells expressed Green Fluorescence. **(B)** Green protein expressing cells were cell-sorted using flow cytometry.

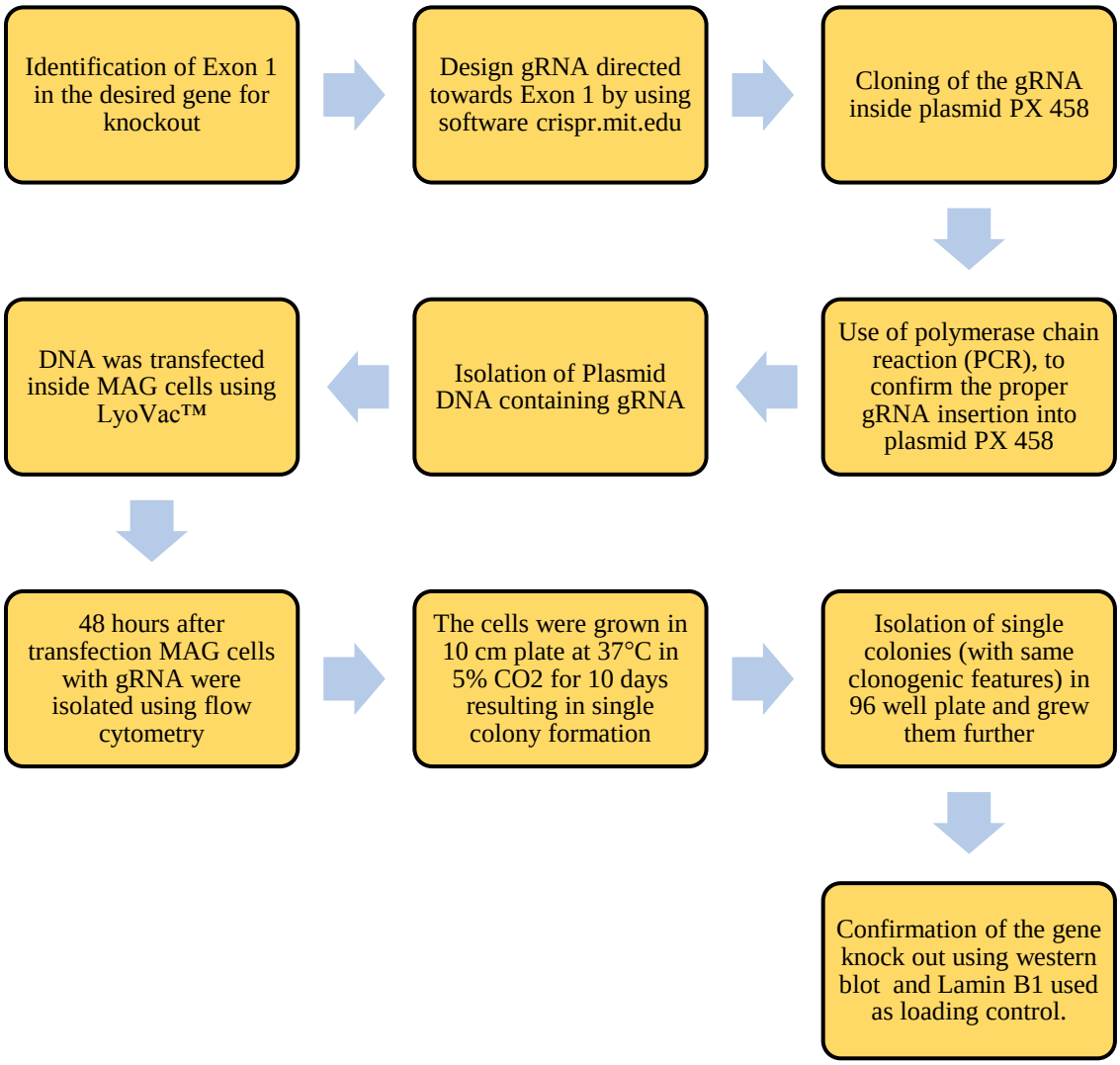


Figure 2.2.4: Scheme of the generation how XLF and XRCC4 knockout cell lines using CRISPR Cas9.

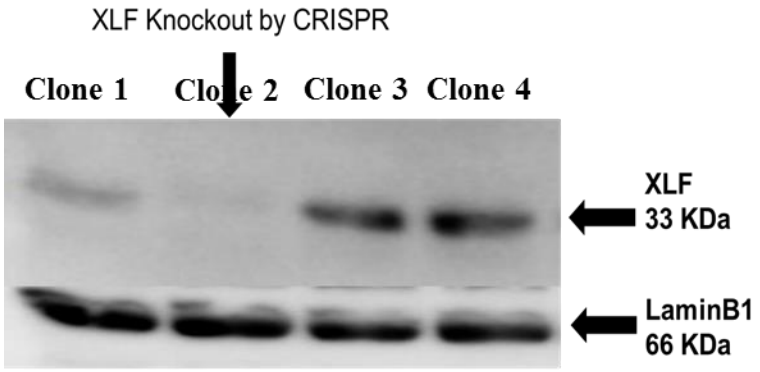


Figure 2.2.5: CRISPR/Cas9 of XLF led to MAG cells with XLF Knockout expression. Briefly, following CRISPR/Cas9 of XLF nuclear protein expression was assessed by Western Blot using Lamin B1 as lading control.

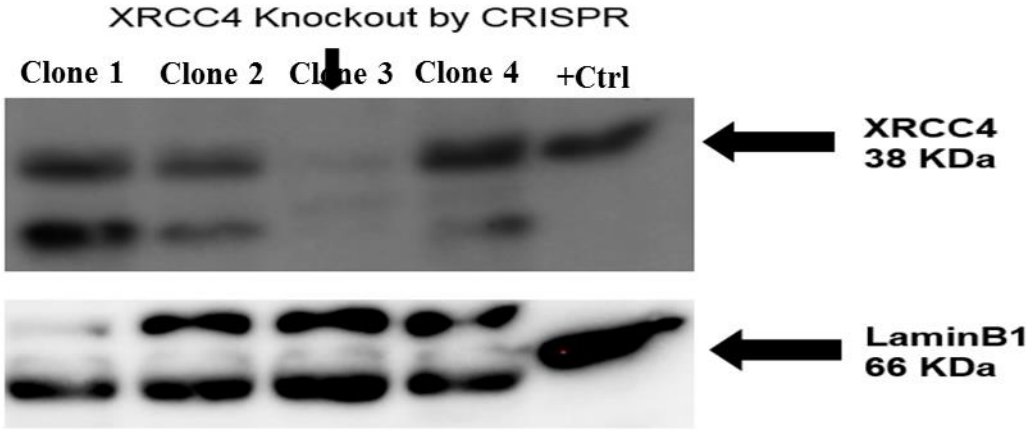


Figure 2.2.6: CRISPR/Cas9 of XRCC4 led to MAG cells with XRCC4 Knockout expression. Briefly, following CRISPR/Cas9 of XRCC4 nuclear protein expression was assessed by Western Blot using Lamin B1 as lading control.

2.3 Treatment of CRISPR knockout MAG cell lines with genistein and quercetin

After generation of XLF (-) and XRCC4 (-) MAG cell lines, cells were cultured in similar conditions as parental cells. These cell lines were individually treated with genistein (50 and 100 µM) and quercetin (50 and 100 µM) each. Protein expressions were measured following treatment as in parental cells as described above in figure 2.1.

2.4 Extraction of nuclear proteins

Nuclear Proteins were extracted using the NE-PER kit (Thermo Scientific). Briefly, immediately before use, protease inhibitors were added to CER I and NER solutions. All steps were completed at 4°C.

Cells ($5x10^6$) from 10 cm plate were trypsinized (3 ml trypsin) for 20 minutes at 37°C and in 5% CO_2 to detach cells from plate. 3 ml media (ES media or DMEM) were added to the plate after 20 minutes to neutralize trypsin. This 6 ml were transferred into the 15 ml tube and centrifuged at 1000rpm for 10 minutes at 4°C.Then the supernatant was discarded and cells were washed by suspending the cell pellet with 1 ml DPBS. This 1 ml solution were transferred into a 1.5 ml micro centrifuge tube and pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C. Supernatant was discarded, leaving the cell pellet as dry as possible. Ice cold CER I added to the cell pellet and the cell pellet resuspended and then incubated on ice for 10 minutes. Next, ice cold CER II solution was added and mixed for 5 seconds followed by incubation on ice for 1 minute. The cells were then further mixed for an additional 5 seconds and centrifugated at maximum speed (16,000g). The cytoplasmic extract (supernatant) was immediately transferred to a clean pre-chilled tube. The insoluble

fraction (pellet), which contains nuclei, was suspended and mixed in ice cold NER solution for ~40min, according to manufacturer (ThermoFisher Scientific, Waltham, MA). Next the nuclei were centrifuged at maximum speed (16000g) for 10 minutes. The Nuclear extract (supernatant) was immediately transferred to a clean pre chilled tube. Extracts were stored at -80°C until use.

2.5 Western blot analysis

Nuclear extracts were assessed by western blotting to identify differences in DNA repair protein expression following different treatments at different time points. 2 microliter of nuclear extract per well were loaded on 4% (for DNA Pk-cs), 6% (For pATM and PolQ) , 8% (for CtIP, PARP and Ligase 4), 12% (For XLF, XRCC4 and Rad 51) and 15% (for yH2AX detection) poly-acrylamide gels and run in SDS-PAGE denaturing conditions at 121 volt for 90 minutes and the proteins were then transferred onto PVDF and nitrocellulose (for yH2AX only) membranes using wet transfer at 100v for 1 hour 30 minutes or semi dry transfer at 23v for 2 hours depending on the size of the protein. Loading of equal protein amounts was assessed by evaluating the even expression of Lamin B1 by immunoblots.

Briefly, after a 1-hr incubation with TBS-T (0.1% Tween 20) containing 5% nonfat milk or 5% BSA (for phosphoproteins) to block non-specific binding, membranes were incubated with antibodies specific for pATM, CtIP, Rad 51 (EMD Millipore), DNA Pk-cs, PolQ, XRCC4, Ligase 4 (Invitrogen) and XLF (Santa Cruz Biotechnology). Following two-hour incubation with the appropriate HRP-conjugated secondary antibody and the

addition of a chemiluminescent substrate (GE), the presence of protein was detected using a bio chemiluminescent imaging system (Biorad). Differences in protein expression were determined by densitometry using Quantity One software (Biorad, Hercules, CA) following normalization to Lamin B1 expression.

2.6 Analysis of proteins phosphorylation

Phosphorylation status of ATM, DNA-Pkcs, Rad51, XLF and Pol Q were analyzed using Phos tag gel as previously [81]. MAG cells were treated with Radiation 10GY, genistein 100 μM, quercetin 100 μM and incubated for 24 hours. Nuclear protein was extracted from these samples and were run in a Phos-Tag™ gel (VWR). Similarly to western blotting, proteins were transferred from Phos-Tag gel to 45-micron PVDF membrane and blocked with 5% BSA. The phosphorylation status was analyzed by using antibodies for each specific protein.

2.7 Identification of frequency of chromosomal Translocation

After treatment of MAG, XLF (-) MAG, XRCC4 (-) MAG cells with genistein and quercetin at different doses (Figure 2.1), cell-seeded plates were incubated further at 37°C, 5% CO_2 and examined daily for GFP expression. Four day after the treatment, cells presented GFP positive colonies. Untreated MAG, XLF (-) MAG, XRCC4 (-) MAG cells were used as negative control. The number of positive colonies per plate (10 cm diameter plate) was recorded.

2.8 Statistical Analyses

All data are presented as means ±SD/SEM. All the experiments were performed in triplicate as biological replicates (n=3). All Statistical analyses were performed using Prism v 7.0 software (Graph Pad) using ANOVA and Dunett post-hoc test with a priori $p<0.05$ considered significant. Significance of the group difference was symbolized as follows: *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

CHAPTER 3: Potential for genistein to induce DNA DSBs, DDR and to influence repair pathway choice

Previous studies have shown that the potent TOPOII inhibitor etoposide can cause DNA DSBs, initiate DDR, and promote chromosomal translocations in mammalian cells [82]**.** Since the biochemical structure of the isoflavone genistein is similar to etoposide, it leads to the hypothesis that genistein can act as DNA damaging agent as well [77]. Genistein inhibits TOPOII activity [83], induces cleavage in the *MLL* gene locus, and results in some detection of MLL rearrangements [72, 77, 84] .However, genistein and other bioflavonoids have pleiotropic effects on cells and their direct role in any single DNA damage or repair pathway is not understood [85]. In this chapter my experiments demonstrate that exposure to genistein induces DNA DSBs and DDR in the murine embryonic stem cell line MAG. These experiments also show that exposure to genistein influences DNA repair pathway proteins that support the idea that genistein promotes repair by the Alt-NHEJ pathway.

3.1 Genistein induces DNA DSBs and DDR

In this set of experiments, I wanted to determine if treatment with genistein can initiate DNA DSBs and DDR in MAG cells by assessing the protein level of phosphorylated H2AX (γH2AX) and phosphorylated ATM (pATM).

MAG cells were treated with genistein at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 4 and 24 hr. post-treatment for Western blotting. DSBs were

assessed by detecting levels of ɣH2AX (serine139) [5]. Initiation of DDR was assessed by detecting levels of pATM (serine1981) since previous studies have shown ATM is phosphorylated at s1981 in response to DNA DSBs by multiple DNA damaging agents including ionizing radiation, chemotherapeutic agent such as TOPO II inhibitor and various chemicals [86]. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

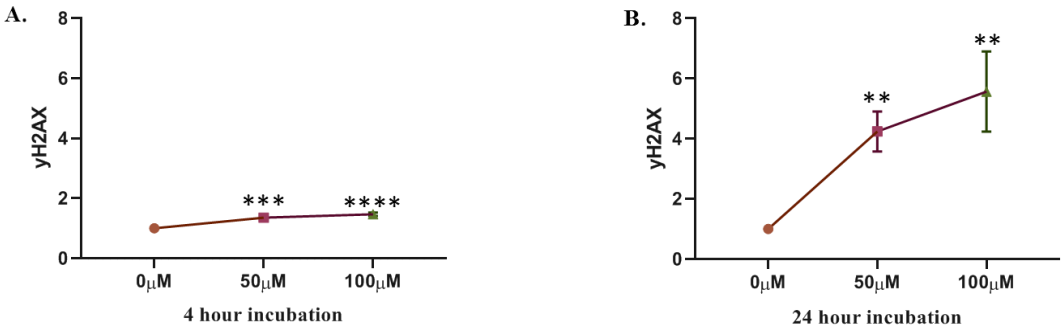


Figure 3.1.1: Genistein induces DNA DSBs (A) Densitometry analysis of western blot showing significantly elevated level of γH2AX in 50 and 100 µM genistein treated group at 4 hr. time point compared to no treatment group. (B) Densitometry analysis of western blot showing significantly elevated level of γH2AX in 50 and 100 µM genistein treated group at 24 hr. time point compared to no treatment group. (****p<0.0001, ***p=0.0003, **p=0.0065, **p=0.0011)

MAG cells treated with genistein showed an increase in the level of ɣH2AX. ɣH2AX levels start to increase at 4 hr. and remain elevated 24 hr. post-treatment compared to untreated MAG cells. Densitometry analysis of immunoblots showed significantly elevated levels of γH2AX induced by 50 µM (p=0.0003) and 100 µM (p<0.0001) genistein at 4hr post treatment compared to the no treatment group. The higher levels of γH2AX persisted at

24hr post treatment; densitometry analysis shows γH2AX remained significantly elevated in 50 µM (p=0.0065) and 100 µM (p=0.0011) genistein treated groups compared to no treatment group. These data shows genistein can significantly induce DNA DSBs in MAG cells in a dose dependent manner. The short time point to detect the DSBs supports the idea that this bioflavonoid directly, rather than indirectly, induces the DSBs detected.

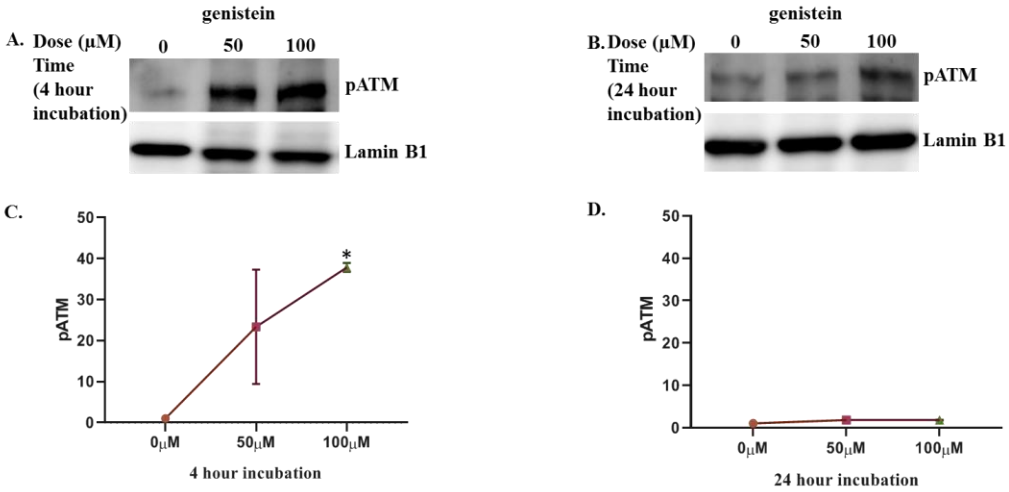


Figure 3.1.2: Genistein can generate DNA damage response (A) Representative immunoblot showing elevated level of pATM in mouse ES cell following treatment with genistein compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing level of pATM in mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of pATM in 100 µM genistein treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no significant change in the level of pATM in genistein treated group at 24 hr. time point compared to no treatment group. (*p=0.0397)

Following treatment with genistein pATM is detected by 4 hr. post-treatment and remains elevated at 24 hr. post-treatment compared to the no treatment groups. Densitometry analysis of immunoblots shows significantly elevated level of pATM in 100 µM (p=0.0397) genistein treated group compared to 50 µM genistein treated group and no treatment groups at 4hr. At 24 hr. post-treatment, the level of pATM remain elevated compared to untreated group but treated and untreated group doesn't show any significant

difference. These data show that genistein can significantly induce DDR in MAG cells in a time and dose dependent manner.

3.2 Genistein suppresses levels of DNA repair proteins of the C-NHEJ pathway

DNA-PKcs is an essential initial component of C-NHEJ pathway [87]. XLF, XRCC4 and DNA ligase 4 form a ligation complex which facilitates end stages of C-NHEJ pathway [72].

Treatment of mammalian cells with the potent TOPOII inhibitor etoposide results in breaks with a 4 bp 5'-overhang and covalently attached TOPOII protein. The large majority of these breaks are repaired rapidly by NHEJ [88]. However, it is not known whether C-NHEJ or Alt-EJ is involved in the repair process. Studies have shown that treatment of mammalian cells with topo II inhibitor and Bioflavonoid genistein can increase chromosomal translocation [77]. Usually in chromosomal translocation Alt-EJ is the preferred pathway [89]. Additionally, studies have shown that suppression of components involved in C-NHEJ pathway (Ku, DNA-PKcs, XLF, and LIGIV) can activate Alt-EJ pathway[90] [91]. In this set of experiments, I wanted to determine if treatment with genistein can influence level of DNA-PKcs, XLF and XRCC4 proteins in MAG cells as a marker of inducing the C-NHEJ pathway of DSB repair.

MAG cells were treated with genistein at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear

membrane protein Lamin B1 was used as a loading control. Protein levels of DNA-PKcs, XRCC4 and XLF were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

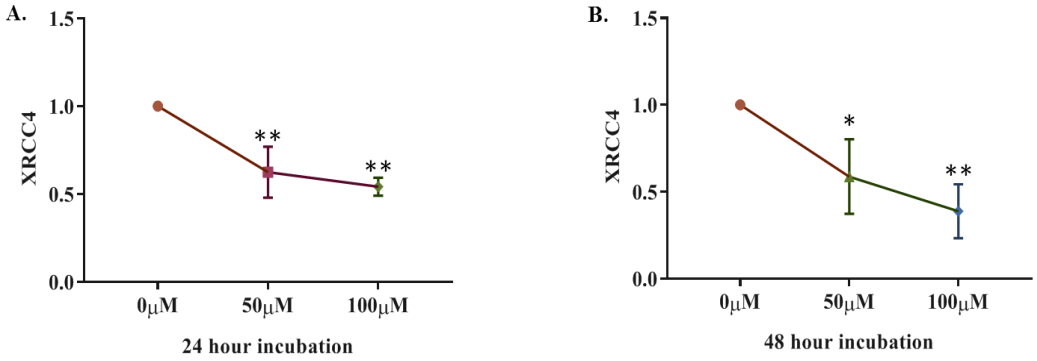


Figure 3.2.1: Genistein suppresses levels of XRCC4 proteins of the C-NHEJ pathway (A) Densitometry analysis of western blot showing significantly decreased level of XRCC4 in 50 and 100 µM genistein treated group at 24 hr. time point compared to no treatment group. (B) Densitometry analysis of western blot showing significantly decreased level of XRCC4 in 50 and 100 µM genistein treated group at 48 hr. time point compared to no treatment group.(**p=0.0037, **p=0.0013, **p=0.0064, *p=0.0371)

MAG cells treated with genistein showed a significant decrease in the level of XRCC4 compared to untreated cells. XRCC4 levels are significantly decreased at 24 hr. post-treatment and remain decreased at 48 hr. compared to untreated cells. Densitometry analysis of immunoblot showed significantly decreased level of XRCC4 after treatment with 100 µM (p=0.0013) and 50 µM (p=0.0037) genistein compared to no treatment groups. In the 48 hr. post treatment group XRCC4 protein levels remain decreased significantly in 100 µM (p=0.0064) and 50 µM (p=0.0371) genistein treated group

compared to no treatment groups. These data show genistein can significantly affect XRCC4 protein level in MAG cells in a dose and time dependent manner.

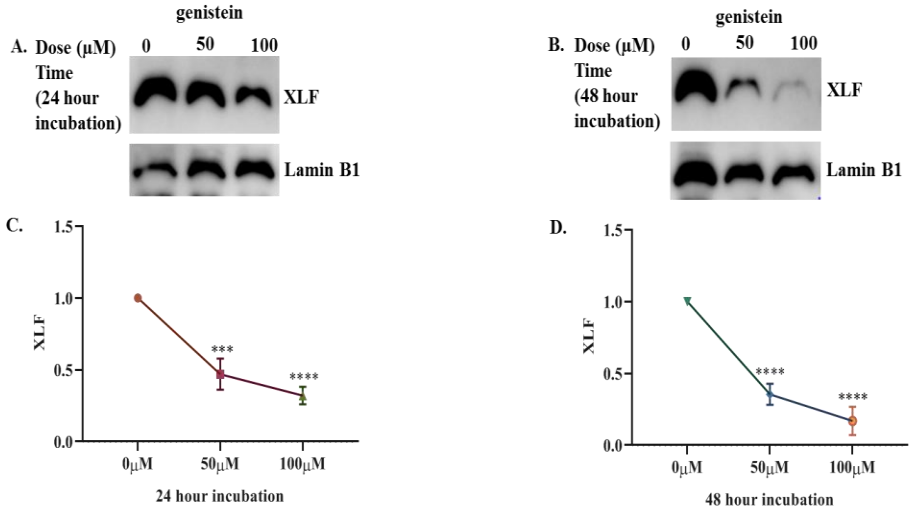


Figure 3.2.2: Genistein suppresses levels of XLF proteins of the C-NHEJ pathway (A) Representative immunoblot showing decreased level of XLF in mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing decreased level of XLF in mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly decreased level of XLF in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group. (C) Densitometry analysis of western blot showing significantly decreased level of XLF in 50 and 100 μM genistein treated group at 48hr. time point compared to no treatment group.(****p<0.0001,***p=0.0002).

MAG cells treated with genistein showed a decrease in the level of XLF compared to untreated cells. In genistein treated cells XLF levels start to decrease at 24 hr. and remain decreased at 48 hr. post treatment compared to untreated cells. Densitometry analysis of immunoblots showed significantly decreased level of XRCC4 following both 50μM ($p=0.0002$) and 100 μM ($p<0.0001$) genistein treatment compared to the no treatment group. In the 48hr post treatment group densitometry analysis shows XLF protein levels remain decreased in both 50 and 100 μM ($p<0.0001$) genistein treated groups compared to no treatment group. These data shows genistein can significantly affect XLF protein level in MAG cells.

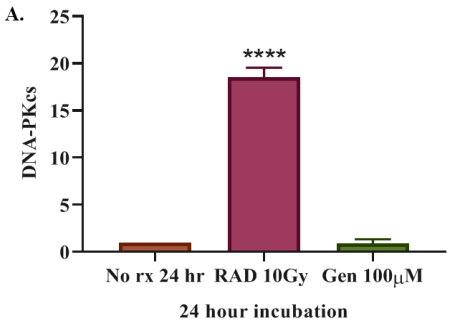


Figure 3.2.3: Genistein doesn't affects levels of DNA-PKcs proteins of the C-NHEJ pathway (A) Densitometry analysis of western blot showing no significant change in DNA-PKcs level between 100 μM genistein treated group and untreated group. Cells treated with 10Gy radiation shows significantly increased level of DNA-PKcs at 24 hr. time point compared to no treatment group.(****p<0.0001).

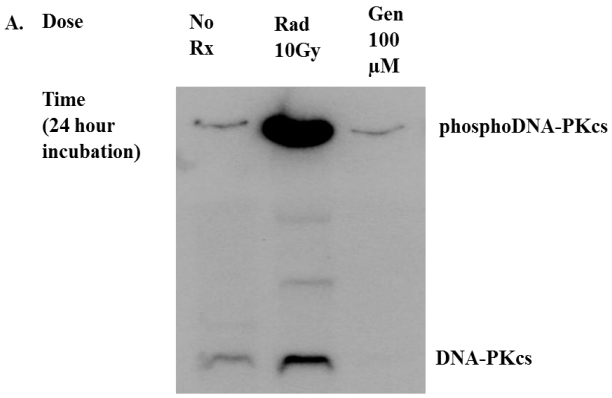


Figure 3.2.4: Genistein and DNA-PKcs phosphorylation shown in a PhosTag™ Gel system (A) Representative immunoblot showing elevated level of DNA-PKcs phosphorylation in mouse ES cell following treatment with 10Gy radiation compared to 100 μM Genistein treated and no treatment group at 24 hr. time point.

Exposure to genistein altered protein levels of XRCC4 and XLF compared to untreated groups. However, no change in the levels of DNA-PKcs was observed at 24 hr. post-treatment with 100 μM genistein compared to untreated cells. This result contrasts with treatment of MAG cells with another DNA damaging agent radiation (10Gy) that induced significantly increased levels of DNA-PKcs ($p<0.0001$) at 24 hr. post-treatment These

results demonstrate specificity of different DNA damaging agents on the functionality of DNA NHEJ repair sub-pathways.

Phosphorylation of DNA-PKcs is mechanistically essential for effective C-NHEJ [92]. MAG cells treated with radiation shows highly increased level of DNA-PKcs phosphorylation whereas 100 μM genistein treated MAG cells showed equal or slightly less phosphorylation of DNA-PKcs in comparison to untreated cells. This findings suggests that genistein mechanistically can impact the C-NHEJ pathway by influencing DNA-PKcs phosphorylation status in MAG cells.

3.3 Genistein promotes increased levels of DNA repair proteins of the Alt-EJ pathway

Polθ and CtIP play important roles in promoting the Alt-EJ repair pathway [93]. Recent studies in mice indicated that Polθ is associated with micro homology mediated end joining (MMEJ) that is one form of Alt-EJ, leading to fusions of dysfunctional telomeres and chromosomal translocations [94]. Previous studies demonstrate that CtIP is a crucial factor for efficient chromosomal translocation formation by micro homology-prone Alt-EJ [89].

In this set of experiments, I wanted to determine whether treatment with genistein can influence the levels of CtIP and Polθ proteins involved in Alt-EJ pathway.

MAG cells were treated with genistein at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of CtIP and Polθ

were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

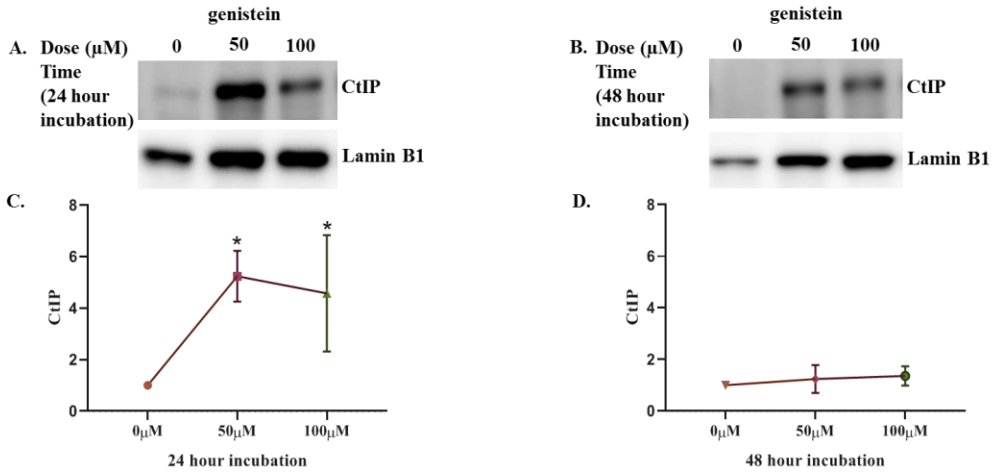


Figure 3.3.1: Genistein promotes CtIP protein level of Alt-EJ pathway in MAG cells (A) Representative immunoblot showing elevated level of CtIP in mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of CtIP in mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of CtIP in 50 and 100 µM genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no change in the level of CtIP in genistein treated group at 48 hr. time point compared to no treatment group. (*p=0.0250, *p=0.0495)

MAG cells treated with genistein showed a significant increase in the levels of CtIP compared to untreated cells. Following treatment with genistein, levels of CtIP are significantly elevated at 24 hr. post-treatment compared to untreated cells and starts to level of 48 hr. post treatment with untreated cells. Densitometry analysis of immunoblot showed significantly increased level of CtIP following both 50 µM (p=0.0250) and 100 µM (p=0.0495) genistein compared to no treatment groups at 24 hr. post-treatment. At the 48 hr. post-treatment densitometry analysis showed no significant difference in the level of CtIP between treated and untreated MAG cells. These data show that genistein transiently affects the levels of CtIP protein.

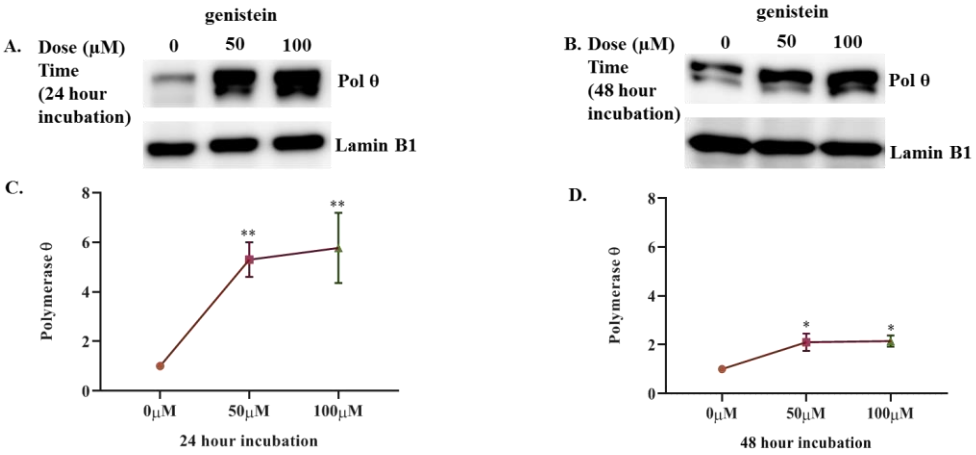


Figure 3.3.2: Genistein promotes Pol θ protein level of Alt-EJ pathway in MAG cells (A) Representative immunoblot showing elevated level of Pol θ in mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of Pol θ in mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 50 and 100 μM genistein treated group at 48 hr. time point compared to no treatment group. (**p=0.0021,**p=0.0012, *p=0.0324, *p=0.0292)

MAG cells treated with genistein also showed a significant increase in the levels of Pol θ compared to untreated cells. Following treatment with genistein, levels of Pol θ are significantly elevated at 24 hr. post-treatment and remain at the observed increased levels 48-hr post-treatment compared to untreated cells. Densitometry analysis of immunoblots showed significantly increased levels of Pol θ following both 50 μM (p= 0.0021) and 100 μM (p=0.0012) genistein treatment compared to the no treatment group. At 48hr post-treatment densitometry analysis shows Pol θ protein level remain significantly increased following both 50 μM (p=0.0324) and 100 μM (p=0.0292) genistein compared to the no treatment group. These data show that genistein significantly affects the levels of Pol θ protein.

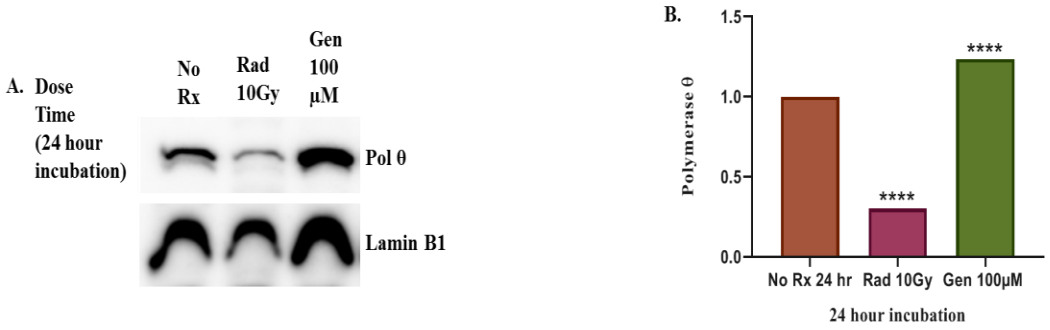


Figure 3.3.3: Effect of radiation on Pol θ protein level of Alt-EJ pathway in MAG cells (A) Representative immunoblot showing elevated level of Pol θ in mouse ES cell following treatment with genistein and quercetin compared to radiation treated group and no treatment group at 24 hr. time point. Immunoblot also shows level of Pol θ in mouse ES cell decreases following treatment with radiation compared to genistein treated, quercetin treated and no treatment group at 24 hr. time point. (B) Densitometry analysis of western blot showing significantly decreased levels of Pol θ in radiation treated MAG cells at 24 hr. post-treatment compared to untreated cells and also compared to cells treated with 100 µM genistein and 100 µM quercetin. (****p<0.0001)

Exposure to genistein induced elevated protein levels of Pol θ compared to untreated cells. By contrast, treatment of MAG cells with another DNA damaging agent radiation (10Gy) showed significantly ($p<0.0001$) decreased levels of Pol θ at 24 hr post-treatment compared to untreated cells and also compared to cells treated with 100 µM genistein. Although reports in the literature demonstrate that radiation can promote chromosomal translocations [95], my findings indicate specificity of different DNA damaging agents to influence levels of individual DNA repair pathway proteins. My data support the hypothesis that genistein increases the amount of proteins involved in Alt-EJ repair.

3.4 Genistein does not alter levels of the HR protein Rad51

Rad 51 is a well-regarded protein central to the process of HR. Literature suggests that NHEJ and HR are competing pathways of repair. In this set of experiments, I wanted to identify whether treatment with genistein can influence levels of Rad 51 protein to indicate

whether genistein globally impacts al DNA repair pathways, or if genistein specifically alters NHEJ.

MAG cells were treated with genistein at multiple doses (0, 50,100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of Rad51were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

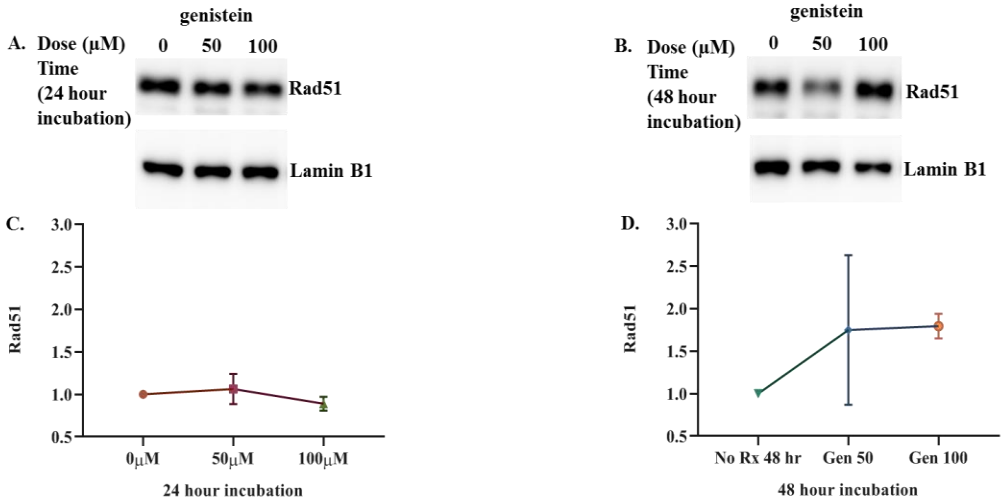


Figure 3.4.1: Genistein does not alter levels of the HR protein Rad51 (A) Representative immunoblot showing no change in the level of Rad51 in mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of Rad51 in mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing no significant change in the level of Rad51 in genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no significant change in the level of Rad51 in genistein treated group at 48 hr. time point compared to no treatment group (p=0.99)

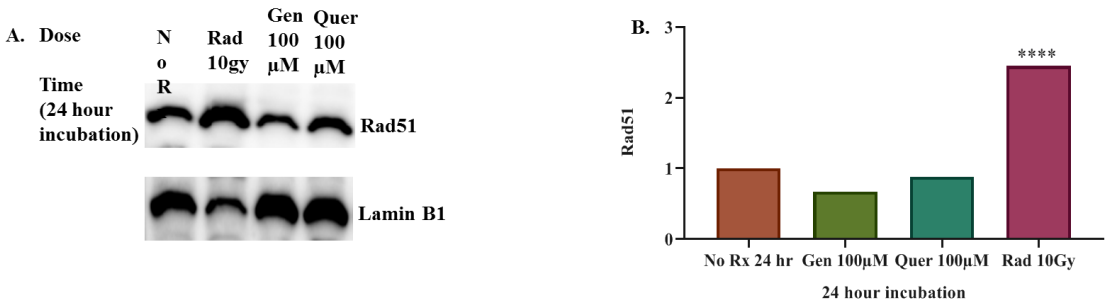
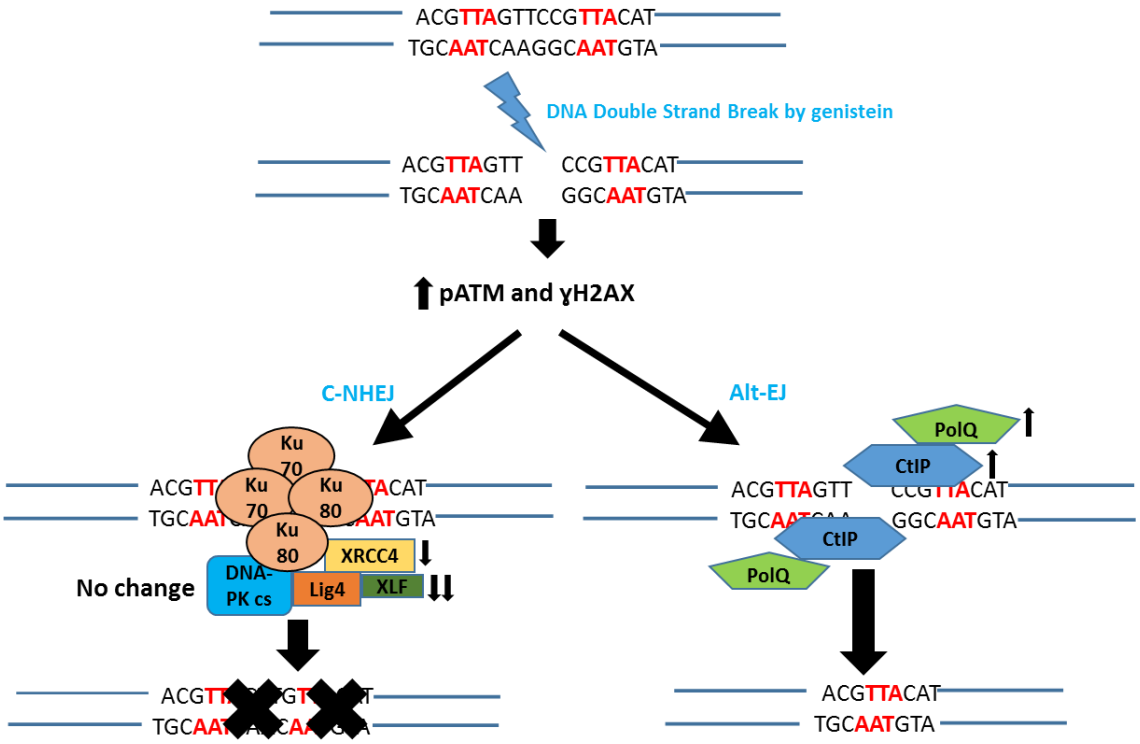


Figure 3.4.2: Effect of radiation on Rad51 protein level of HR pathway in MAG cells (A) Representative immunoblot showing elevated level of Rad51 in mouse ES cell following treatment with radiation compared to genistein and quercetin treated group and no treatment group at 24 hr. time point. Immunoblot also shows level of Rad51 in mouse ES cell shows no significant change following treatment with genistein, quercetin compared no treatment group at 24 hr. time point. (B) Densitometry analysis of western blot showing significantly increased levels of Rad51 in radiation treated MAG cells at 24 hr. post-treatment compared to untreated cells and also compared to cells treated with 100 µM genistein and 100 µM quercetin. (****$p<0.0001$)

MAG cells treated with both 50 µM 100 µM genistein showed no significant changes in Rad51 protein levels between treated and untreated groups ($p = 0.99$). At both 24 and 48 hr post-treatment. By contrast, MAG cells treated with 10Gy radiation (10 Gy) showed significantly elevated levels of Rad51 protein compared to both the genistein treated groups and untreated group ($p < 0.0001$) at 24 hr post-treatment. Elevated levels of Rad51 indicate radiation-induced DNA damage has the potential to be repaired by HR; however, genistein more specifically alters proteins only within the NHEJ sub-pathways. These findings further support the specificity of cellular response to different DNA damaging agents.

3.5 Discussion and Conclusion



In this chapter I have described that genistein can induce DNA DSBs and induce a DDR signaling pathway. Subsequently, I demonstrated that genistein can suppress C-NHEJ pathway proteins and promote Alt-EJ pathway protein levels. These findings are consistent with the hypothesis that genistein can influence DNA repair pathway choice by its impact of protein levels responsible for each of these two competing pathways.

CHAPTER 4: Potential for quercetin to induce DNA DSBs, DDR and to influence repair pathway choice

Previous studies have shown etoposide, a TOPOII inhibitor can cause DNA DSBs and initiate DDR and promotes chromosomal translocation in mammalian cells [82]**.** Since the biochemical structure of the flavonol quercetin is similar to etoposide, it leads to the hypothesis that quercetin can act as DNA damaging agent as well [77]. Quercetin inhibits TOPOII activity [83] induces cleavage in the *MLL* gene locus, and results in some detection of *MLL* rearrangements [72, 77, 84]. However, quercetin and other bioflavonoids have pleiotropic effects on cells and their direct role in any single DNA damage or repair pathway is not understood [85]. In this chapter my experiments demonstrate that exposure to quercetin induces DNA DSBs and DDR in the murine embryonic stem cell line MAG. These experiments also show that exposure to quercetin influences DNA repair pathway proteins that support the idea that quercetin promotes repair by the Alt-NHEJ pathway.

4.1 Quercetin induces DNA DSBs and DDR

In this set of experiments, I wanted to determine if treatment with quercetin can initiate DNA DSBs and DDR in MAG cells by assessing the protein level of γH2AX and pATM.

MAG cells were treated with quercetin at multiple doses (0, 50,100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 4 and 24 hr post-treatment for Western blotting. DSBs were assessed by detecting levels of γH2AX protein (serine139) [5]. Initiation of DDR was

assessed by detecting levels of pATM (serine1981) since previous studies have shown ATM is phosphorylated at s1981 in response to DNA DSBs by multiple DNA damaging agents including ionizing radiation, chemotherapeutic agent such as TOPO II inhibitor and various chemicals [86]. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

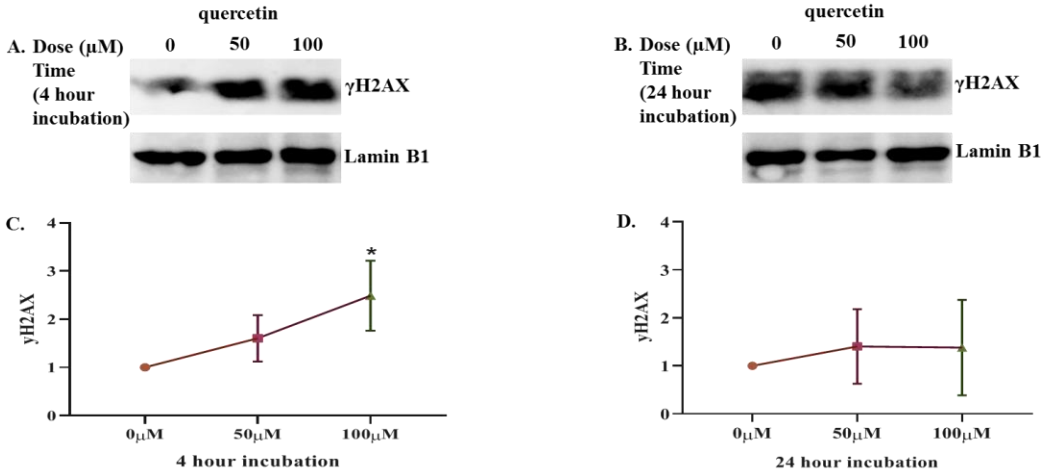


Figure 4.1.1: Quercetin induces DNA DSBs (A) Representative immunoblot showing elevated level of γH2AX in mouse ES cell following treatment with quercetin compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing level of γH2AX in mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of γH2AX in 100 μM quercetin treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing level of γH2AX in 50 and 100 μM quercetin treated group at 24 hr. time point compared to no treatment group with no significant change. (*p=0.0195)

MAG cells treated with quercetin showed an increase in the level of ɣH2AX as an indication of DNA damage. ɣH2AX levels start to increase at 4 hr post-treatment; densitometry analysis of immunoblots showed significantly elevated levels of γH2AX induced by 100 μM (p=0.0195) quercetin compared to the 50 μM quercetin treated group and no treatment group. The levels of γH2AX remain elevated at 24 hr post-treatment;

densitometry analysis shows elevated levels of γH2AX in both 50 µM (p=0.7324) and 100 µM (p=0.7580) quercetin treated groups compared to no treatment group but it is not significant.

These data show quercetin can induce DNA DSBs in MAG cells in a dose and time dependent manner. The short time point to detect the DSBs supports the idea that this bioflavonoid directly, rather than indirectly, induces the DSBs detected.

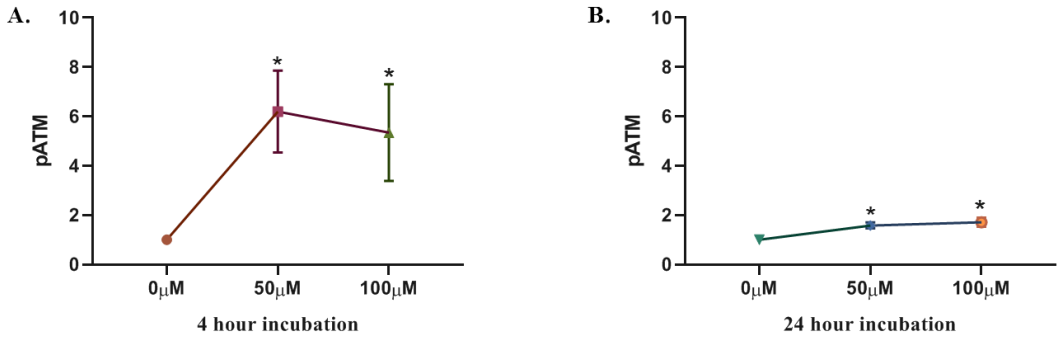


Figure 4.1.2: Quercetin can generate DNA damage response (A) Densitometry analysis of western blot showing significantly elevated level of pATM in 50 µM and 100 µM quercetin treated group at 4 hr. time point compared to no treatment group. (B) Densitometry analysis of western blot showing significantly elevated level of pATM in 50 µM and 100 µM quercetin treated group at 24 hr. time point compared to no treatment group. (*p=0.0122,*p=0.0267,*p=0.0404, *p=0.0235)

Following treatment with quercetin pATM is detected by 4 hr. post-treatment and remain elevated at 24 hr. post-treatment compared to untreated cells. At 4 hr. post-treatment densitometry analysis of immunoblots show significantly elevated level of pATM following 50 µM (p=0.0122) and 100 µM (p=0.0267) quercetin compared to the no treatment group. By 24 hr. post-treatment levels of pATM remain significantly elevated in 50 µM (p=0.0404) and 100 µM (p=0.0235) quercetin compared to the no treatment group.

These data shows quercetin can significantly induce DDR signaling in MAG cells in a time and dose dependent manner.

4.2 Quercetin has a non-specific effect on C-NHEJ pathway protein levels

DNA-PKcs is an essential initial component of C-NHEJ pathway [87]. XLF, XRCC4 and DNA ligase 4 form a ligation complex which facilitates end stages of the C-NHEJ pathway [72].

Treatment of mammalian cells with the potent TOPOII inhibitor etoposide results in breaks with a 4 bp 5'-overhang and covalently attached TOPOII protein. The large majority of these breaks are repaired rapidly by NHEJ [88]. However, it is not known whether C-NHEJ or Alt-EJ is involved in the repair process. Studies have shown that treatment of mammalian cells with TOPOII inhibitor quercetin can increase chromosomal translocation [77]. Usually in chromosomal translocation Alt-EJ is the preferred pathway [89]. Additionally, studies have shown that suppression of components involved in C-NHEJ pathway (Ku, DNA-PKcs, XLF, and LIGIV) can activate Alt-EJ pathway[90] [91]. In this set of experiments, I wanted to determine if treatment with quercetin can influence level of DNA-PKcs, XLF and XRCC4 proteins in MAG cells as a marker of inducing the C-NHEJ pathway of DSB repair.

MAG cells were treated with quercetin at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of DNA-PKcs, XRCC4 and XLF were analyzed by densitometry using Quantity One software (Bio-Rad).

Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

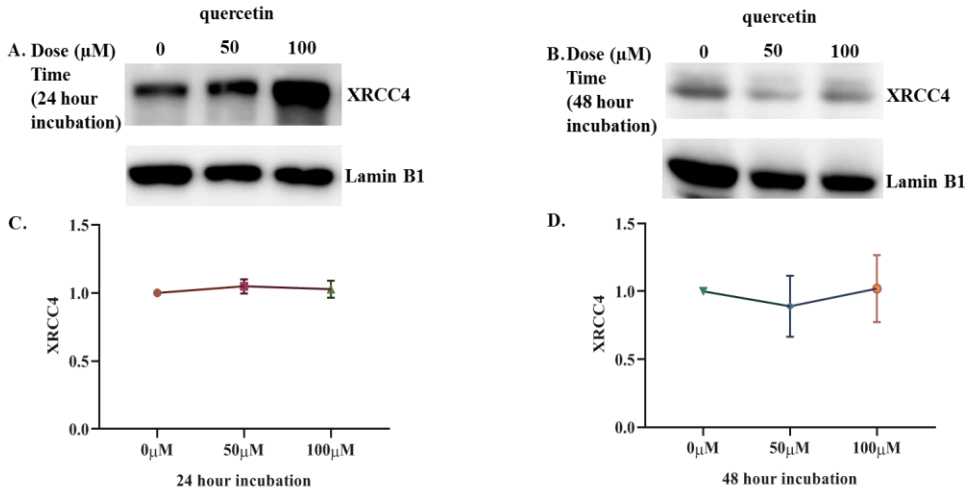


Figure 4.2.1: Quercetin shows no effect on the levels of XRCC4 proteins of the C-NHEJ pathway (A) Representative immunoblot showing level of XRCC4 in mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of XRCC4 in mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing no significant change in the level of XRCC4 in 50 and 100 μM quercetin treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no significant change in the level of XRCC4 in 50 and 100 μM quercetin treated group at 48 hr. time point compared to no treatment group.

MAG cells treated with quercetin showed no change in the level of XRCC4 protein at either 50 μM or 100 μM doses at both 24 and 48 hr. post-treatment compared to untreated cells. Densitometry analysis of immunoblots at 24 hr. post-treatment showed no significant difference in the level of XRCC4 following 50 μM ($p=0.9976$) and 100 μM ($p=0.9998$) quercetin compared to the no treatment group. Densitometry analysis of immunoblots at 48 hr. post-treatment showed XRCC4 protein levels remain unchanged following 50 μM ($p= 0.9239$) and 100 μM ($p>0.9999$) quercetin compared to the no treatment group. These data show quercetin does not affect XRCC4 protein levels in MAG cells. These results are in contrast to genistein treatment which resulted in significantly decreased levels of XRCC4 protein (Chapter 3).

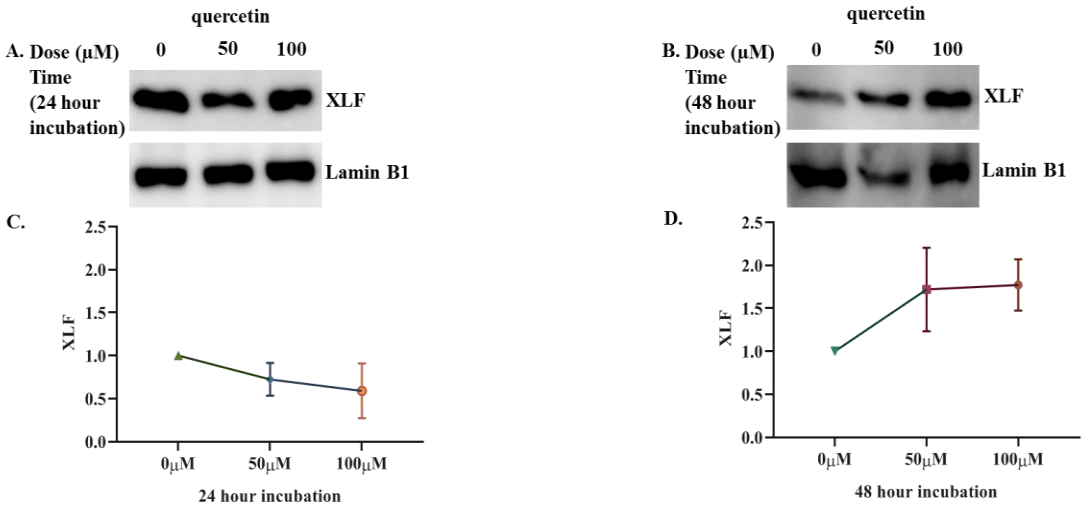


Figure 4.2.2: Quercetin does not affects levels of XLF proteins of the C-NHEJ pathway (A) Representative immunoblot showing level of XLF in mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of XLF in mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing no significant change in the level of XLF in quercetin treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no significant change in the level of XLF in quercetin treated group at 48 hr. time point compared to no treatment group

MAG cells treated with quercetin showed no significant change in the level of XLF compared to untreated MAG cells. Densitometry analysis of immunoblots at 24 hr. post-treatment showed no change in XLF protein levels after 50 and 100 µM quercetin compared to the no treatment group. In the 48hr post treatment group also densitometry analysis shows no significant change in XLF protein level in 50 and 100 µM quercetin treated group compared to no treatment group. These data shows quercetin does not affect XLF protein level in MAG cells. These results are in contrast to genistein treatment which resulted in consistently significantly ($p<0.0001$) decreased levels of XLF protein over 24 and 48 hr. time period compared to untreated group. (Chapter 3).

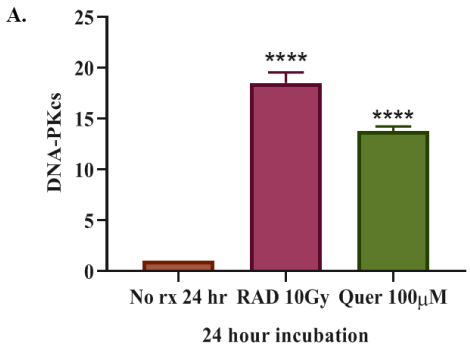


Figure 4.2.3: Quercetin promotes levels of DNA-PKcs proteins of the C-NHEJ pathway (A) Densitometry analysis of western blot showing significant change in DNA-PKcs level between 100 μM quercetin treated group and untreated group at 24 hr. time point. Cells treated with 10Gy radiation also shows significantly increased level of DNA-PKcs at 24 hr. time point compared to no treatment group.(****p<0.0001).

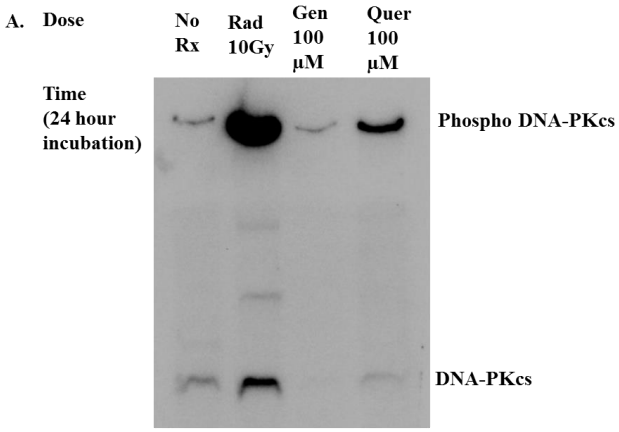


Figure 4.2.4: Quercetin and DNA-PKcs phosphorylation shown in a PhosTag™ Gel system (A) Representative immunoblot showing elevated level of DNA-PKcs phosphorylation in mouse ES cell following treatment with 10Gy radiation compared to 100 μM quercetin treated and no treatment group at 24 hr. time point.

Exposure to quercetin did not show much significant effect on the protein levels of XRCC4 and XLF compared to untreated cells. However, there was a significant increase in the levels of DNA-PKcs protein at 24 hr. post-treatment following 100 μM ($p < 0.0001$) quercetin compared to untreated cells. These results are in contrast to genistein treatment that did not alter the levels of DNA-PKcs.

Phosphorylation of DNA-PKcs is mechanistically essential for effective C-NHEJ [92]. MAG cells treated with radiation shows highly increased level of DNA-PKcs phosphorylation whereas 100 µM quercetin treated MAG cells showed little more phosphorylation of DNA-PKcs in comparison to untreated cells. Though DNA-PKcs level increased significantly following treatment with 100 µM quercetin, similar to radiation treatment, the level of DNA-PKcs phosphorylation is not as pronounced as radiation treated cells. This findings suggests that quercetin mechanistically can impact the C-NHEJ pathway by influencing DNA-PKcs phosphorylation status in MAG cells.

These data show quercetin can influence DNA-PKcs protein but does not affect the levels of XLF and XRCC4 proteins involved in C-NHEJ pathway. These findings altogether indicate the bioflavonoids genistein and quercetin show specificity of activity and impact on the levels of DNA repair proteins.

4.3 Quercetin promotes increased levels of DNA repair proteins of the Alt-EJ pathway

Polθ and CtIP plays important roles in promoting the Alt-EJ repair pathway [93]. Recent studies in mice indicated that Polθ is associated with micro homology mediated end joining (MMEJ) that is one form of Alt-EJ, leading to fusions of dysfunctional telomeres and chromosomal translocations [94]. Previous studies demonstrate that CtIP is a crucial factor for efficient chromosomal translocation formation by micro homology-prone Alt-EJ [89].

In this set of experiments, I wanted to determine whether treatment with quercetin can influence the levels of CtIP and Polθ proteins involved in Alt-EJ pathway.

MAG cells were treated with genistein at multiple doses (0, 50,100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77] .I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of CtIP and Polθ were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

MAG cells treated with quercetin showed a significant increase in the levels of CtIP compared to untreated cells. Following treatment with quercetin, levels of CtIP are significantly elevated at 24 hr. post-treatment and remains at the observed elevated levels 48 hr. post-treatment compared to untreated cells. At 24 hr. post-treatment densitometry analysis of immunoblots showed significantly increased levels of CtIP following 100 µM ($p=0.0197$) quercetin compared to the no treatment group at 24 hr. time point. At 48 hr. post-treatment, densitometry analysis showed significantly increased levels of CtIP following ($p=0.0089$) quercetin compared to the no treatment group. These data shows quercetin can significantly elevate CtIP protein levels. This finding is similar to genistein that also promoted an increase in levels of CtIP 24 and 48 hr. post-treatment.

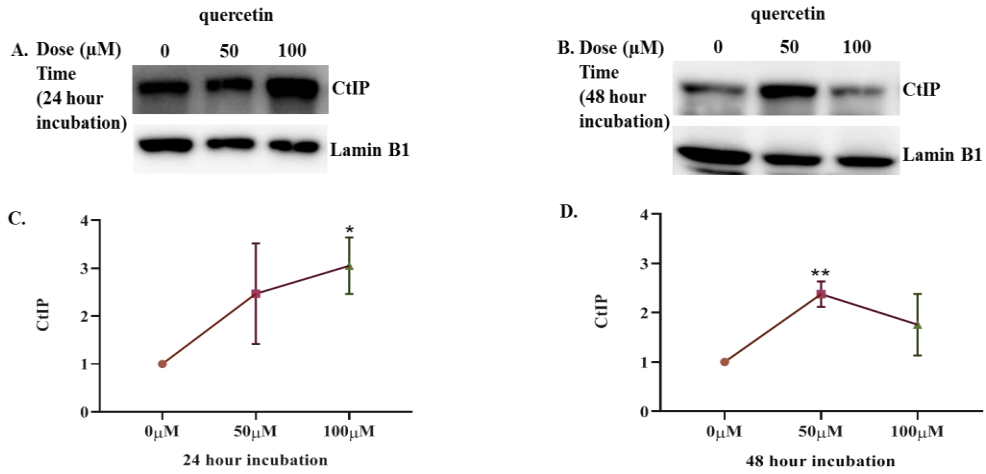


Figure 4.3.1: Quercetin promotes CtIP protein level of Alt-EJ pathway in MAG cells (A) Representative immunoblot showing elevated level of CtIP in mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of CtIP in mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of CtIP in 100 μM quercetin treated group at 24 hr. time point compared to 50 μM treated group and no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of CtIP in 50 μM quercetin treated group at 48 hr. time point compared to 100 μM treated group and no treatment group. (*p=0.0197,**p=0.0089)

MAG cells treated with quercetin also showed a significant increase in the levels of Pol θ compared to untreated MAG cells. Following treatment with quercetin, levels of Pol θ are significantly elevated at 24 hr. post-treatment and remain at the observed increased levels 48 hr. post-treatment compared to untreated cells. At 24 hr. post-treatment, densitometry analysis of immunoblots showed significantly increased levels of Pol θ following both 50 μM (p= 0.0157) and 100 μM (p=0.0196) quercetin compared to no treatment group. At 48 hr. post-treatment, densitometry analysis of immunoblots showed that Pol θ protein levels remained significantly increased following 100 μM (p=0.0244) quercetin compared to the no treatment group. However, at the lower dose of 50 μM quercetin, levels of Pol θ had returned to basal level (p=0.0914) compared to the no treatment group. These data show quercetin can significantly promote increased levels of

Pol θ protein level in MAG cells in a dose and time dependent manner similar to genistein that also promoted increased levels of Pol θ at 24 and 48 hr. post-treatment.

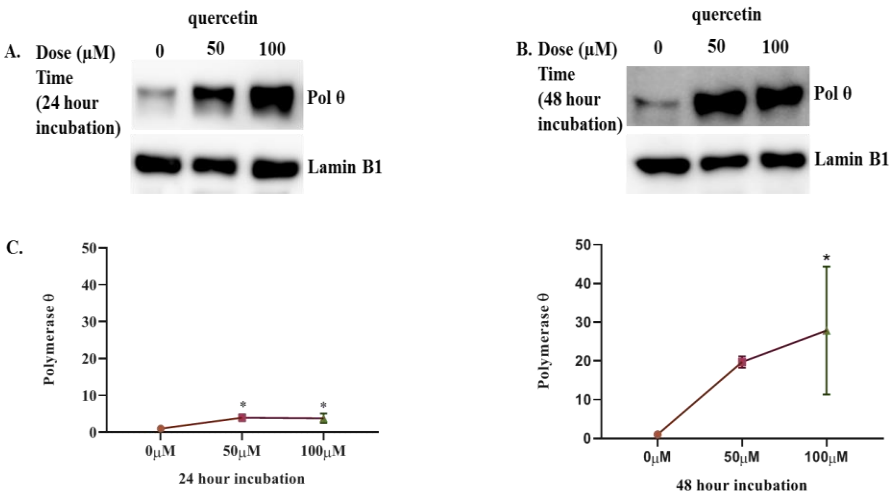


Figure 4.3.2: Quercetin promotes Pol θ protein level of Alt-EJ pathway in MAG cells (A) Representative immunoblot showing elevated level of Pol θ in mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of Pol θ in mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 50 and 100 μM quercetin treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 100 μM quercetin treated group at 48 hr. time point compared to no treatment group. (*p=0.0157,*p=0.0196, *p=0.0244)

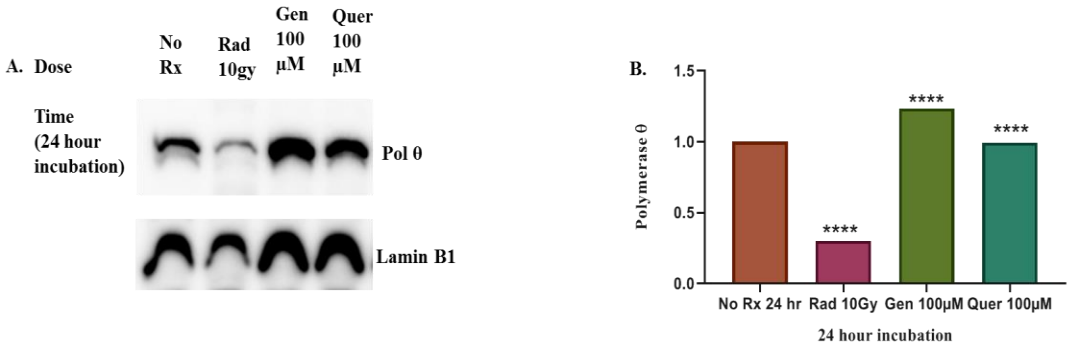


Figure 4.3.3: Effect of radiation on Pol θ protein level of Alt-EJ pathway in MAG cells (A) Representative immunoblot showing elevated level of Pol θ in mouse ES cell following treatment with genistein and quercetin compared to radiation treated group and no treatment group at 24 hr. time point. Immunoblot also shows level of Pol θ in mouse ES cell decreases following treatment with radiation compared to genistein treated, quercetin treated and no treatment group at 24 hr. time point. (B) Densitometry analysis of western blot showing significantly decreased levels of Pol θ in radiation treated MAG cells at 24 hr. post-treatment compared to untreated cells and also compared to cells treated with 100 µM genistein and 100 µM quercetin. (****p<0.0001)

Exposure to quercetin altered protein levels of Pol θ compared to the untreated group. By contrast, treatment of MAG cells with another DNA damaging agent radiation (10Gy) showed significantly ($p<0.0001$) decreased levels of Pol θ at 24hr post treatment compared to untreated cells, and also compared to cells treated with either genistein or quercetin. Although reports in the literature demonstrate that radiation can promote chromosomal translocations [95], my findings indicate specificity of different DNA damaging agents to influence levels of individual DNA repair pathway proteins. My data support the hypothesis that quercetin increases the amount of proteins involved in Alt-EJ repair.

4.4 Quercetin does not alter levels of the HR protein Rad51

Rad 51 is a well-regarded protein central to the process of HR. Literature suggests that NHEJ and HR are competing pathways of repair. In this set of experiments, I wanted to identify whether treatment with genistein can influence levels of Rad 51 protein to indicate

whether genistein globally impacts al DNA repair pathways, or if genistein specifically alters NHEJ.

MAG cells were treated with quercetin at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of Rad51were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

MAG cells treated with both 50 μM and 100 μM quercetin showed no significant changes in Rad51 protein levels between treated and untreated groups ($p = 0.99$) at both 24 and 48 hr. post-treatment. By contrast, MAG cells treated with 10Gy radiation (10 Gy) showed significantly elevated levels of Rad51 protein compared to both the genistein treated groups and untreated group ($p < 0.0001$) at 24 hr. post-treatment. Elevated levels of Rad51 indicate radiation-induced DNA damage has the potential to be repaired by HR; however, genistein more specifically alters proteins only within the NHEJ sub-pathways. These findings further support the specificity of cellular response to different DNA damaging agents.

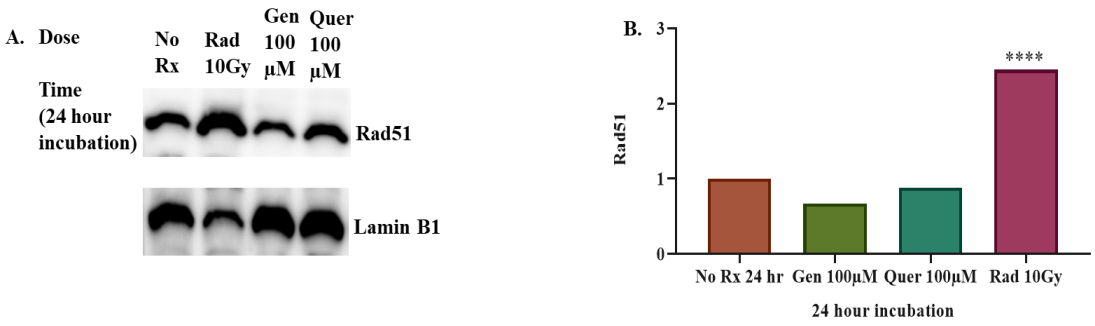
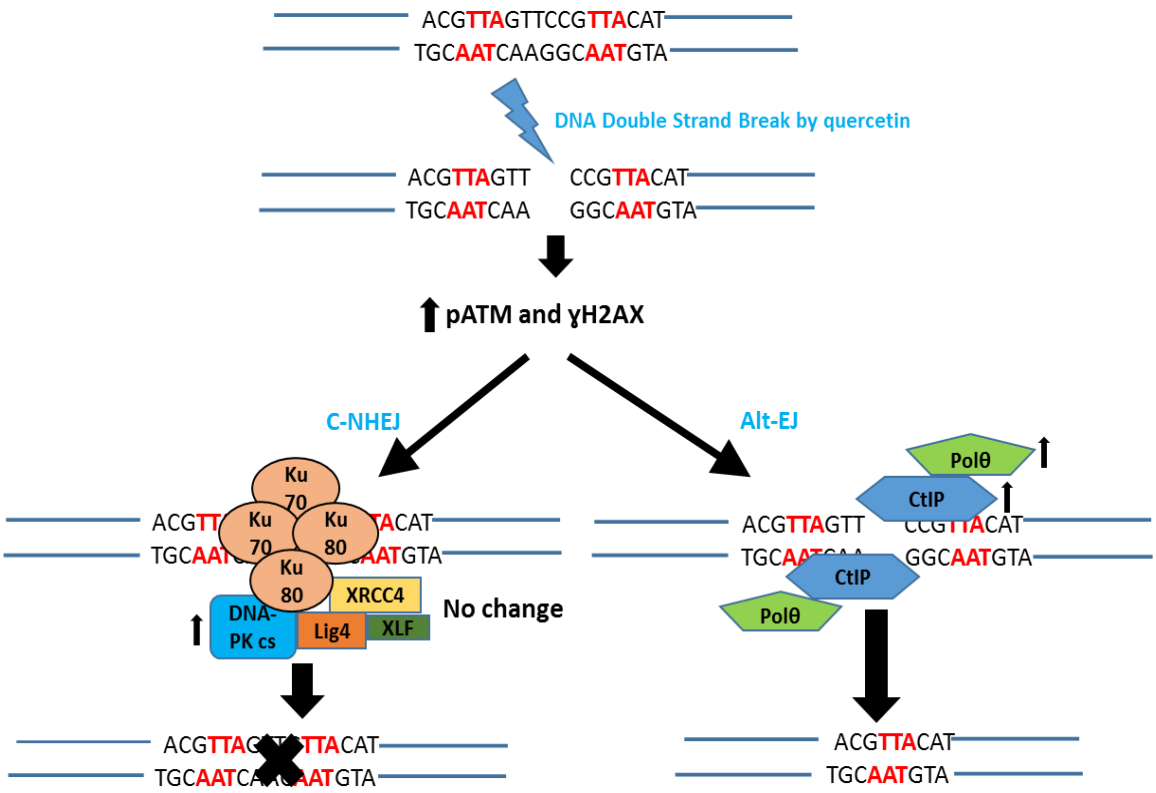


Figure 4.4.2: Effect of radiation on Rad51 protein level of HR pathway in MAG cells (A) Representative immunoblot showing elevated level of Rad51 in mouse ES cell following treatment with radiation compared to genistein and quercetin treated group and no treatment group at 24 hr. time point. Immunoblot also shows level of Rad51 in mouse ES cell shows no significant change following treatment with genistein, quercetin compared no treatment group at 24 hr. time point. (B) Densitometry analysis of western blot showing significantly increased levels of Rad51 in radiation treated MAG cells at 24 hr. post-treatment compared to untreated cells and also compared to cells treated with 100 µM genistein and 100 µM quercetin. (****p<0.0001)

4.5 Discussion and Conclusion



In this chapter I have described that quercetin can induce DNA DSBs and induce a DDR signaling pathway. Subsequently, I demonstrated that quercetin can promote increased Alt-EJ pathway protein levels. These findings are consistent with the hypothesis that quercetin can influence DNA repair pathway choice by its impact of protein levels involved in one specific pathway.

CHAPTER 5: Potential for genistein to induce DNA DSBs, DDR and to influence repair pathway choice in XLF (-) cells

Previous studies have shown that the potent TOPOII inhibitor etoposide can cause DNA DSBs, initiate DDR, and promote chromosomal translocations in mammalian cells [82]**.** Since the biochemical structure of genistein is similar to etoposide, it leads to the hypothesis that genistein can act as DNA damaging agent as well [77]. In Chapter 3 I demonstrated that exposure to genistein induces DNA DSBs and DDR in MAG cells. Those experiments also showed that exposure to genistein influences DNA repair pathway proteins that support the idea that genistein promotes repair by the Alt-NHEJ repair pathway.

XLF is an important component of C-NHEJ pathway. XLF, XRCC4 and DNA ligase 4 form a ligation complex which facilitates end stages of C-NHEJ pathway [72]. In this chapter I used CRISPR/Cas9-mediated XLF (-) MAG cells for my experiments (see Chapter 2 for details on generation of this cell line). I demonstrate that exposure to genistein in the absence of XLF can induce DNA DSBs and initiate DDR. These experiments also show that treatment with genistein in the absence of XLF further promotes levels of proteins involved in the Alt-NHEJ repair pathway, and influences DNA repair pathway choice and translocation frequency in XLF (-) MAG cells.

5.1 Genistein induces DNA DSBs and DDR in XLF (-) cells

In Chapter 3 I showed that both 50 and 100 µM genistein can induce DNA DSBs and initiate DDR signaling in parental MAG cells. In this set of experiments, I wanted to determine if treatment with genistein can initiate DNA DSBs and DDR in XLF (-) MAG cells by assessing the protein levels of γH2AX and pATM. Because XLF's activity is known to be primarily during the later stages of NHEJ repair, I hypothesized that loss of XLF would not inhibit DSB induction or the initiation of DDR.

XLF (-) MAG cells were treated with genistein at multiple doses (0, 50,100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 4 and 24 hr. post-treatment for Western blotting. DSBs were assessed by detecting levels of ɣH2AX (serine139) [5]. Initiation of DDR was assessed by detecting levels of pATM (serine1981) since previous studies have shown ATM is phosphorylated at s1981 in response to DNA DSBs by multiple DNA damaging agents including (please list some) [86]. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

XLF (-) MAG cells treated with genistein showed an increase in the level of ɣH2AX as an indication of DNA damage. ɣH2AX levels start to increase at 4 hr. and are significantly elevated 24 hr. post-treatment compared to untreated cells. Although slightly elevated amounts of γH2AX were observed at 4 hr. post-treatment, densitometry analysis of immunoblots did not show a significant difference. By 24 hr. post-treatment densitometry

analysis of immunoblots showed significantly elevated levels of γH2AX following 100 μM ($p=0.0325$) genistein compared to the no treatment group. These data show genistein can significantly DNA DSBs in XLF (-) MAG cells in a time and dose dependent manner.

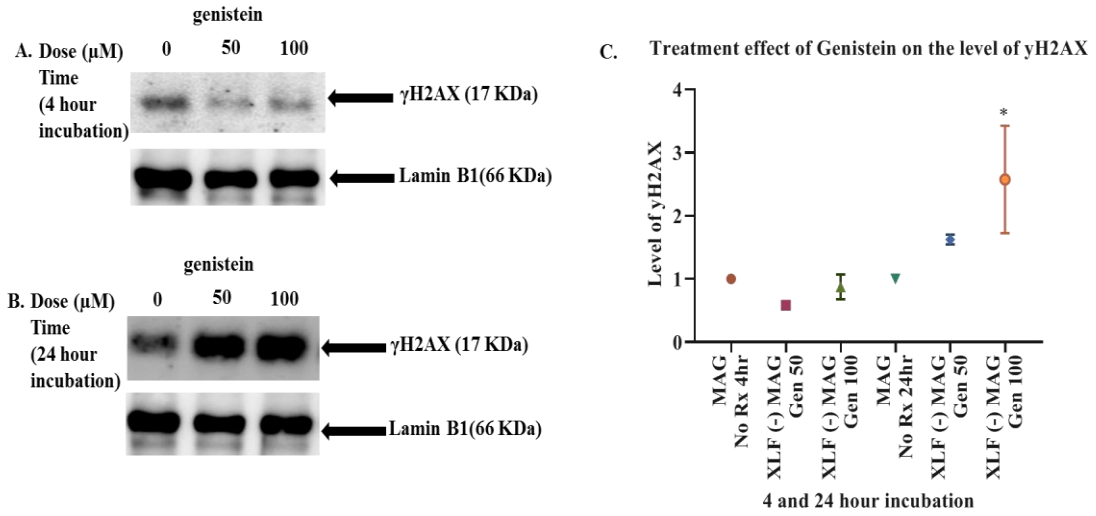


Figure 5.1.1: Genistein induces DNA DSBs in XLF(-) cells (A) Representative immunoblot showing level of γH2AX in XLF(-) mouse ES cell following treatment with genistein compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing elevated level of γH2AX in XLF(-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing level of γH2AX in 50 and 100 μM genistein treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of γH2AX in 100 μM genistein treated group at 24 hr. time point compared to no treatment group .(*p=0.0325)

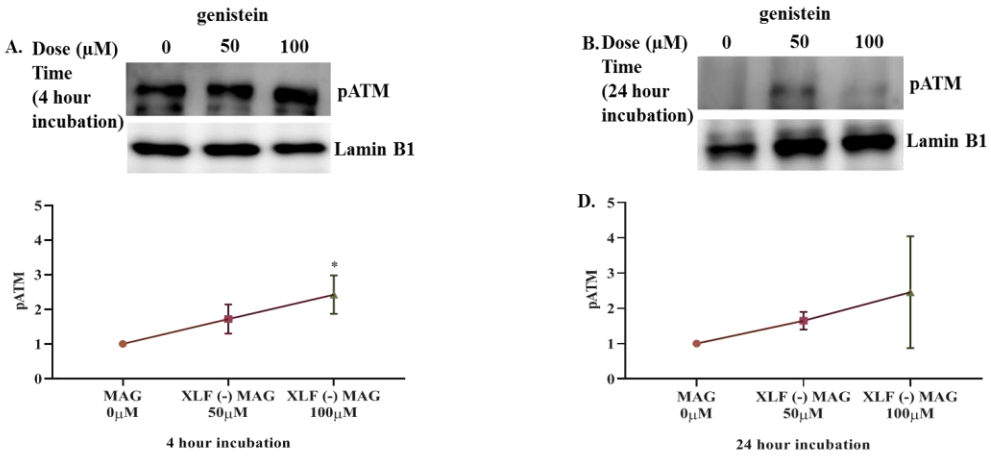


Figure 5.1.2: Genistein can generate DNA damage response in XLF(-) cells (A) Representative immunoblot showing elevated level of pATM in XLF(-) mouse ES cell following treatment with genistein compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing level of pATM in XLF (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of pATM in 100 μM genistein treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no significant change in the level of pATM in genistein treated group at 24 hr. time point compared to no treatment group.(*p=0.0116)

In XLF (-) MAG cells following treatment with genistein, pATM is detected by 4 hr. post-

treatment and remain elevated at 24 hr. post-treatment compared to untreated cells. At 4 hr. post-treatment densitometry analysis of immunoblots showed significantly elevated levels of pATM following 100 μM ($p=0.0116$) genistein compared to the no treatment group. At 24 hr. post-treatment although the pATM level showed trend towards elevation, no significant ($p= 0.1718$) difference is present between genistein treated and untreated group.

These data show genistein can significantly induce DDRs in XLF (-) MAG cells in a dose dependent manner. supporting the idea that XLF does not play a significant role in DSB induction or initiation of DDR following treatment with genistein since treatment with genistein produces similar effect in both parental MAG and XLF(-) MAG cells.

5.2 Genistein suppresses C-NHEJ pathway protein levels in XLF (-) cells

XLF, XRCC4 and DNA ligase 4 forms a ligation complex which facilitates the repair of DSBs by the C-NHEJ pathway [96]. Loss of XLF should impair the formation of a functional ligation complex for C-NHEJ. In Chapter 3 I demonstrated that exposure to genistein suppresses protein levels of both XLF and XRCC4 in parental MAG cells. In this set of experiments, I wanted to determine whether treatment of XLF (-) cells with genistein can further suppress levels of XRCC4.

XLF (-) MAG cells were treated with genistein at multiple doses (0, 50, 100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Levels of nuclear membrane protein Lamin B1 was used as a loading control. XRCC4 protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical

analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

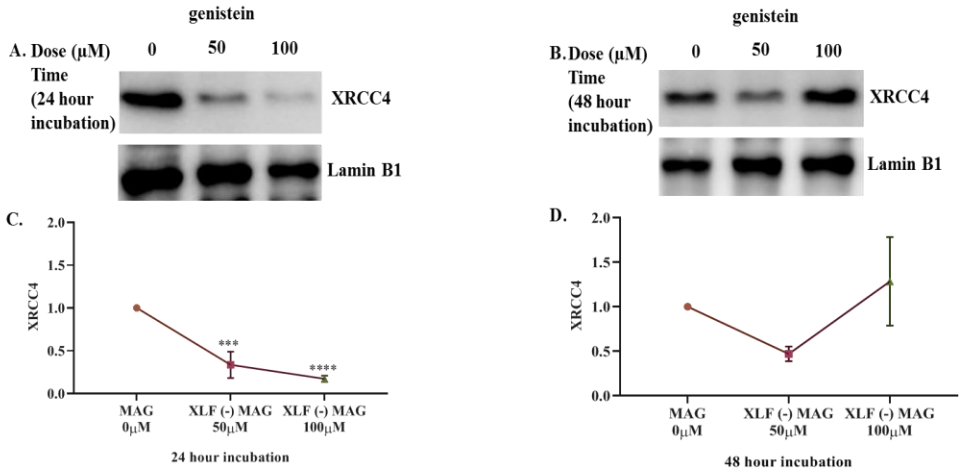


Figure 5.2.1: Genistein suppresses XRCC4 protein levels in XLF (-) cells (A) Representative immunoblot showing decreased level of XRCC4 in XLF(-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of XRCC4 in XLF(-) mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significant decrease in the level of XRCC4 in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group.(D) Densitometry analysis of western blot showing no significant change in the level of XRCC4 in 50 and 100 μM genistein treated group at 48 hr. time point compared to no treatment group. (***p=0.0002, ****p<0.0001)

Following treatment with genistein, the levels of XRCC4 decrease significantly following both 50 μM (p=0.0002) and 100 μM (p<0.0001) by 24 hr. post-treatment in XLF (-) MAG cells compared to the no treatment group. However, by 48 hr. post-treatment XRCC4 protein levels return to baseline and similar to the no treatment group. Similarly, in parental MAG cells following treatment with genistein XRCC4 decrease significantly by 24 hr. post-treatment compared to untreated group. It is notable that 100 μM genistein induced a larger decrease in of XRCC4 protein levels in XLF (-) MAG cells (p<0.0001) than in parental MAG cells (p=0.0013). These data support the idea that absence of XLF and simultaneous treatment with genistein can affect XRCC4 levels more adversely in XLF (-) MAG cells in comparison to genistein treated parental MAG cells.

5.3 Genistein promotes levels of DNA repair proteins of the Alt-EJ pathway in XLF (-) cells

Polθ and CtIP play important roles in promoting the Alt-EJ repair pathway [93]. Recent studies in mice indicated that Polθ is associated with micro homology mediated end joining (MMEJ) that is one form of Alt-EJ, leading to fusions of dysfunctional telomeres and chromosomal translocations [94]. Previous studies demonstrate that CtIP is a crucial factor for efficient chromosomal translocation formation by micro homology-prone Alt-EJ [89]. In Chapter 3 I demonstrated that exposure to genistein promotes a significant increase in protein levels of CtIP and Polθ in parental MAG cells. In this set of experiments, I wanted to identify whether treatment with genistein can influence level CtIP and Polθ in XLF (-) MAG cells in a time and dose dependent manner. Loss of XLF should impair the formation of a functional ligation complex for C-NHEJ and promote the use Alt-NHEJ for repair. Thus, I hypothesized that absence of XLF can result in a further increase in Alt-NHEJ protein levels.

XLF (-) MAG cells were treated with genistein at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of CtIP and Polθ were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

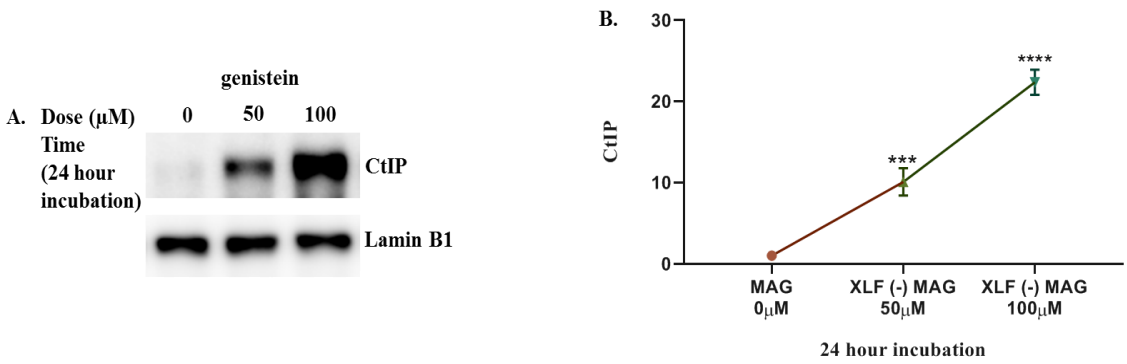


Figure 5.3.1: Genistein promotes levels of CtIP proteins of the Alt-EJ pathway in XLF (-) (A) Representative immunoblot showing elevated level of CtIP in XLF (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Densitometry analysis of western blot showing significantly elevated level of CtIP in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group. (***p=0.0003, ****p<0.0001)

XLF (-) MAG cells treated with genistein showed a significant increase in the levels of CtIP compared to untreated cells. In genistein treated XLF (-) MAG cells CtIP levels are significantly elevated by 24 hr post treatment compared to untreated cells. Densitometry analysis of immunoblots showed significantly increased levels of CtIP following both 50 μM ($p=0.0003$) and 100 μM ($p<0.0001$) genistein compared to the no treatment group. These data show genistein significantly increases CtIP protein levels in XLF (-) MAG cells. These results are similar to results observed in genistein treated parental MAG cells. It is notable that 100 μM genistein induced a larger increase in of CtIP protein levels in XLF (-) MAG cells ($p<0.0001$) than in parental MAG cells ($p=0.0348$) consistent with my hypothesis that absence of the C-NHEJ protein XLF can result in a further increase in Alt-NHEJ protein levels.

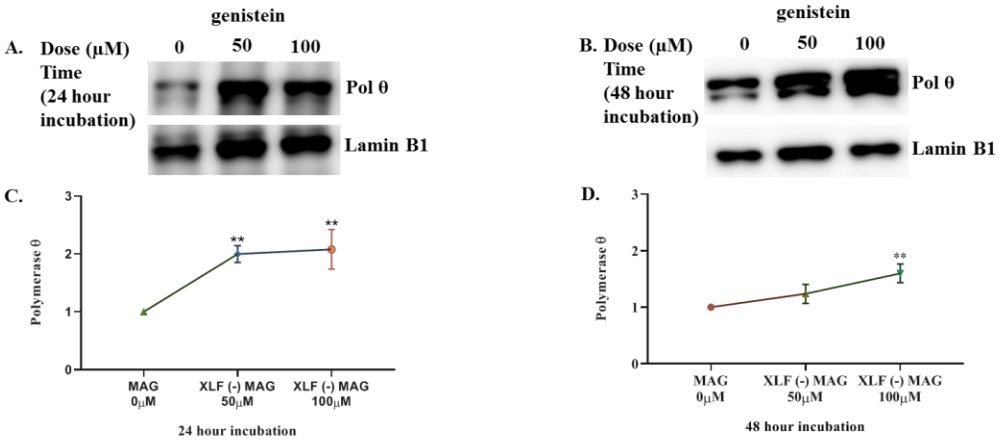


Figure 5.3.2: Genistein promotes levels of Pol θ proteins of the Alt-EJ pathway in XLF (-) cells (A) Representative immunoblot showing elevated level of Pol θ in XLF (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing elevated level of Pol θ in XLF(-) mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 100 μM genistein treated group at 48 hr. time point compared to no treatment group. (**p=0.0022,**p=0.0015, **p=0.0031)

XLF (-) MAG cells treated with genistein showed an increase in the levels of Pol θ compared to untreated cells. In genistein treated XLF (-) MAG cells Pol θ protein levels are significantly elevated by 24 hr. post-treatment and remain elevated 48-hr post treatment compared to untreated MAG cells. Densitometry analysis of immunoblots showed significantly increased levels of Pol θ following 50 μM (p=0.0022) and 100 μM (p=0.0015) genistein compared to the no treatment group. At 48 hr. post-treatment group densitometry analysis shows Pol θ protein levels remain significantly increased following 100 μM (p=0.0031) genistein compared to the no treatment group. These data show genistein can significantly affect Pol θ protein level in XLF (-) MAG cells and are similar to results observed in genistein treated parental MAG cells. It is notable that 100 μM genistein, induced a larger increase of Pol θ levels in XLF (-) MAG cells (p=0.0015) than in parental MAG cells (p=0.0292) consistent with my hypothesis that absence of the C-NHEJ protein XLF can result in a further increase in Alt-NHEJ protein levels.

5.4 Genistein suppresses levels of the HR protein Rad51 in XLF (-) cells

Rad 51 is a well-regarded protein central to the process of HR. Literature suggests that NHEJ and HR are competing pathways of repair. In this set of experiments, I wanted to identify whether treatment of XLF (-) cells with genistein can influence levels of Rad 51 protein to indicate whether genistein globally impacts al DNA repair pathways, or if genistein specifically alters NHEJ.

XLF (-) MAG cells were treated with genistein at multiple doses (0, 50,100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of Rad51were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

XLF (-) MAG cells treated with 50 µM or 100 µM genistein show no significant difference between treated and untreated group ($p = 0.99$) by 24 hr post-treatment. However, XLF (-) MAG cells treated with 50 µM ($p=0.0365$) and 100 µM ($p=0.0006$) genistein show a significant decrease in the level of Rad51 by 48 hr post-treatment compared to untreated group. It is notable that parental MAG cells showed no change in

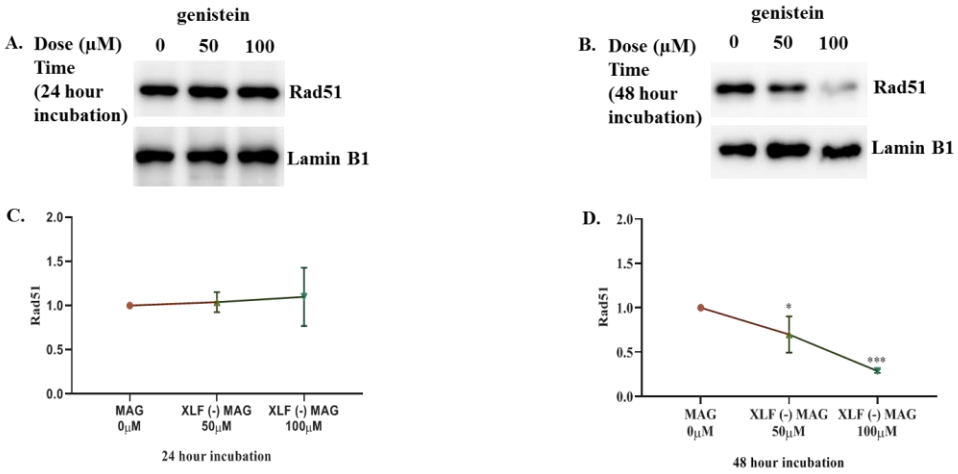


Figure 5.4.1: Genistein suppresses levels of the HR protein Rad51 in XLF (-) cells (A) Representative immunoblot showing no change in the level of Rad51 in XLF (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing decreased level of Rad51 in XLF (-) mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing no significant change in the level of Rad51 in genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly decreased level of Rad51 in genistein treated group at 48 hr. time point compared to no treatment group. (***p=0.0006, *p=0.0365)

level of Rad51 at 50 and 100 μM and at 24, 48-hour time points. This result suggest absence of XLF may have some negative effect on Rad51 protein and HR.

Additionally, parental MAG cells treated with 10Gy radiation leads to significantly elevated levels of Rad 51 compared to 100 μM genistein treated XLF (-) MAG cells and untreated cells ($p < 0.0001$) at 24-hour post treatment. This elevated Rad51 indicate radiation induced DNA damage has a propensity to be repaired by HR compared to genistein treated DNA damage. My findings indicate specificity of different DNA damaging agents to influence levels of individual DNA repair pathway proteins.

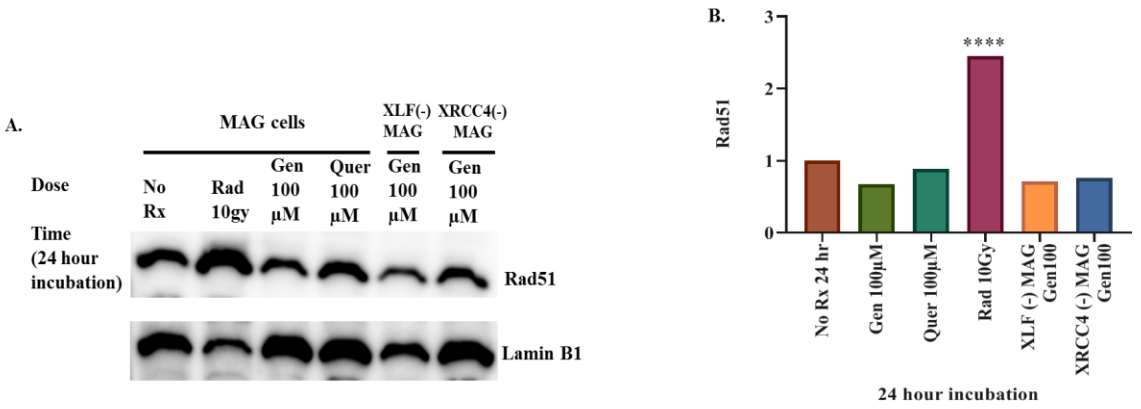


Figure 5.4.2: Effect of radiation on Rad51 protein level of HR pathway in MAG cells (A) Representative immunoblot showing elevated level of Rad51 in mouse ES cell following treatment with radiation compared to genistein and quercetin treated group and no treatment group at 24 hr. time point. Immunoblot also shows level of Rad51 in mouse ES cell does not change following treatment with genistein, quercetin compared no treatment group at 24 hr. time point. (B) Densitometry analysis of western blot showing significantly increased levels of Rad51 in radiation treated MAG cells at 24 hr. post-treatment compared to untreated cells and also compared to cells treated with 100 µM genistein and 100 µM quercetin. (****p<0.0001)

5.5 Genistein promotes an increased number of chromosomal translocations in XLF (-) cells

Previous studies from Richardson Lab showed MAG cells treated with genistein can promote the formation of chromosomal translocations during DSB repair between the MLL and AF9 bcr transgene reporter constructs in the parental MAG cells. Detection and quantification of translocations is possible since a translocation brings together engineered exons 1 and 2 of a Green Fluorescent Protein gene (GFP) onto a single DNA helix thus allowing for identification by expression of the green fluorescent protein by inverted microscopy by 96 hr. post-treatment [77].

Loss of XLF should impair the formation of a functional ligation complex for C-NHEJ and promote the use Alt-NHEJ for repair. I demonstrated that in the absence of XLF genistein treatment promotes elevated levels of proteins involved in the Alt-NHEJ repair pathway.

In this set of experiments, I wanted to determine if in the absence of XLF genistein treatment also promotes an increase in the number of detectable chromosomal translocations in the XLF (-) MAG cells and compared to parental MAG cells [77].

After treatment of parental MAG and XLF (-) MAG cells with genistein at different doses (0, 50 and 100 µM), the cells were maintained at 37 °C, 5% CO2 and were examined every 24 hr. for GFP expression by an inverted microscopy. By 96 hr. post-treatment GFP+ colonies were detectable. Untreated parental MAG and untreated XLF (-) MAG cells were used as negative controls.

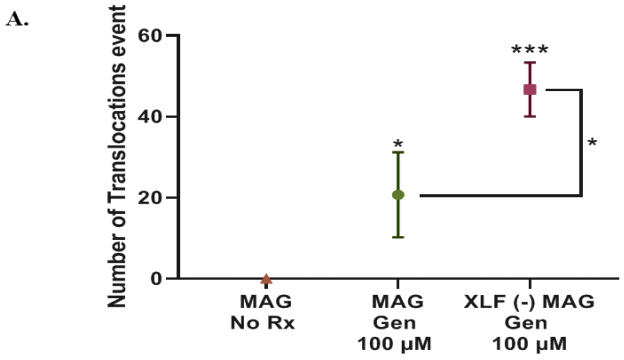
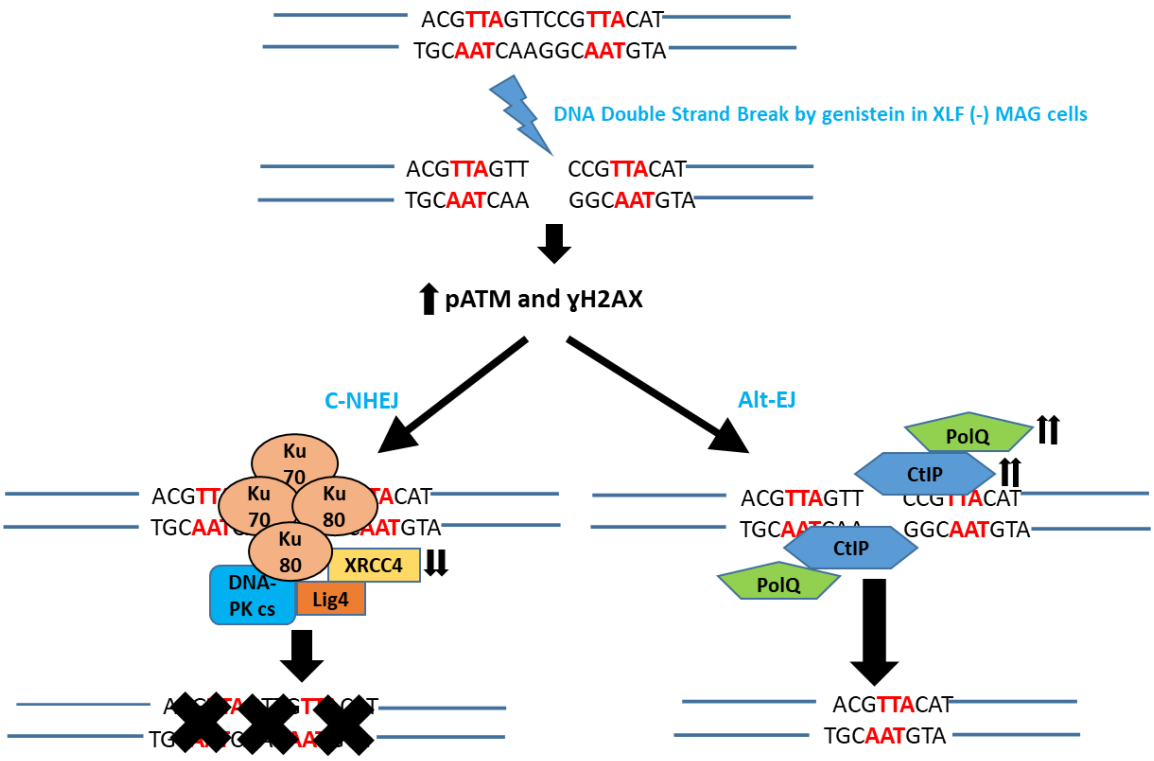


Figure 5.5.1: Genistein promotes an increased number of chromosomal translocations in XLF (-) cells
(A) Analysis of number of GFP positive colony showing significantly elevated level of translocation frequency in 100 µM genistein treated XLF (-) MAG group compared to 100 µM genistein treated MAG group and no treatment group. (***p=0.0005, *p=0.0310, *p=0.0105)

MAG cells treated with genistein 100 µM (p=0.0310) showed significantly increased number of GFP positive colonies indicating high translocation frequency compared to untreated MAG cells. Similarly, XLF (-) MAG cells treated with genistein 100 µM (p=0.0005) showed significantly increased translocation frequency compared to untreated

MAG and XLF (-) MAG cells. In comparison to genistein treated MAG cells XLF (-) MAG cells showed significantly increased translocation frequency at 100 µM (p=0.0105) dosage confirming my hypothesis that XLF (-) MAG cells are more susceptible to chromosomal translocation event following exposure to genistein.

5.6 Discussion and Conclusion



In this chapter I described that genistein can induce DNA DSBs, initiate DDR and can influence DNA repair protein levels in XLF (-) MAG cells. My data support the hypothesis that genistein promotes Alt-EJ repair. Moreover, absence of the XLF protein involved in C-NHEJ pathway drives the repair more robustly towards Alt-EJ pathway over both C-NHEJ and HR.

CHAPTER 6: Potential for quercetin to induce DNA DSBs, DDR and to influence repair pathway choice in XLF (-) cells

Previous studies have shown that the potent TOPOII inhibitor etoposide can cause DNA DSBs, initiate DDR, and promote chromosomal translocations in mammalian cells [82]**.** Since the biochemical structure of quercetin is similar to etoposide, it leads to the hypothesis that quercetin can act as DNA damaging agent as well [77]. In Chapter 3 I demonstrated that exposure to quercetin induces DNA DSBs and DDR in MAG cells. Those experiments also showed that exposure to quercetin influences DNA repair pathway protein levels that support the idea that quercetin promotes repair by the Alt-NHEJ repair pathway.

XLF is an important component of C-NHEJ pathway. XLF, XRCC4 and DNA ligase 4 form a ligation complex which facilitates end stages of C-NHEJ pathway [72]. In this chapter I used CRISPR/Cas9-mediated XLF (-) MAG cells for my experiments (see Chapter 2 for details on generation of this cell line). I demonstrate that exposure to quercetin in the absence of XLF can induce DNA DSBs and initiate DDR. These experiments also show that treatment with quercetin in the absence of XLF further promotes levels of proteins involved in the Alt-NHEJ repair pathway, and influences DNA repair pathway choice and promotes the formation of chromosomal translocations in XLF (-) MAG cells

6.1 Quercetin induces DNA DSBs and DDR in XLF (-) cells

In Chapter 3 I showed that both 50 and 100 µM quercetin can induce DNA DSBs and initiate DDR signaling in parental MAG cells. In this set of experiments, I wanted to determine if treatment with quercetin can initiate DNA DSBs and DDR in XLF (-) MAG cells by assessing the protein levels of γH2AX and pATM. Because XLF's activity is known to be primarily during the later stages of NHEJ repair, I hypothesized that loss of XLF would not inhibit DSB induction or the initiation of DDR.

XLF (-) MAG cells were treated with quercetin at multiple doses (0, 50,100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 4 and 24 hr. post-treatment for Western blotting. DSBs were assessed by detecting levels of ɣH2AX (serine139) [5]. Initiation of DDR was assessed by detecting levels of pATM (serine1981) since previous studies have shown ATM is phosphorylated at s1981 in response to DNA DSBs by multiple DNA damaging agents including ionizing radiation, chemotherapeutic agent such as TOPO II inhibitor and various chemicals [86]. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

XLF (-) MAG cells treated with quercetin showed an increase in the level of ɣH2AX as an indication of DNA damage. ɣH2AX levels start to increase at 4 hr. post-treatment; Densitometry analysis of immunoblots showed significantly elevated levels of γH2AX induced by 100 µM ($p=0.0038$) quercetin compared to the 50 µM quercetin treated group

and no treatment group. The levels of γH2AX come back to basal level at 24 hr. post-treatment. Densitometry analysis shows no significant change in the levels of γH2AX in both 50 µM (p=0.6180) and 100 µM (p=0.4422) quercetin treated groups compared to no treatment group at 24 hr. time point. . The short time to detect the DSBs supports the idea that quercetin directly, rather than indirectly, induces the DSBs detected.

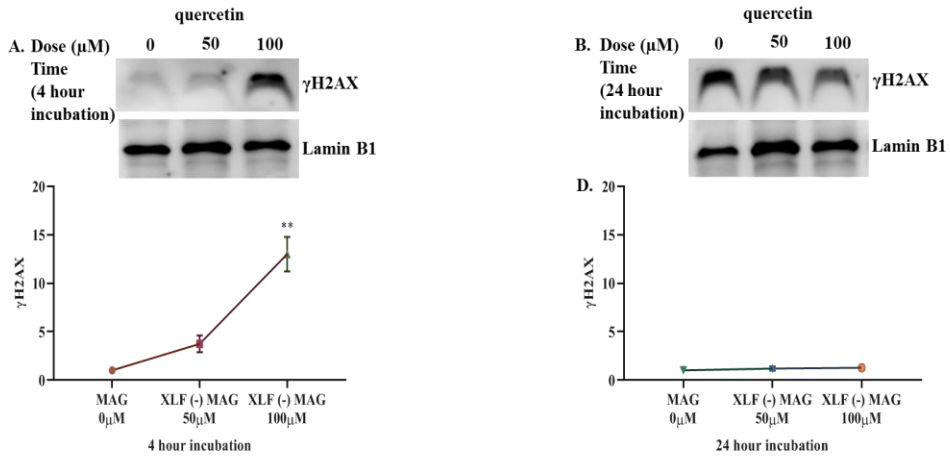


Figure 6.1.1: Quercetin induces DNA DSBs in XLF (-) cells (A) Representative immunoblot showing elevated level of γH2AX in XLF (-) mouse ES cell following treatment with quercetin compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing level of γH2AX in XLF (-) mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of γH2AX in 100 µM quercetin treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot also shows level of γH2AX in 50 and 100 µM quercetin treated group at 24 hr. time point compared to no treatment group with no significant change. (**p=0.0038)

In XLF (-) MAG cells following treatment with quercetin pATM is detected by 4 hr. post-treatment and its level increases significantly by 24 hr. post-treatment compared to the untreated group. At 24 hr. post-treatment densitometry analysis of immunoblots show significantly elevated level of pATM in 50 µM (p=0.0037) and 100 µM (p=0.0010) quercetin treated group compared to the no treatment group. These data shows quercetin can significantly induce DDR signaling in MAG cells in a time and dose dependent manner.

These data show quercetin can significantly induce DDRs in XLF (-) MAG cells supporting the idea that XLF does not play a significant role in DSB induction or initiation of DDR

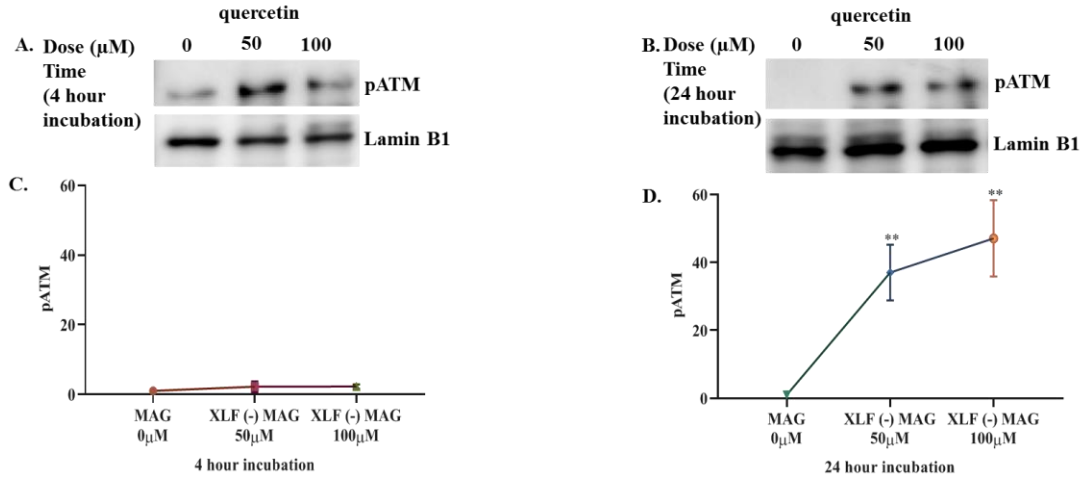


Figure 6.1.2: Quercetin induces DDR in XLF (-) cells (A) Representative immunoblot showing level of pATM in XLF (-) mouse ES cell following treatment with quercetin compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing elevated level of pATM in XLF (-) mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing no significant change in level of pATM in 50 and 100 μM quercetin treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of pATM in 50 and 100 μM quercetin treated group at 24 hr. time point compared to no treatment group.(**p=0.0037,**p=0.0010)

following treatment with quercetin since treatment with quercetin produces similar effect in both parental MAG and XLF(-) MAG cells.

6.2 Quercetin does not alter C-NHEJ pathway protein levels in XLF (-) cells

XLF, XRCC4 and DNA ligase 4 forms a ligation complex which facilitates the repair of DSBs by the C-NHEJ pathway [96]. Loss of XLF should impair the formation of a functional ligation complex for C-NHEJ. In Chapter 4 I demonstrated that exposure to quercetin suppresses protein levels of both XLF and XRCC4 in parental MAG cells. In this set of experiments, I wanted to determine whether treatment of XLF (-) cells with quercetin can further suppress levels of XRCC4.

XLF (-) MAG cells were treated with quercetin at multiple doses (0, 50, 100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Levels of nuclear membrane protein Lamin B1 was used as a loading control. XRCC4 protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

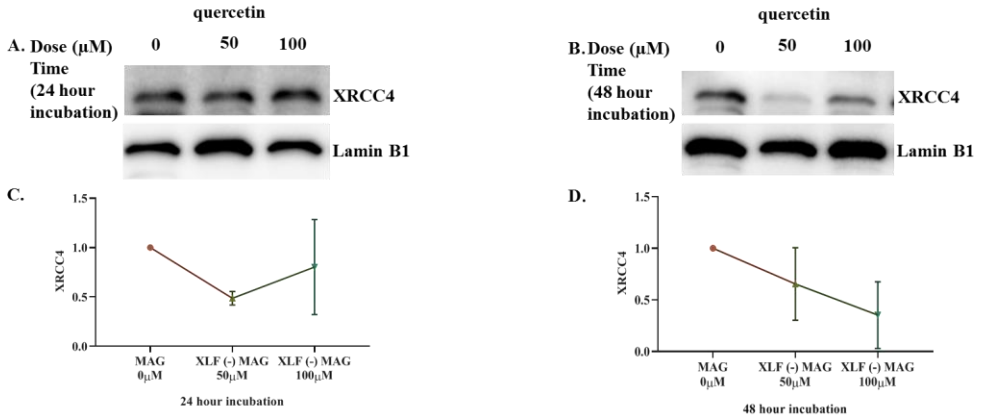


Figure 6.2.1: Quercetin does not alter XRCC4 protein levels in XLF (-) cells (A) Representative immunoblot showing level of XRCC4 in XLF(-) mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing level of XRCC4 in XLF(-) mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing no significant change in the level of XRCC4 in 50 and 100 µM quercetin treated group at 24 hr. time point compared to no treatment group.(D) Densitometry analysis of western blot showing no significant change in the level of XRCC4 in 50 and 100 µM quercetin treated group at 48 hr. time point compared to no treatment group.(p=0.1578, p=0.0741)

Following treatment with 100uM quercetin, the levels of XRCC4 were decreased slightly by 24 and 48 hr. post treatment in XLF (-) MAG cells compared to no treatment group although not to a significant level (p=0.0741) compared to untreated cells. These results contrast to detected protein levels following exposure to genistein that suppressed levels of C-NHEJ proteins in XLF (-) cells.

6.3 Quercetin promotes levels of DNA repair proteins of the Alt-EJ pathway in XLF (-) cells

Polθ and CtIP play important roles in promoting the Alt-EJ repair pathway [93]. Recent studies in mice indicated that Polθ is associated with micro homology mediated end joining (MMEJ) that is one form of Alt-EJ, leading to fusions of dysfunctional telomeres and chromosomal translocations [94]. Previous studies demonstrate that CtIP is a crucial factor for efficient chromosomal translocation formation by micro homology-prone Alt-EJ [89]. In Chapter 4 I demonstrated that exposure to quercetin promotes a significant increase in protein levels of CtIP and Polθ in parental MAG cells. In this set of experiments, I wanted to identify whether treatment with quercetin can influence levels CtIP and Polθ in XLF (-) MAG cells in a time and dose dependent manner. Loss of XLF should impair the formation of a functional ligation complex for C-NHEJ and promote the use Alt-NHEJ for repair. Thus, I hypothesized that absence of XLF can result in a further increase in Alt-NHEJ protein levels.

XLF (-) MAG cells were treated with quercetin at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of CtIP and Polθ were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

XLF (-) MAG cells treated with quercetin showed a significant increase in the levels of CtIP compared to untreated cells. In quercetin treated XLF (-) MAG cells CtIP levels are

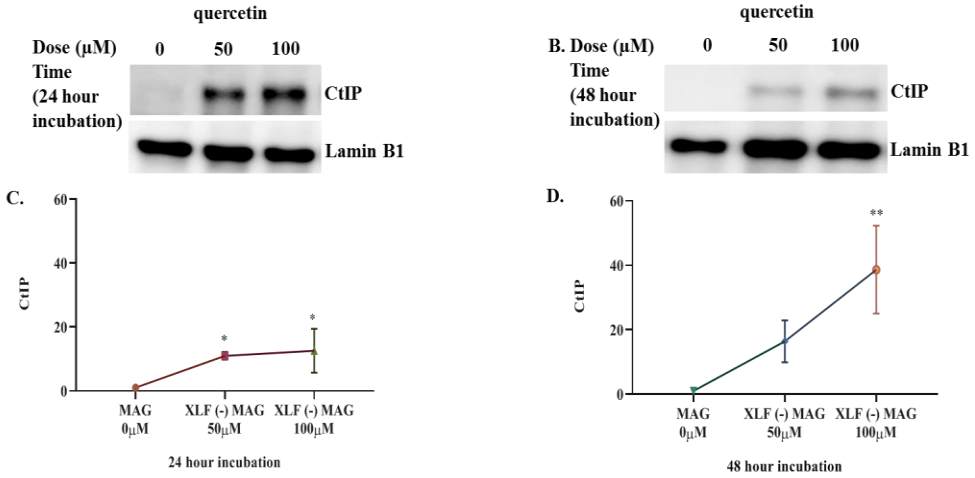


Figure 6.3.1: Quercetin promotes levels of CtIP proteins of the Alt-EJ pathway in XLF (-) cells (A) Representative immunoblot showing elevated level of CtIP in mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing elevated level of CtIP in mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of CtIP in 50 and 100 µM quercetin treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of CtIP in 100 µM quercetin treated group at 48 hr. time point compared to 50 µM treated and no treatment group. (*p=0.0396,*p=0.0219,**p=0.0044)

significantly elevated by 24 hr. and remain elevated 48 hr. post-treatment compared to untreated cells. Densitometry analysis of immunoblots showed significantly increased levels of CtIP following 50µM (p=0.0396) and 100 µM (p=0.0219) quercetin treatment at 24 hr. time point compared to the no treatment group. By 48 hr. post-treatment densitometry analysis showed CtIP level remain significantly increased in 100 µM (p=0.0044) quercetin treated group in comparison to untreated group. These results are similar to results observed in genistein treated parental MAG cells. These data show quercetin significantly increases CtIP protein levels in XLF (-) MAG cells consistent with my hypothesis that absence of the C-NHEJ protein XLF can result in a further increase in Alt-NHEJ protein levels.

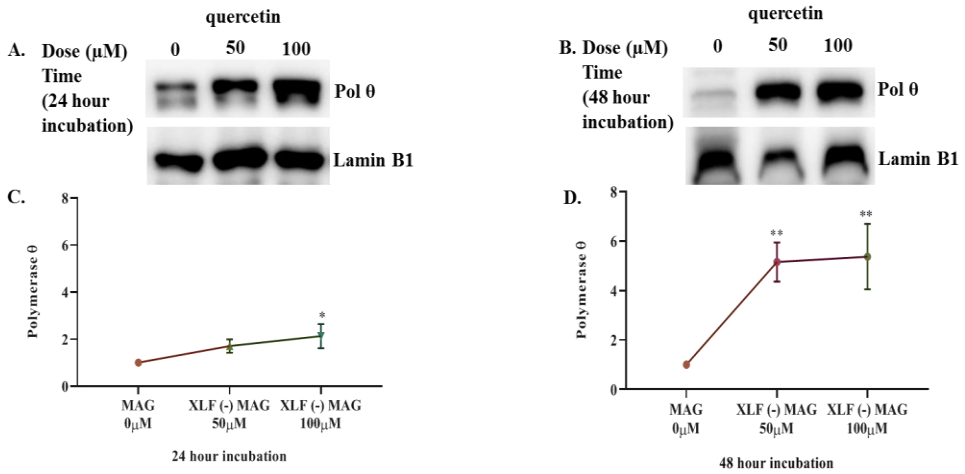


Figure 6.3.2: Quercetin promotes levels of Pol θ proteins of the Alt-EJ pathway in XLF (-) cells (A) Representative immunoblot showing elevated level of Pol θ in XLF (-) mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing elevated level of Pol θ in XLF(-) mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 100 µM quercetin treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of Pol θ in both 50 and 100 µM quercetin treated group at 48 hr. time point compared to no treatment group. (*p=0.0118,**p=0.0022, **p=0.0017)

XLF (-) MAG cells treated with quercetin showed an increase in the levels of Pol θ compared to untreated cells. In quercetin treated XLF (-) MAG cells Pol θ protein levels are significantly elevated by 24 hr. post-treatment and remain elevated 48-hr post treatment compared to untreated MAG cells. Densitometry analysis of immunoblots 24 hr. post-treatment showed significantly increased levels of Pol θ following 100 µM (p=0.0118) quercetin compared to the no treatment group. At 48 hr. post-treatment group densitometry analysis shows Pol θ protein levels remain significantly increased following both 50 µM (p=0.0022) and 100 µM (p=0.0017) quercetin compared to the no treatment group. These results are similar to results observed in quercetin treated parental MAG cells. It is notable that 100 µM quercetin induced a larger increase in of Pol θ protein levels in XLF (-) MAG cells (p=0.0017) than in parental MAG cells (p=0.0244) at 48-hour time point. These data show quercetin can significantly affect Pol θ protein level in XLF (-) MAG cells and are

consistent with my hypothesis that absence of the C-NHEJ protein XLF can result in a further increase in Alt-NHEJ protein levels.

6.4 Quercetin suppresses levels of the HR protein Rad51 in XLF (-) cells

Rad 51 is a well-regarded protein central to the process of HR. Literature suggests that NHEJ and HR are competing pathways of repair. In this set of experiments, I wanted to identify whether treatment of XLF (-) cells with quercetin can influence levels of Rad 51 protein to indicate whether quercetin globally impacts al DNA repair pathways, or if quercetin specifically alters NHEJ.

XLF (-) MAG cells were treated with quercetin at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of Rad51were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

XLF (-) MAG cells treated with 50 μM quercetin showed significantly ($p=0.0314$) decreased level of Rad51 protein level compared to 100 μM treated group and untreated group by 24 hr. post-treatment. At 48 hr. post treatment XLF (-) MAG cells treated with 100 μM genistein show a significant ($p=0.0251$) decrease in the level of Rad51 compared to 50 μM treated and untreated group. It is notable that parental MAG cells showed no

change in level of Rad51 at 50 and 100 µM quercetin treatment and at 24, 48-hour time points. This result suggest absence of XLF may have some negative effect on Rad51 protein and HR.

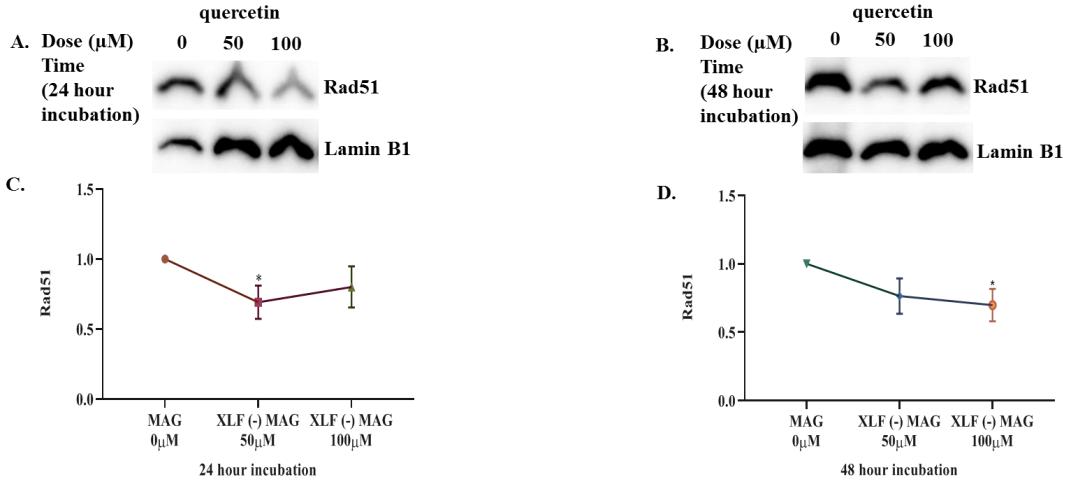


Figure 6.4.1: Quercetin suppresses levels of the HR protein Rad51 in XLF (-) cells (A) Representative immunoblot showing change in the level of Rad51 in XLF (-) mouse ES cell following treatment with quercetin compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing decreased level of Rad51 in mouse ES cell following treatment with quercetin compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly decreased level of Rad51 in 50 µM quercetin treated group at 24 hr. time point compared to no treatment group.(D) Densitometry analysis of western blot showing significantly decreased level of Rad51 in 100 µM quercetin treated group at 48 hr. time point compared to no treatment group (*p=0.0314,*p=0.0251)

6.5 Quercetin promotes an increased number of chromosomal translocations in XLF (-) cells

Previous studies from Richardson Lab showed MAG cells treated with quercetin can promote the formation of chromosomal translocations during DSB repair between the MLL and AF9 bcr transgene reporter constructs in the parental MAG cells. Detection and quantification of translocations is possible since a translocation brings together engineered exons 1 and 2 of a Green Fluorescent Protein gene (GFP) onto a single DNA helix thus

allowing for identification by expression of the green fluorescent protein by inverted microscopy by 96 hr. post-treatment [77].

Loss of XLF should impair the formation of a functional ligation complex for C-NHEJ and promote the use Alt-NHEJ for repair. I demonstrated that in the absence of XLF quercetin treatment promotes elevated levels of proteins involved in the Alt-NHEJ repair pathway. In this set of experiments, I wanted to determine if in the absence of XLF, quercetin treatment also promotes an increase in the number of detectable chromosomal translocations in the XLF (-) MAG cells and compared to parental MAG cells [77].

After treatment of parental MAG and XLF (-) MAG cells with quercetin at different doses (0, 50 and 100 µM), the cells were maintained at 37 °C, 5% CO2 and were examined every 24 hr. for GFP expression by an inverted microscopy. By 96 hr. post-treatment GFP+ colonies were detectable. Untreated parental MAG and untreated XLF (-) MAG cells were used as negative controls.

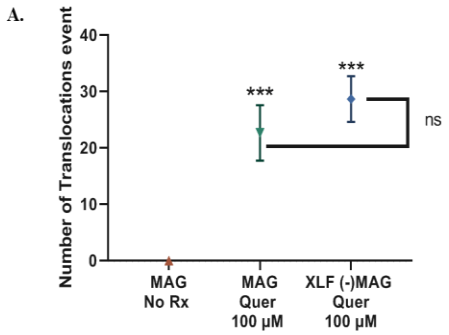
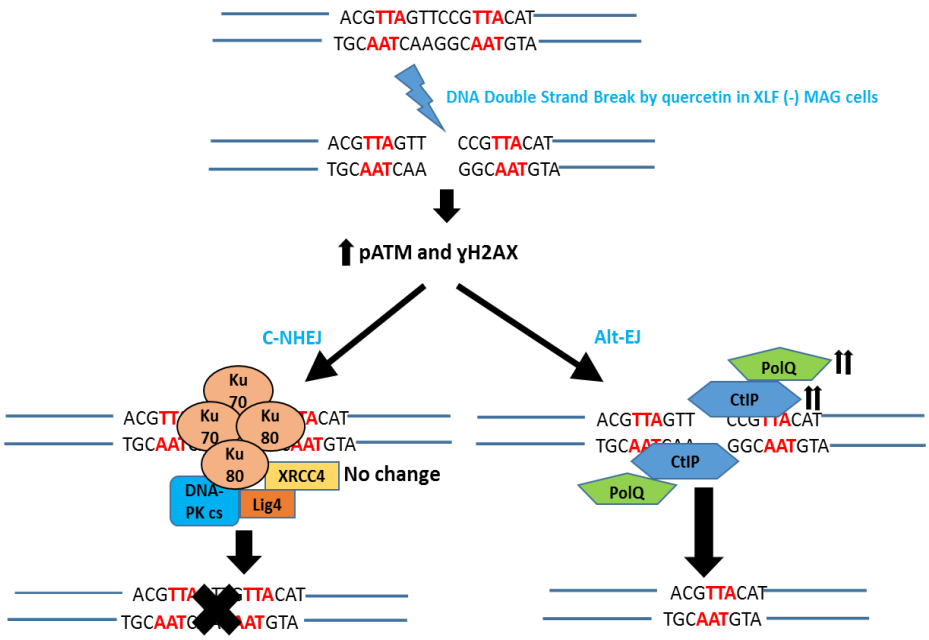


Figure 6.5.1: Quercetin promotes an increased number of chromosomal translocations in XLF (-) cells
(A) Analysis of number of GFP positive colony showing significantly elevated level of translocation frequency in 100 µM quercetin treated MAG group and 100 µM XLF (-) MAG group compared to no treatment group. However translocation frequency between 100µM quercetin treated MAG and 100µM quercetin treated XLF (-) MAG are not statistically significant. (***p=0.0007, ***p=0.0002).

MAG cells treated with quercetin 100 µM (p=0.0007) showed significantly increased translocation frequency compared to untreated MAG cells. Similarly, XLF (-) MAG cells treated with genistein 100 µM (p=0.0002) showed significantly increased translocation frequency compared to untreated MAG and XLF (-) MAG cells. However, in comparison to quercetin treated MAG cells XLF (-) MAG cells doesn't show any significantly increased translocation frequency at 100 µM dosage. Though not statistically significant but the upward trend of increasing translocation frequency in XLF (-) quercetin treated cells confirms my hypothesis that XLF (-) MAG cells are more susceptible to chromosomal translocation event following exposure to quercetin.

6.6 Discussion and Conclusion



In this chapter I have described in XLF (-) MAG cells quercetin can induce DSBs and initiate DDR. In addition the absence of XLF protein promotes Alt-EJ DNA repair pathway choice by altering the level of proteins involved. Moreover, blocking of C-NHEJ repair pathway by elimination of XLF pathway drives the repair process more towards Alt-EJ pathway which becomes evident by an increased number of chromosomal translocations.

CHAPTER 7: Potential for genistein to induce DNA DSBs, DDR and to influence repair pathway choice in XRCC4 (-) cells

Previous studies have shown that the potent TOPOII inhibitor etoposide can cause DNA DSBs, initiate DDR, and promote chromosomal translocations in mammalian cells [82]. Since the biochemical structure of genistein is similar to etoposide, it leads to the hypothesis that genistein can act as DNA damaging agent as well [77]. In Chapter 3 I

demonstrated that exposure to genistein induces DNA DSBs and DDR in MAG cells. Those experiments also showed that exposure to genistein influences DNA repair pathway proteins that support the idea that genistein promotes repair by the Alt-NHEJ repair pathway.

XLF, XRCC4 and DNA ligase 4 form a ligation complex which facilitates end stages of C-NHEJ pathway [72]. In Chapter 5 I demonstrated that absence of XLF additionally augments the levels of proteins that facilitate Alt-NHEJ. In this chapter I used CRISPR/Cas9-mediated XRCC4 (-) MAG cells for my experiments (see Chapter 2 for details on generation of this cell line). Since both XLF and XRCC4 are part of the same complex for C-NHEJ these experiments would show if the effects observed in XLF (-) cells are more likely to be due to loss of a cohesive functional complex or instead specific to XLF alone and its activity. I demonstrate that exposure to genistein in the absence of XRCC4 can induce DNA DSBs and initiate DDR. These experiments also show that treatment with genistein in the absence of XRCC4 further promotes levels of proteins involved in the Alt-NHEJ repair pathway in XRCC4 (-) MAG cells.

7.1 Genistein induces DNA DSBs and DDR in XRCC4 (-) cells

In Chapter 3 I showed that both 50 and 100 µM genistein can induce DNA DSBs and initiate DDR signaling in parental MAG cells. In Chapter 5 I showed that both 50 and 100 µM genistein can induce DNA DSBs and initiate DDR signaling in XLF (-) MAG cells. In this set of experiments, I wanted to determine if treatment with genistein can initiate DNA DSBs and DDR in XRCC4 (-) MAG cells by assessing the protein levels of γH2AX

and pATM. Because XRCC4's activity is known to be primarily during the later stages of NHEJ repair, I hypothesized that loss of XRCC4, similar to loss of XLF, would not inhibit DSB induction or the initiation of DDR.

XRCC4 (-) MAG cells were treated with genistein at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 4 and 24 hr. post-treatment for Western blotting. DSBs were assessed by detecting levels of ɣH2AX (serine139) [5]. Initiation of DDR was assessed by detecting levels of pATM (serine1981) since previous studies have shown ATM is phosphorylated at s1981 in response to DNA DSBs by multiple DNA damaging agents including ionizing radiation, chemotherapeutic agent such as TOPO II inhibitor and various chemicals [86]. Nuclear membrane protein Lamin B1 was used as a loading control throughout except for figure 7.1.2 A, ATM was used as loading control. Protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

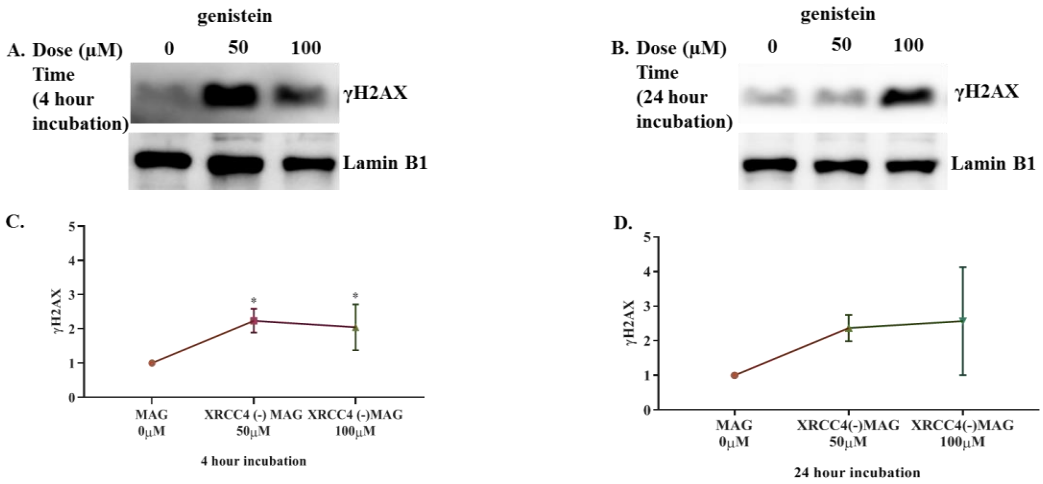


Figure 7.1.1: Genistein induces DNA DSBs in XRCC4 (-) cells (A) Representative immunoblot showing elevated level of γH2AX in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing level of γH2AX in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of γH2AX in 50 and 100 μM genistein treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing level of γH2AX in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group with no significant change. (*p=0.0232,*p=0.0449)

The XRCC4 (-) MAG cells treated with genistein showed an increase in the level of γH2AX as an indication of DNA damage. γH2AX levels start to increase at 4 hr. post-treatment; densitometry analysis of immunoblots showed significantly elevated levels of γH2AX induced by 50 μM (p=0.0232) and 100 μM (p=0.0449) genistein compared to the no treatment group. The levels of γH2AX remain elevated at 24 hr. post-treatment; densitometry analysis shows elevated levels of γH2AX in both 50 μM (p=0.1998) and 100 μM (p=0.1417) genistein treated groups compared to no treatment group but it is not significant and levels were declining toward baseline amounts. These data shows genistein can significantly induce DNA DSBs in XRCC4 (-) MAG cells in a time and dose dependent manner. These results are similar to those observed in parental MAG cells and XLF (-) cells.

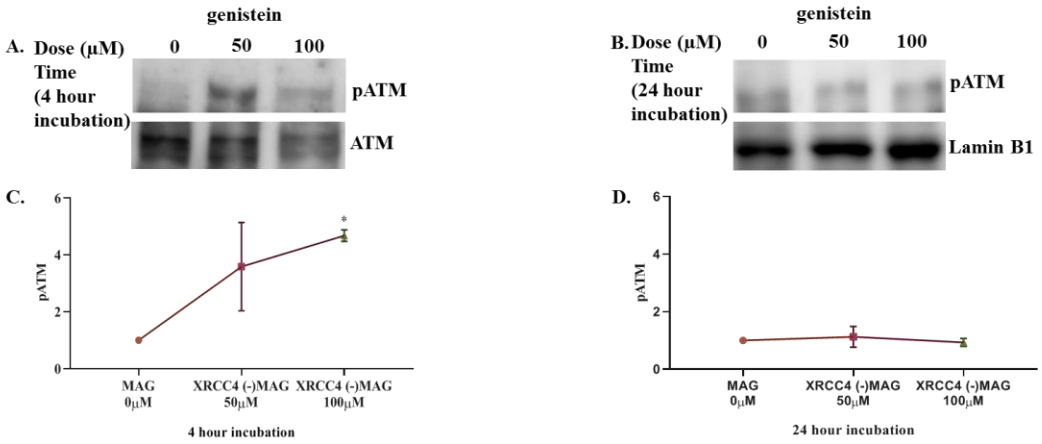


Figure 7.1.2: Genistein can generate DNA damage response in XRCC4 (-) cells (A) Representative immunoblot showing elevated level of pATM in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 4 hr. time point. (B) Representative immunoblot showing level of pATM in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of pATM in 100 μM genistein treated group at 4 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no significant change in the level of pATM in genistein treated group at 24 hr. time point compared to no treatment group.(*p=0.0437)

Following treatment with genistein pATM is elevated by 4 hr. post-treatment and then returns to baseline levels by 24 hr. post-treatment in XRCC4 (-) MAG cells compared to untreated cells. At 4 hr. post-treatment densitometry analysis of immunoblots showed significantly elevated levels of pATM following 100 μM (p=0.0437) genistein compared to the no treatment group. By 24 hr. post-treatment no significant difference is present between genistein treated and untreated group (p= 0.9126). Similarly, in parental MAG cells following treatment with genistein pATM increased significantly by 4 hr. post-treatment and returned to basal levels by 24 hr. post-treatment.

These data show that genistein can significantly induce DSBs and initiate DDR signaling in XRCC4 (-) MAG cells in a dose dependent manner with a similar effect in both parental MAG and XRCC4 (-) MAG cells.

7.2 Genistein suppresses C-NHEJ pathway protein levels in XRCC4 (-) cells

XLF, XRCC4 and DNA ligase 4, these 4 proteins forms a ligation complex which facilitates classic NHEJ pathway [96]. Loss of XRCC4 should impair the formation of a functional ligation complex for C-NHEJ. In Chapter 3 I demonstrated that exposure to genistein suppresses protein levels of XRCC4 and XLF in MAG cells. In this set of experiments, I wanted to determine whether treatment of XRCC4 (-) cells with genistein can further suppress levels of XLF.

XRCC4 (-) MAG cells were treated with genistein at multiple doses (0, 50, 100 µM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Levels of nuclear membrane protein Lamin B1 was used as a loading control. XLF protein levels were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

XRCC4 (-) MAG cells treated with genistein showed a decrease in the level of XLF compared to untreated cells. In genistein treated cells XLF levels start to decrease at 24 hr. and remain decreased at 48 hr. post treatment compared to untreated cells. Densitometry analysis of immunoblots showed significantly decreased level of XLF following both 50 and 100 µM ($p<0.0001$) genistein treatment compared to the no treatment group. In the 48hr post treatment group densitometry analysis shows XLF protein levels remain decreased in 100 µM ($p<0.0280$) genistein treated groups compared to no treatment group. These data shows genistein can significantly affect XLF protein level in XRCC4 (-) MAG

cells. These results are similar results observed in parental MAG cells following treatment with genistein.

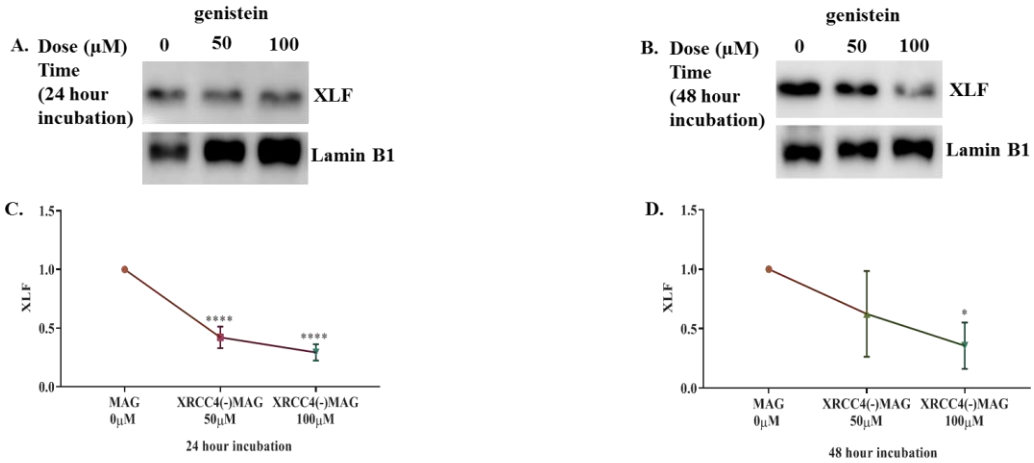


Figure 7.2.1: Genistein suppresses XLF protein levels in XRCC4 (-) cells (A) Representative immunoblot showing decreased level of XLF in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing decreased level of XLF in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly decreased level of XLF in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly decreased level of XLF in 100 μM genistein treated group at 48 hr. time point compared to 50μM and no treatment group.(****$p<0.0001$,*$p=0.0280$)

From chapter 3 and 5 I showed that in XLF (-) MAG cells 100 μM genistein induced a larger decrease of XRCC4 protein levels ($p<0.0001$) than in parental MAG cells ($p=0.0013$). These data support the idea that absence of XLF and simultaneous treatment with genistein can affect XRCC4 levels more adversely in XLF (-) MAG cells in comparison to genistein treated parental MAG cells. But in XRCC4 (-) MAG cells 100 μM genistein produce similar level ($p<0.0001$) of decrease in XLF in comparison to parental MAG cells. These data support the idea that absence of XRCC4 and simultaneous treatment with genistein can affect XLF levels similarly in XRCC4 (-) MAG cells in comparison to genistein treated parental MAG cells. Further these results mirror the impact

of loss of protein levels involved in ligation complex formation supporting the hypothesis that creation of a functional complex may play a role in stability of these proteins.

7.3 Genistein promotes levels of DNA repair proteins of the Alt-EJ pathway in XRCC4 (-) cells

Polθ and CtIP play important roles in promoting the Alt-EJ repair pathway [93]. Recent studies in mice indicated that Polθ is associated with micro homology mediated end joining (MMEJ) that is one form of Alt-EJ, leading to fusions of dysfunctional telomeres and chromosomal translocations [94]. Previous studies demonstrate that CtIP is a crucial factor for efficient chromosomal translocation formation by micro homology-prone Alt-EJ [89]. In Chapter 3 I demonstrated that exposure to genistein promotes a significant increase in protein levels of CtIP and Polθ in parental MAG cells. In Chapter 5 I demonstrated that exposure of XLF (-) cells to genistein promotes a significant increase in protein levels of CtIP and Polθ. In this set of experiments, I wanted to identify whether treatment with genistein can influence level CtIP and Polθ in XRCC4 (-) MAG cells in a time and dose dependent manner. Loss of XRCC4 should impair the formation of a functional ligation complex for C-NHEJ and promote the use Alt-NHEJ for repair. Thus, I hypothesized that absence of XRCC4 can result in a further increase in Alt-NHEJ protein levels.

XRCC4 (-) MAG cells were treated with genistein at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting. Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of CtIP and Polθ were analyzed by densitometry using Quantity One software (Bio-Rad).

Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

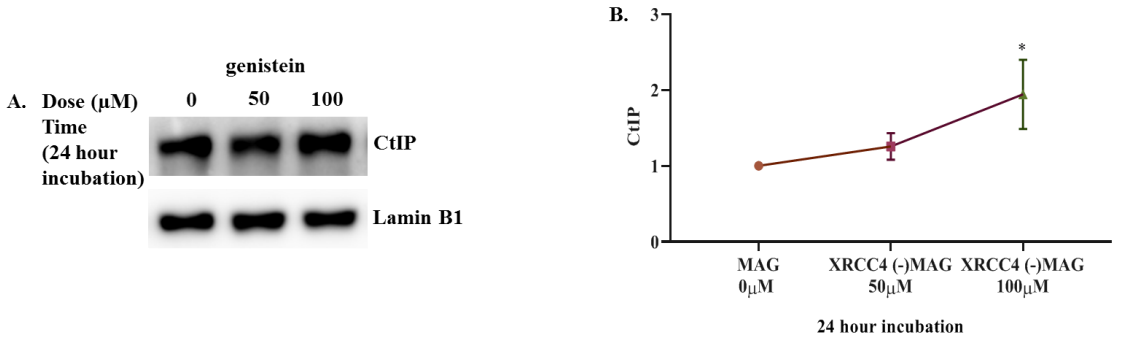


Figure 7.3.1: Genistein promotes levels of CtIP proteins of the Alt-EJ pathway in XRCC4 (-) (A) Representative immunoblot showing elevated level of CtIP in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Densitometry analysis of western blot showing significantly elevated level of CtIP in 100 µM genistein treated group at 24 hr. time point compared to no treatment group. (*p=0.0113)

XRCC4 (-) MAG cells treated with genistein showed a significant increase in the level of CtIP compared to untreated cells. By 24 hr. post-treatment densitometry analysis of immunoblots showed significantly increased level of CtIP following 100 µM (p=0.0113) genistein compared to the no treatment group. By 48 hr. post-treatment the levels of CtIP returned to basal levels and no difference was observed between treated and untreated cells. These data show genistein can significantly affect CtIP protein level in XRCC4 (-) MAG cells in a time dependent manner. Similarly, in parental MAG cells following treatment with genistein CtIP increased significantly by 24 hr. post-treatment and returned to basal levels by 48 hr. post-treatment. It is notable that 100 µM genistein induced a greater increase in CtIP levels in XRCC4 (-) MAG cells (p=0.0113) than in parental MAG cells (p=0.0348) consistent with my hypothesis that absence of the C-NHEJ protein XRCC4 can result in further increase in Alt-EJ protein levels.

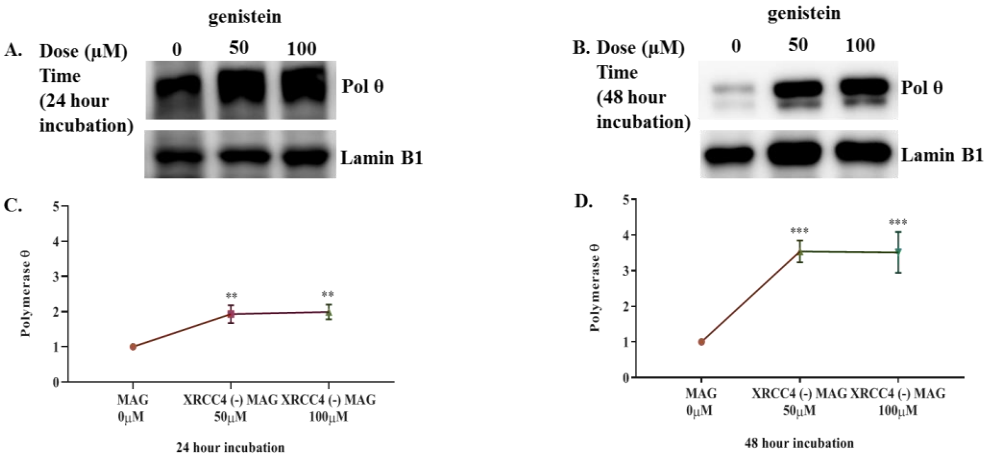


Figure 7.3.2: Genistein promotes levels of Pol θ proteins of the Alt-EJ pathway in XRCC4 (-) cells (A) Representative immunoblot showing elevated level of Pol θ in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing elevated level of Pol θ in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 50 and 100 μM genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing significantly elevated level of Pol θ in 50 and 100 μM genistein treated group at 48 hr. time point compared to no treatment group. (**p=0.0017,**p=0.0012, ***p=0.0003)

XRCC4 (-) MAG cells treated with genistein showed an increase in the level of Pol θ compared to untreated cells. In genistein treated XRCC4 (-) MAG cells Pol θ protein levels are significantly elevated by 24 hr. post-treatment and remain elevated 48 hr. post-treatment compared to untreated cells. Densitometry analysis of immunoblots showed significantly increased levels of Pol θ following 50 μM (p=0.0017) and 100 μM (p=0.012) genistein compared to the no treatment group. In the 48 hr. post-treatment group densitometry analysis shows Pol θ protein levels remain significantly increased following both 50 μM (p=0.0003) and 100 μM (p=0.0003) genistein compared to the no treatment group. These data show genistein can significantly affect Pol θ protein level in XRCC4 (-) MAG cells and are similar to results observed in parental MAG cells and in XLF (-) cells. It is notable that 100 μM genistein induced a larger increase Pol θ levels in XRCC4 (-) MAG cells (p=0.0003) than in parental MAG cells (p=0.0292) consistent with my

hypothesis that absence of the C-NHEJ protein XRCC4 can result in a further increase in Alt-NHEJ protein levels.

7.4 Genistein does not impact levels of the HR protein Rad51 in XRCC4 (-) cells

Rad51 is a well-regarded protein central to the process of HR. Literature suggests that NHEJ and HR are competing pathways of repair. In this set of experiments, I wanted to identify whether treatment of XRCC4 (-) cells with genistein can influence levels of Rad 51 protein to indicate whether genistein globally impacts al DNA repair pathways, or if genistein specifically alters NHEJ.

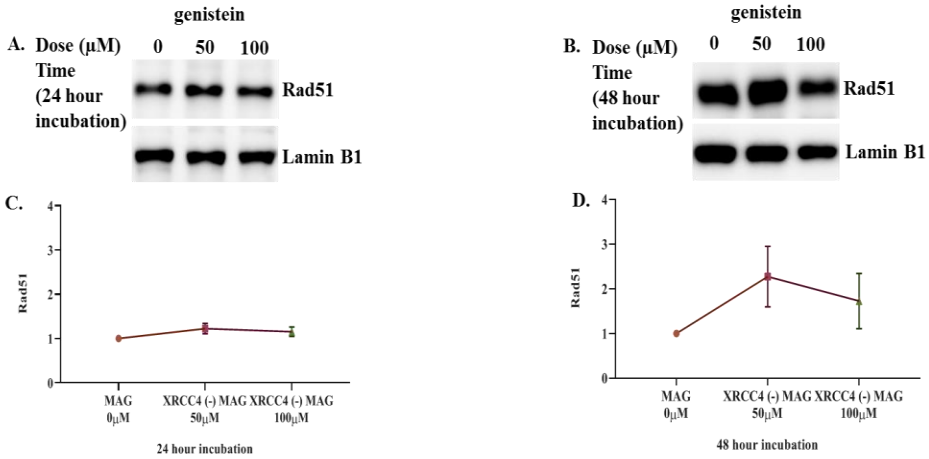


Figure 7.4.1: Genistein does not impact levels of the HR protein Rad51 in XRCC4 (-) cells (A) Representative immunoblot showing no change in the level of Rad51 in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 24 hr. time point. (B) Representative immunoblot showing decreased level of Rad51 in XRCC4 (-) mouse ES cell following treatment with genistein compared to no treatment group at 48 hr. time point. (C) Densitometry analysis of western blot showing no significant change in the level of Rad51 in genistein treated group at 24 hr. time point compared to no treatment group. (D) Densitometry analysis of western blot showing no significant change in the level of Rad51 in genistein treated group at 48 hr. time point compared to no treatment group.

XRCC4 (-) MAG cells were treated with genistein at multiple doses (0, 50,100 μM) for 1 hr. The doses were determined by the LD 50 value of the compound [77]. I collected cells and isolated nuclear protein extracts at 24 and 48 hr. post-treatment for Western blotting.

Nuclear membrane protein Lamin B1 was used as a loading control. Protein levels of Rad51were analyzed by densitometry using Quantity One software (Bio-Rad). Statistical analysis was performed using Graph Pad prism software. All the experiments were performed in triplicate as biological replicates (n=3).

XRCC4 (-) MAG cells treated with 50 µM or 100 µM genistein show no significant difference between treated and untreated group (p =0.99) by 24 or 48 hr. post-treatment. Similarly parental MAG cells showed no change in level of Rad51 at 50 and 100 µM and at 24 hr. and 48hr post-treatment.

7.5 Genistein promotes an increased number of chromosomal translocations in XRCC4 (-) cells

Previous studies from Richardson Lab showed MAG cells treated with genistein can promote the formation of chromosomal translocations during DSB repair between the MLL and AF9 bcr transgene reporter constructs in the parental MAG cells. Detection and quantification of translocations is possible since a translocation brings together engineered exons 1 and 2 of a Green Fluorescent Protein gene (GFP) onto a single DNA helix thus allowing for identification by expression of the green fluorescent protein by inverted microscopy by 96 hr. post-treatment [77].

Loss of XRCC4 should impair the formation of a functional ligation complex for C-NHEJ and promote the use Alt-NHEJ for repair. I demonstrated that in the absence of XRCC4 genistein treatment promotes elevated levels of proteins involved in the Alt-NHEJ repair pathway. In this set of experiments, I wanted to determine if in the absence of XRCC4

genistein treatment also promotes an increase in the number of detectable chromosomal translocations in the XRCC4 (-) MAG cells and compared to parental MAG cells [77].

After treatment of parental MAG and XRCC4 (-) MAG cells with genistein at different doses (0, 50 and 100 µM), the cells were maintained at 37 °C, 5% CO2 and were examined every 24 hr. for GFP expression by an inverted microscopy. By 96 hr. post-treatment GFP+ colonies were detectable. Untreated parental MAG and untreated XRCC4 (-) MAG cells were used as negative controls.

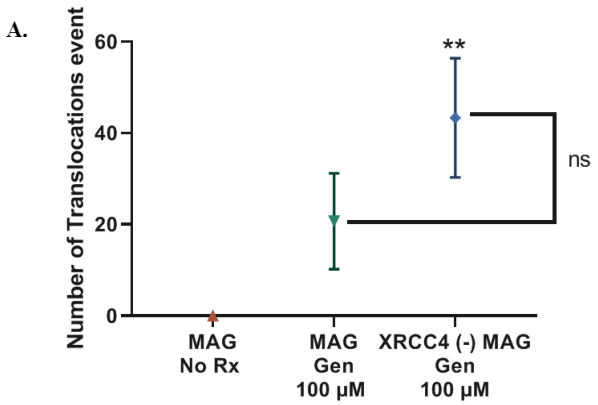
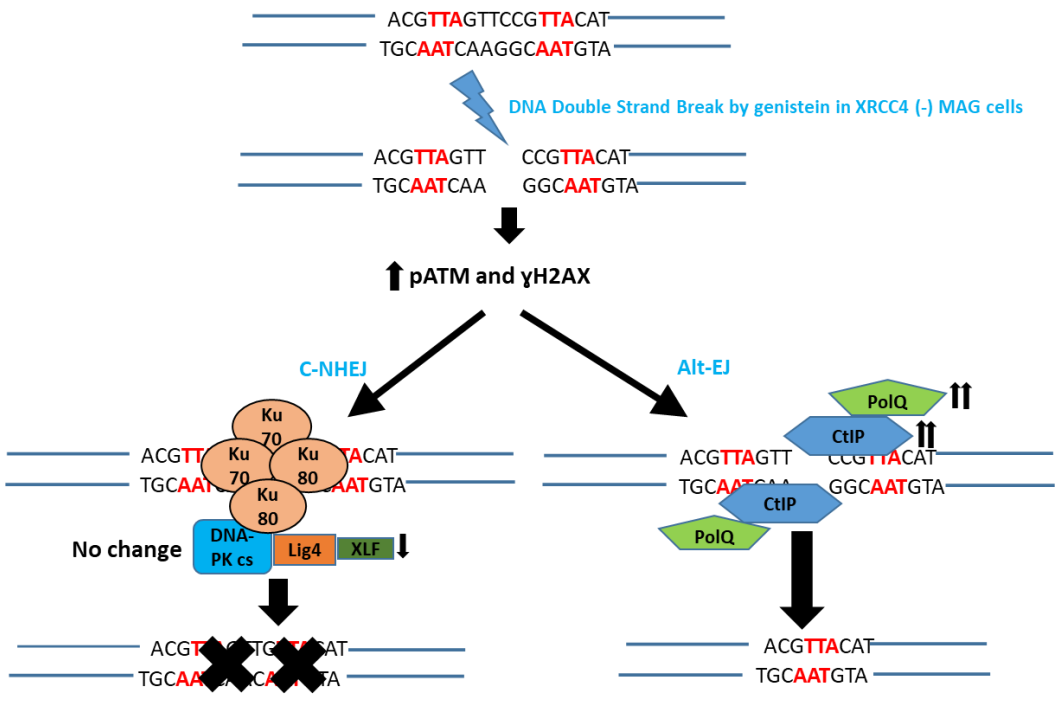


Figure 7.5.1: Genistein promotes an increased number of chromosomal translocations in XRCC4(-) cells
(A) Analysis of number of GFP positive colony showing significantly elevated level of translocation frequency in 100 µM genistein treated XRCC4 (-) MAG group compared to no treatment group. (**p=0.0037)

XRCC4 (-) MAG cells treated with genistein 100 µM (p=0.0037) showed significantly increased translocation frequency compared to untreated MAG and XRCC4 (-) MAG cells. In comparison to genistein treated MAG cells XRCC4 (-) MAG cells showed increased translocation frequency (though not significant) at 100 µM (p=0.0639) dosage confirming

my hypothesis that XRCC4 (-) MAG cells are more susceptible to chromosomal translocation event following exposure to genistein.

7.6 Discussion and Conclusion



In this chapter I have described that genistein can induce DNA DSBs, initiate DDR and can influence DNA repair protein levels in XRCC4 (-) MAG cells .My data support the hypothesis that genistein promotes Alt-EJ repair and that absence of the XRCC4 protein involved in C-NHEJ pathway drives the repair process more robustly towards Alt-EJ pathway.

CHAPTER 8: Discussion and Future Directions

Studies have shown that the bioflavonoids genistein and quercetin have beneficial effects on health [97, 98] [99]. However, bioflavonoids have been shown to act biochemically as TOPO II inhibitors [83, 100, 101] and can induce cleavage in the *MLL* gene bcr. Additional recent evidence from Richardson lab shows that these compounds along with other bioflavonoids have the potential to generate *MLL*bcr-*AF9*bcr translocations further bolstering the hypothesis that they contribute to infant leukemia [67, 77, 102].

In this thesis I have demonstrated that bioflavonoids genistein and quercetin have the ability to induce DNA DSBs and initiate DDR in parental MAG, XRCC4 (-) MAG and XLF (-) MAG cells. I also demonstrated that genistein and quercetin can have variable influences over different DNA repair proteins involved in the competing C-NHEJ and Alt-EJ pathways that can impact the formation of chromosomal translocations. Further the biologic impact of the compounds on DSBs, the initiation of DDR, and pathway choice is further augmented in XRCC4 (-) MAG and XLF (-) MAG cells. Using the unique MAG *MLL-AF9* bcr GFP reporter gene system I showed how protein levels involved in different DNA repair pathways can correlate with translocation formation. The results shown here are the first to demonstrate that in embryonic stem cells bioflavonoids genistein and quercetin can influence levels of different DNA repair proteins and subsequently play role in DNA DSB repair pathway choice.

DNA DSBs repaired by Alt-EJ are characterized by more extensive end-resection, activation of a unique set of repair proteins, and a higher potential to result in chromosomal translocations than DSBs repaired by C-NHEJ [57], and some specific DNA repair proteins

such as CtIP and Pol θ play significant roles in this process[103] [89, 104, 105]. Previous studies have shown DNA DSBs induced by etoposide are repaired by NHEJ with a requirement for the MRN complex and CtIP [106] which may promote the translocations associated with etoposide exposure observed in therapy-related leukemias [89]. My studies are the first to elucidate the influence of genistein and quercetin on specific DNA repair proteins in stem cells to fill the gap in understanding repair pathway choice and repair outcome frequency. Moreover, my studies are the first to investigate how absence of a protein in the C-NHEJ pathway and subsequent exposure to genistein and quercetin can impact the repair pathway proteins. Overall, I demonstrated that genistein has a clear suppressive effect on the C-NHEJ proteins and promoting effect on Alt-EJ proteins in parental MAG cells as well as in XLF (-) and XRCC4 (-) MAG cells. Quercetin has a weaker suppressive effect on C-NHEJ pathway proteins but does have a strong promoting effect on Alt-EJ pathway proteins in parental MAG cells as well as in XLF (-) and XRCC4 (-) MAG cells.

8.1 Genistein and quercetin can induce DNA DSBs and initiate DDR

My studies demonstrate that treatment with either genistein or quercetin leads to H2AX phosphorylation at serine 139 or ATM phosphorylation at serine 1981 in parental MAG cells as an indication of DNA damage and DNA damage response. These results are consistent with other studies that have shown the presence of γH2AX foci following exposure to these and other bioflavonoids (D. Goodenow and C. Richardson personal communication) [77, 107]. In addition, these studies are consistent with other studies that show activation of ATM signaling following exposure to bioflavonoids [108, 109]. These

results provided a baseline of comparison for treatment of cells deficient in the C-NHEJ repair proteins XRCC4 and XLF.

Treatment of XLF (-) MAG cells with genistein or quercetin showed elevated levels of γH2AX and pATM indicating absence of XLF protein does not impact the DSBs and DDR in embryonic stem cells. Similarly, treatment of XRCC4 (-) MAG cells with genistein or quercetin also showed elevated levels of γH2AX and pATM indicating absence of XLF protein does not impact the DSBs and DDR in embryonic stem cells. These results indicate that the two C-NHEJ proteins XLF and XRCC4 play roles downstream of initial DNA breakage and initial DDR signaling. Instead, their role in cellular response to these two bioflavonoids is restricted to later steps of EJ repair in cells. Given that bioflavonoids have pleiotropic effects on cells, these approaches were important to demonstrate that the bioflavonoids do not alter in some unknown way an early function in cellular response or DDR of these two specific repair proteins.

8.2. Genistein and quercetin and the C-NHEJ pathway

In my experiments here, I demonstrate that genistein has a clear inhibitory effect on the C-NHEJ pathway. XLF and XRCC4 protein levels decreased following exposure to genistein. Additionally, overall DNA-PKcs protein levels were not increased, and significantly the phosphorylation status of DNA-PKcs did not change following exposure to genistein.

Genistein has pleotropic effect in cells. Depending on cell type its effect can be variable [77]. The observed decreased levels of XLF and XRCC4 following treatment with genistein may be the result of similar mechanism to its inhibition of other signaling pathways. Genistein has inhibitory activity on several different pathways such as NF-κB

and Akt signaling pathways and has negative effect on cyclins and CDKs as well. For example, genistein inhibits translocation of NF-κB to the nucleus, preventing NF-κB from binding to its target DNA and thereby inhibiting the transcription of NF- κB downstream genes [110] [111]. It is possible that genistein similarly suppresses XLF and XRCC4 transport to the nucleus, since I used nuclear extract throughout.

XLF and XRCC4 are part of the C-NHEJ ligation complex and presence of all the components are necessary for the stability. Studies have shown absence of XRCC4 adversely affect the stability of ligase4 protein and disrupts the function of ligation complex [112]. Genistein is known to induce post-translational modifications such as enhancing ubiquitination of MDM2 [113] resulting in its downregulation and enhancing proteosomal degradation of Top2β [114]. The decreased levels of XLF and XRCC4 could be due to similar impact on post-translational modification. In XLF (-) MAG cells following treatment with genistein, XRCC4 levels decrease by 24 hr. post-treatment more significantly compared to the decrease observed in parental MAG cells. In XRCC4 (-) MAG cells following treatment with genistein, XLF levels decrease by 24 hr. post-treatment similarly as observed in parental MAG cells but remains decreased for a longer time. Both of these findings support the idea that each protein stabilizes the other through binding interactions and simultaneous absence of each and exposure to genistein may more adversely affect the levels of the other.

Quercetin treatment induced less specific effects on the levels of C-NHEJ proteins. In MAG parental cells treated with quercetin, XLF protein levels are decreased by 24 hr. post-treatment but then increases significantly by 48 hr. post-treatment compared to untreated

cells. This finding suggests altered kinetics of cellular response to quercetin, with no direct early effect, but instead an indirect later stimulation Although quercetin treated MAG cells show decrease in the levels of XLF protein by 24 hr. post-treatment, it is not as significant as observed in genistein treated cells. In XLF (-) MAG cells, treatment with quercetin shows a trend towards decreasing but no significant change in XRCC4 levels compared to untreated cells. It is possible XLF has more favorable structure for binding quercetin in comparison to XRCC4 [115].

DNA-PKcs is stimulated following DSBs and cellular stresses. DNA DSBs bound to Ku recruits DNA-PKcs and activates DN-PKcs kinase activity DNA-PKcs is auto-phosphorylated at the ABCDE cluster Thr2609 and PQR cluster at Ser2056, and studies have shown that phosphorylation at S2056 limits end-resection [116, 117]. Further, kinase-dead DNA-PKcs inhibits end-ligation [118]. In my studies, cells treated with genistein showed no change in the total levels of DNA-PKcs. Additionally, cells treated with genistein showed no or possibly decreased amount of DNA-PKcs phosphorylation, while cells treated with quercetin showed a small induction of DNA-PKcs phosphorylation. These results were in strong contrast to substantial increase in DNA-PKcs phosphorylation following IR treatment. IR creates DNA end structures which require DNA-PKcs mediated repair but other end structures or breaks might not recruit DNA-PKcs effectively [26]. Genistein might be creating simple broken DNA ends which are not stimulating DNA-PKcs levels. Mechanistically, lack of DNA-PKcs phosphorylation will enhance shuttling of broken ends towards increased resection and Alt-EJ repair which fully support the idea that exposure to bioflavonoids promotes DSBs repaired by specific DSB repair pathway. Interestingly, pATM levels were increased in response to both bioflavonoids. In addition

to multiple other substrates, pATM can phosphorylate DNA-PKcs although at lower levels and only at Thr2609 which promotes end-resection and may promote genome alterations [117, 119]. These findings altogether indicate genistein and quercetin suppress C-NHEJ pathway by affecting multiple DNA repair proteins in the pathway although each shows some specificity of action and variable impact.

8.3 Genistein, quercetin and Alt-EJ pathway

Here I demonstrate that both genistein and quercetin have a stimulatory effect on the Alt-EJ pathway. CtIP and Pol θ protein levels significantly increased following exposure to either genistein or quercetin. CtIP and Pol θ has the propensity to bind to specific type of broken DNA ends [104]. It is possible DSBs induced by genistein create specific overhangs appropriate for binding of CtIP and Pol θ and as they are utilized for joining, and their stability and protein levels increase.

Genistein has pleotropic effect in cells. Depending on cell type its effect can be variable [77]. Genistein is known to have positive effects on proteins involved in different regulatory pathways [120, 121]. Studies have shown that genistein enhances endothelial nitric oxide protein through up-regulating mRNA transcription in human aortic endothelial cells [121]. In addition, genistein belongs to the polyphenolic compound, and studies have shown that plant polyphenols can inhibit ubiquitin proteosomal degradation system [121]. The observed increases level of CtIP and Polθ could additionally be attributed to genistein preventing their degradation.

Quercetin has both suppressing and inducing effects. It can inhibit production of tumor necrosis factor alpha (TNF-α), interleukin 8 (IL-8) and can induce the production of interferon gamma (IFN-ɣ) [122]. In a similar way quercetin can enhance the level of CtIP and Pol θ in MAG cells. Quercetin may transcriptionally influence the level of CtIP and Pol θ or may prevent their degradation.

Studies have shown absence of components in C-NHEJ pathway enhances the activity of proteins involved in other repair pathways [57, 91]. My results are consistent with those findings. In both XLF (-) MAG and XRCC4 (-) cells following treatment with genistein, CtIP levels increase by 24 hr. post-treatment more significantly compared to the increase observed in parental MAG cells. In XLF (-) MAG cells following treatment with genistein, Pol θ levels increase by 24 hr. post-treatment and by 48 hr. post-treatment the increase is more significant compared to the increase observed in parental MAG cells. In XRCC4 (-) MAG cells Pol θ protein levels are significantly elevated as well. Similarly, in XLF (-) MAG cells following treatment with quercetin, both CtIP and Pol θ levels increase, the increase of Pol θ levels are more significant compared to the increase observed in parental MAG cells .These results indicate that downregulation of proteins involved in C-NHEJ pathway further enhances the level of proteins involved in Alt-EJ pathway following exposure to these bioflavonoids.

Overall these results indicate that genistein and quercetin has a propensity towards promoting the proteins involved in Alt-EJ pathway in stem cells. Additionally, in absence of a C-NHEJ protein, subsequent exposure to genistein or quercetin produces more Alt-EJ proteins CtIP and Pol θ.

8.4 Genistein, quercetin and promotion of chromosomal translocations

Using genetically engineered MAG cell line, the Richardson Lab previously demonstrated that genistein and quercetin can promote EJ-directed *MLL*bcr-*AF9*bcr chromosomal translocations scored as GFP+ colonies [77]. Since absence of protein involved in C-NHEJ pathway and subsequent exposure to genistein or quercetin led to an observed augmented increase in Alt-EJ proteins, it is critical to demonstrate that this series of observations is biologically relevant and correlates with increased stimulation of chromosomal translocations. Results are consistent with a biological relevance to the observed protein level changes, XLF (-) MAG cells treated with genistein or quercetin and XRCC4 (-) MAG cells treated with genistein showed an increased number of GFP+ colonies compared to parental MAG cells. Although all showed consistently increased number of GFP+ colonies compared to treated parental MAG cells, only in XLF (-) MAG cells treated with genistein did the difference achieve statistical significance. In the other two cohorts, the difference was smaller and did not reach statistical significance. Future experiments can increase the sample size to determine if significance can be achieved or to show if there is specificity in biological outcome between different mutant cell lines or from different compound treatments. The subtle differences between impact of genistein and quercetin treatment on protein levels and phosphorylation may translate to the slight differences observed in stimulation of translocations.

Previous studies in the Richardson lab demonstrated that genistein and quercetin promote chromosomal translocations in both embryonic stem and isogenic hematopoietic stem cells but lower numbers of translocations in more differentiated hematopoietic cell types [77]. Future studies can examine how the loss of XLF or XRCC4 compares with those findings.

8.5 Significance and future directions

In this thesis I have demonstrated that bioflavonoids genistein and quercetin have the ability to induce DNA DSBs and initiate DDR. I also demonstrated that genistein and quercetin can have variable influences over different DNA repair proteins involved in the competing C-NHEJ and Alt-EJ pathways that can impact the formation of chromosomal translocations. Further the biologic impact of the compounds on DSBs, the initiation of DDR, and pathway choice is further augmented in XRCC4 (-) MAG and XLF (-) MAG cells. Using the unique MAG *MLL-AF9* bcr GFP reporter gene system I showed how protein levels involved in different DNA repair pathways can correlate with translocation formation.

The results shown here are the first to demonstrate that in embryonic stem cells bioflavonoids genistein and quercetin can influence levels of different DNA repair proteins and subsequently play role in DNA DSB repair pathway choice. This study further emphasizes that absence of C-NHEJ protein can more adversely affect DNA repair pathway choice. Bioflavonoids are common in foods and used as dietary supplements in a widespread manner. Previous studies reported average adult soy intake to be 0.15-3.0 mg/day [123], but daily intake averages can vary and increase to 8.6mg/day in women and

7.5mg/day in men [124]. Foods contain multiple bioflavonoids, and bioflavonoids are bio-accumulative which likely increases plasma concentrations [125].

In the era of personalized medicine, decreased expression of C-NHEJ proteins in some individuals could be determined as a risk factor for bioflavonoid-induced genome rearrangements. Variant alleles of DNA repair proteins may promote susceptibility to flavonoid-induced translocations, although to date no variants on the proteins that I examined have been reported in this context. Both heterozygous and homozygous mutants of the DNA damage response gene *Atm* exposed prenatally to flavonoids exhibited detectable numbers of *MLL* rearrangements [126]. In addition, inhibition or mutation of multiple DNA repair proteins potentiates cytotoxicity of topoII inhibitors, and MRE11 plays a direct mechanistic role in removal of topoII-DNA complexes in yeast and mammals [127, 128].

Study of the potential for these compounds to induce infant leukemias is relevant since they cross the placental barrier as shown with both genistein and quercetin [126, 129]. Exposure *in utero* is likely more damaging due to differences in metabolic and excretion rates of mother and fetus [130] as well as rapidly developing and proliferating fetal cells that are more sensitive to topoII inhibiting agents [131].

Finally, I adapted the genetically engineered MAG cell line model system developed in the Richardson lab to directly address the impact of CRISPR engineered loss of specific DNA repair proteins on pathway choice and the stimulation of chromosomal translocations following exposure to two specific bioflavonoid compounds. This model system can systematically screen a large panel of compounds, even those not yet suspected

to have mutagenic potential, in combination with each other to understand their additive, synergistic, or even quenching effects on the potential to promote Alt-NHEJ and chromosomal rearrangements and potentially, infant leukemias.

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