Role of TopBP1-Msh2 interaction in ATR-Chk1 pathway

A thesis submitted in partial fulfillment of the requirements

Of the degree of

Doctor of Philosophy

By

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CERTIFICATE

I hereby certify that the work incorporated in the thesis "**Role of TopBP1-Msh2 interaction in ATR-Chk1 pathway**" submitted by Ms **Payal Arya** was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

Dr. Mayurika Lahiri

(Supervisor)

Date:

Declaration

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Dedication

To my mother “Kusum” for her name itself suggests to bloom like a flower…..

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Synopsis

Title: Role of TopBP1-Msh2 interaction in ATR-Chk1 pathway

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Introduction

In order to survive successfully, organisms must accurately replicate and transmit their genetic material to the progeny. Failure of cellular machinery to correct errors encountered during replication and other spontaneous changes cause mutations in the genome. The eukaryotic genome faces threats during internal processes like replication and also from external sources like irradiation and chemicals. Mutations in the proto oncogenes and tumor suppressor genes result in uncontrolled division of cells leading to tumorigenesis and malignancy. Cells have evolved DNA damage response (DDR) pathways, which are elaborate mechanisms that detect and correct genomic errors ensuring genomic integrity. If the errors are not rectified by these mechanisms, checkpoint proteins to prevent tumorous growth arrest the cell's replication. Defects in DNA repair and genome checkpoints have been implicated in increasing genomic instability (Bartek and Lukas 2001, Hoeijmakers 2001). DDR pathways operate in close coordination between checkpoint and DNA repair pathways.

Components of DNA damage response pathways

The DNA damage signaling cascade in terms of time and space can be categorized into sensors, transducers and the effectors. DNA damage is signaled initially to the two apical kinases-Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3 related (ATR) which then relay the information to transducers like Mdc1, TopBP1, Claspin and finally the information reaches the effector kinases –Chk2 and Chk1 which delay the cell cycle progression and promote DNA repair or apoptosis in case of irreparable damage (Abraham 2001).

Both ATM and ATR share substrates and phosphorylate serine, threonine residues followed by glutamine. ATR is essential for cell viability in human and mouse cells while ATM is not (Brown and Baltimore 2000, de Klein, Muijtjens et al. 2000, Cortez, Guntuku et al. 2001). ATM functions in response to double strand breaks while ATR is activated in response to single strand breaks or stalled replication forks (Nyberg, Michelson et al. 2002). Humans with mutated ATM or MRN complex are predisposed to cancer development possibly due to genomic instability resulting from defects in checkpoint activation and recombination repair. Although no such defects are associated with loss of function of ATR and Chk1 as both the proteins are required for cell proliferation and survival. The loss of function of ATR and chk1 therefore results in cell death rather survival with mutations.

ATR signaling at a glance

Different types of DNA lesions like intra-strand crosslinks, base adducts, replication stress are capable of activating ATR kinase. Common to all these lesions is presence of single stranded region of DNA that seems to be sufficient and necessary to activate ATR. The single stranded DNA is bound by Replication protein A (RPA), which recruits ATR – ATRIP complex to the damaged site (Costanzo, Shechter et al. 2003, Zou and Elledge 2003, Fanning, Klimovich et al. 2006). Another trimeric ring shaped complex- 9-1-1 (Rad9, Hus1, Rad1) is loaded with the help of clamploader-Rad17 onto the RPA

coated single stranded region adjacent to ATR-ATRIP (Ellison and Stillman 2003, Zou, Liu et al. 2003, Parrilla-Castellar, Arlander et al. 2004).

To achieve ATR kinase activation, a mediator protein, TopBP1 (Topoisomerase IIβ binding protein) is loaded on to the DNA. TopBP1 interacts with the phosphorylated C-terminal region of Rad9 (a component of the 9-1-1 complex) (Delacroix, Wagner et al. 2007, Ueda, Takeishi et al. 2012). Once on the DNA TopBP1 interacts with ATR through a special domain of TopBP1 called ATR activating domain (AAD) and is able to stimulate its kinase (Burrows and Elledge 2008). Another mediator protein Claspin is phosphorylated by ATR and serves as a platform for recruiting Chk1 to the damaged sites (Kumagai and Dunphy 2000). ATR then phosphorylates Chk1 at multiple SQ/TQ motifs in the C terminal region of chk1 notably at Ser 317 and Ser 345 (Liu, Guntuku et al. 2000). Phosphorylated Chk1 dissociates from chromatin for modifying its substrates such as Cdc25 phosphatases that inhibit the cell cycle transition (Lukas, Lukas et al. 2004).

Cellular response to Alkylating agents

Activation of DNA damage response is dependent on the type of lesion created and the phase of the cell cycle. SN_1-type methylating agents transfer methyl group to the ring nitrogen (N3 and N7) and extra cyclic oxygen (O6) atom of the DNA bases. The methylated bases are processed by various repair mechanisms depending on the dosage and the cell cycle phase involved The N3 methylation of Adenine and N7 methylation of Guanine are higher in number and are removed by nucleotide excision or base excision repair mechanisms.

O6Me-G is a poor substrate for glycosylases involved in base excision repair, therefore remains unrepaired leading to mispairing with thymine in the next replication cycle (Fu, Calvo et al. 2012). Mismatch repair proteins recognize

O6meG/T mispairs and results in activation of DNA damage signaling as well as repair.

Currently there are two hypotheses that explain DNA damage signaling by mismatch repair (MMR) in response to SN_1 type methylating agents. The futile hypotheses suggest that MMR requires replication to repair the O6Me G:T mispairs. During DNA replication, the mismatch repair is directed to the new strand synthesized while the methylated base is in the parental strand, which does not get repaired. The continuous struggle of MMR to repair the mismatch results in multiple excision and resynthesis of the DNA strand. This leads to the generation of gaps and breaks which activate the checkpoint signaling. The second hypothesis suggests direct signaling by MMR, which proposes that MMR proteins recognize the alkylated bases and recruit the ATM/ATR complex for checkpoint signaling. A number of studies in literature support both models of checkpoint signaling by MMR but the mechanism and the protein complexes involved are still under investigation (Yamane, Taylor et al. 2004, Adamson, Beardsley et al. 2005, Wang and Edelmann 2006).

Role of TopBP1 and Msh2 in checkpoint signaling

Human TopBP1 was first identified as an interacting partner of Topoisomerase IIβ. TopBP1 shares sequence homology with Dpb11 (*Saccharomyces cerevisiae*), Rad4/Cut5 (*Schizosacharomyces pombe*), Mus101 (*Drosophila melanogaster*) and Cut5 (*Xenopus*). All the homologues of TopBP1 play an important role in DNA replication and checkpoint activation (Saka, Fantes et al. 1994, Wang and Elledge 1999, Yamane, Wu et al. 2002). Human TopBP1 possesses 9 BRCT domains each having a distinct function. With the help of its BRCT domains TopBP1 is capable of multiple protein-protein interactions and can bind DNA strand breaks. For instance TopBP1 regulates DNA replication initiation. TopBP1can activate the sensor kinase, ATR through its ATR activating domain. In addition it can bind to Rad9, Nbs1 through BRCT1-2 and BACH1 through BRCT7-8 which is required for Chk1 phosphorylation (Wardlaw, Carr et al. 2014). TopBP1 is an essential

scaffolding protein acting as a hub for various protein – protein interactions thereby regulating various cellular process like checkpoint activation and DNA replication. There are no known mutations in TopBP1, which would directly lead to occurrence of certain kind of cancer. However, recently over expression of TopBP1 was observed in at least 60 % of breast cancer patients. It was also related to high tumor grade as well as shorter survival in the patients (Forma, Krzeslak et al. 2012).

MutS homolog 2 (Msh2) is a mismatch repair protein, which is homologous across prokaryotic and eukaryotic organisms. Msh2 forms dimmers with Msh6 (MutSα) and Msh3 (MutSβ) and recognizes base mismatches or insertion deletion loops respectively. (Acharya, Wilson et al. 1996, Marsischky, Filosi et al. 1996). Msh2 plays a critical role in mismatch repair mechanism, which is evident from the fact that mutation in the protein leads to hereditary nonpolyposis colorectal cancer. Msh2 comprises of 4 pfam domains, MutSI, MutSII, MutSd, MutSac (SMART tool). (Guerrette, Wilson et al. 1998). The functions of each of the domain have been well characterized in reference to mismatch repair activity. For example MutSac is an ATPase domain, which confers the ability to bind ATP and hydrolyze to ADP an important step in mismatch repair . Msh2 has also been implicated in checkpoint signaling following alkylation damage. MutSα complex binds O6meG/T mismatches and recruit ATR kinase for checkpoint activation in response to damage caused by cisplatin (Yoshioka, Yoshioka et al. 2006, Pabla, Ma et al. 2011).

Aim of the study

Various protein-protein interactions play a crucial role in DNA damage signaling cascade. In order to find DNA damage specific novel interactors of TopBP1; a GST pull down assay was done which helped in identifying a number of proteins differentially binding to TopBP1. MutSα complex was one of the proteins, which were enriched in the DNA damage fraction when analyzed by mass spectrometry. Later studies (Liu, Fang et al. 2010) also showed that TopBP1 and Msh2 interact following methylation damage caused

by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). However the relevance of such an interaction was not explained.

The aim of the study was to characterize the interaction between TopBP1 and Msh2 structurally and functionally.

Objectives of the study

1. To investigate whether Msh2 and TopBP1 can interact physically in the absence of their auxiliary partners using recombinant proteins
2. To investigate the binding regions in TopBP1 and Msh2 that is critical for their interaction *in vitro*
3. To check whether the interaction between TopBP1 and Msh2 was enhanced in the presence of DNA damage *in vivo*.
4. To investigate the functional relevance of the interaction in the context of checkpoint activation using siRNA mediated knockdown of either of the proteins.

Results and Discussion

***In vitro* interaction studies between TopBP1 and Msh2**

To carry out *in vitro* interaction studies we expressed full length and deletion constructs of TopBP1 and Msh2. Full-length GST-tagged TopBP1 and its different BRCT deletion mutants were expressed in bacteria. Full-length Msh2 was expressed in Sf9 cells and FLAG-tagged deletion mutants were expressed in bacteria. To check if these two proteins could interact *in vitro*, we used far western analysis. In separate experiments both these proteins were used as bait and prey protein. The interaction was ascertained by immunoblotting using antibody against the prey protein. We found that TopBP1 and Msh2 could interact *in vitro* without the presence of DNA or their auxiliary partners. By carrying out interaction studies using deletion mutants, we also identified the domains required for their binding. The MutSd and MutSac of Msh2 and the C-terminal region after BRCT domain VI of TopBP1 were found to be necessary for their binding *in vitro*.

Cellular response to alkylating agents

Once the physical interaction between TopBP1 and Msh2 was confirmed, we wanted to understand the role of this interaction in checkpoint activation following DNA damage. TopBP1 is paramount for checkpoint activation following UV damage, stalled replication forks and irradiation (IR). Earlier studies have shown that the ATR activating domain of TopBP1 is critical for ATR kinase activation (Mordes, Glick et al. 2008, Yan and Michael 2009, Liu, Shiotani et al. 2011). Msh2 can also interact with ATR kinase and cause activation of checkpoint following MNNG (Wang and Qin 2003, Kumagai, Lee et al. 2006). Since both the proteins work in the same ATR-Chk1 pathway we wanted to understand the role of their interaction in the same pathway. We used N-methyl-N-nitrosourea (MNU) to induce damage. We obtained sub-lethal dose of MNU by cytotoxicity assay wherein HeLa cells were treated with a range of concentrations of MNU. The cytotoxicity assay determined that 2 mM MNU was the IC_{50} of the drug at which 50 % of the cells were dead following treatment with MNU. 0.5 mM MNU was chosen to treat HeLa cells so as to determine the cellular response in terms of checkpoint activation and kind of breaks formed.

Single cell gel electrophoresis most commonly known as comet assay was used to determine the presence of single strand and double strand breaks in HeLa cells following treatment with MNU. Within 30 minutes of exposure of MNU, HeLa cells showed breakage in the genome, which is sustained till 48 hours post damage. We wanted to check if the breaks caused by MNU could activate checkpoint signaling. Chk1 phosphorylation at Ser 345 and Chk2 phosphorylation at Thr 68 are used as hallmarks for functional ATR and ATM signaling respectively post damage. Both ATR and ATM signaling were active following MNU exposure in HeLa cells which corroborated with the presence of single and double strand breaks as shown by comet assay. The phosphorylation of Chk1 and Chk2 was observed as early as 30 minutes post MNU exposure and it sustained till 48 hours. But replication protein A (RPA), which binds to the single stranded region, is hyperphosphorylated only at 24 and 48 hours post damage. Previous studies have shown the phosphorylation of ATR, ATM, Chk1 and Chk2 occur in second S phase of cell cycle

following methylation damage. Our results indicate that the cells undergo G2 arrest after second round of replication while the checkpoint signaling is activated with 30 minutes of damage. (Stojic, Mojas et al. 2004). Recent studies have also showed that checkpoints are activated as early as within 3 hours of treatment with Temozolamide (Ito, Ohba et al. 2013). It is possible that the MNU dose might have caused higher number of N3 and N7 mehtylations, which could result in early checkpoint activation and also the increased number of O6 mehtylations, which sustained the phosphorylation till 48 hours.

Response of TopBP1 and Msh2 to alkylation damage

Once we established that checkpoints are activated in HeLa cells following MNU damage. We checked if TopBP1 and Msh2 were recruited at the sites of breakage and form DNA damage response (DDR) foci. TopBP1 was recruited to damaged sites as early as 30 minutes and appeared as foci when visualized under a microscope. At 48 hours post MNU damage about 50 % of cells showed TopBP1 foci probably because of stalled replication forks as the cells were found to be arrested at G2/M phase by flow cytometry. In undamaged cell, Msh2 is distributed in cytoplasm as well as nucleus. DNA damage triggers the accumulation of Msh2 in nucleus post 6 hours of MNU exposure. To investigate if Msh2 or TopBP1 was essential for checkpoint activation following methylation damage, siRNA-mediated knockdown was used. Phosphorylation of Chk1 at Ser 345 and 317 was used as read out for checkpoint activation. The study revealed that absence of TopBP1 abolished checkpoint activation as levels of phospho-Chk1 were reduced. On the other hand knockdown of Msh2 could partially abolish checkpoint activation. TopBP1 is an essential activator of ATR kinase, therefore depletion of TopBP1 abrogated the phosphorylation of Chk1 at Ser 345 and Ser 317. On the other hand depletion of Msh2 leads to disruption of phosphoryltaion of Chk1 at Ser 317 and not Ser 345. Our study shows Msh2 is required for ATR signaling which is not dependent on replication and occurs at early time points following methylation damage but the mechanism of Msh2 dependent ATR activation of Chk1 Ser 317 still needs to be elucidated.

TopBP1 and Msh2 are required for ATR mediated checkpoint activation following MNU damage; therefore we checked if the interaction between these two proteins was enhanced by MNU damage. Immunoprecipitation with whole cell extracts of HeLa S3 cells showed that TopBP1 and Msh2 co-precipitated independent of DNA damage and the interaction was not enhanced upon MNU damage. This suggests that TopBP1 and Msh2-Msh6 are constitutively associated and might be recruited to chromatin, which still needs to be tested. As most of the MMR proteins are recruited to chromatin upon DNA damage, it is possible that TopBP-Msh2-Msh6 complex is also recruited to chromatin upon DNA damage.

Our studies point towards the role of a checkpoint protein, TopBP1 and a mismatch repair protein, Msh2 to be involved in regulation of DNA damage signaling. As the complex between MutSα and TopBP1 is constitutively associated, it is quite possible that the complex could be scanning DNA for any kind of damage irrespective of cell cycle phase. The partial disruption of the checkpoint signaling following Msh2 knockdown suggests that there might be a separate mechanism of phosphorylation of Chk1 at Ser 317, which may or may not be dependent on TopBP1-Msh2 interaction. The study provides new insights into the complex regulation of checkpoint signaling where multiple protein complexes are required for every step in the signaling cascade. The study gives a platform to further explore the recruitment of TopBP1-Msh2 complex and also further characterize the substrate specificity of Msh2 towards activation of Chk1 following methylation damage.

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Contents

List of Figures

Abbreviations

BRCT----- BRCA1 –C-terminal

ATR-------ATM and Rad3 related

ATM------ Ataxia Telangiectasia mutated

9-1-1- -----Rad9-Hus1-Rad1

PIKKs ----Phosphoinositide -3 kinase-related family of kinases

TopBP1---Topoisomerase IIβ binding protein

PCNA-----Proliferating cell nuclear antigen

RFC----- Replication factor C

MRN-----Mre11-Rad50-Nbs1

MDC1--- Mediator of Chk1

RPA----- Replication protein A

53BP1---p53 binding protein

MMR----Mismatch repair

Msh2----MutS homologue 2

Msh6----MutS homologue 6

MNNG-- N-methyl-N'-nitro-N-nitrosoguanidine

MNU----- N-methyl-N-nitrosourea

MGMT--- O(6)-Methylguanine-DNA methyltransferase

NER-------Nucleotide excision repair

BER------Base excision repair

TMZ-----Temozolamide

MTT----- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

IPTG-----Isopropyl β-D-1-thiogalactopyranoside

GST----- Glutathione S-transferase

PVDF----Polyvinylidene fluoride

Chapter1: Introduction

A century ago Theodor Boveri had given a theory on pathogenesis of cancer, which in modern times is considered to have made a profound impact. While studying cell behavior in sea urchin, he proposed that the emergence of malignant tumors could be a result of an abnormal chromosome constitution (Boveri 2008). The assumption he made a century ago, that chromosome instability and chromosomal aberrations are responsible for cancer pathogenesis has been validated to be accurate. In modern biology these causative conditions now collectively called as 'genomic instability' has been found out to be a hallmark of most solid tumors (Hanahan and Weinberg 2011). Genomic instability is marked by presence of abnormal chromosome number and structure resulting from changes in DNA structure due to nucleotide insertion deletion and substitutions. The genetic instability occurs due to DNA damage and accumulation of mutations in the genes that guard the genome. The changes caused in DNA result in activation of oncogenes and inactivation of tumor suppressor genes resulting in cancer. It is extremely important to replicate the genome with accuracy before passing it on to the daughter cell (Ferguson, Chen et al. , Negrini, Gorgoulis et al. 2010, Shen 2011, Abbas, Keaton et al. 2013, Ferguson, Chen et al. 2015). To ensure this, eukaryotic cells have evolved an elaborate DNA damage response mechanism, which maintains the genomic integrity when under threat from exogenous or endogenous sources. DNA damage response pathway is a multifaceted signaling mechanism that coordinates cell cycle progression, DNA repair, chromatin remodeling, transcription activities and even cell death (Zhou and Elledge 2000, Jossen and Bermejo 2013). DNA damage induced checkpoints and DNA repair pathways coordinate the response to different kinds of DNA lesions, arrest the cell cycle till the DNA is repaired and if the damage is too high, trigger apoptotic fate of the cells. The physiological importance of checkpoint pathways is illustrated in patients with Ataxia Telangiectasia disorder in which two mutations in the ATM gene, result in loss of motor

control, immune deficiencies and high risk of cancer [reviewed in (McKinnon 2012)].

1.1 Organization of DNA damage response pathways

Checkpoints serve as surveillance mechanisms which monitor the progression of cell cycle [reviewed in (Weinert and Hartwell 1989)]. DNA damage checkpoints are one of the checkpoints which is activated in response to endogenous or exogenous sources of damage [reviewed in (Hartwell and Kastan 1994)]. The cell cycle is a series of well-defined events that leads to duplication of genetic material before being passed on to the daughter cells. Eukaryotic cell cycle is divided into four phases, G1, S, G2 and M. The G1 phase is the preparatory phase where the cell grows in size and accumulates proteins required for replication and cell division. In S phase the genetic material undergoes replication making exact copy of the genetic material to be equally distributed amongst the two daughter cells. The cells with duplicated genome enters the G2 phase where the cell is divided in to two daughter cells and in the final mitotic phase (M) the duplicated chromosomes are aligned in such a way that the two daughter cells receive the exact copy of the genome [reviewed in (Bartek, Lukas et al. 1999, Houtgraaf, Versmissen et al. 2006)[fig 1.1].

During each of these phases of the cell cycle, the eukaryotic genome is under threat from various sources that can damage the genetic material. The cell cycle is arrested at any phase when faced with damaged DNA structure(s). The four checkpoints that regulate cell cycle progression are G1/S, Intra S, G2/M and the spindle checkpoint [fig 1.1]. The checkpoints are activated in response to plethora of DNA lesions that can occur because of three main reasons. Firstly, there are number of environmental agents like ultraviolet light, ionizing radiations and various genotoxic chemicals that can alter DNA structure. Secondly the DNA can be altered due to byproducts generated from cellular metabolism like reactive oxygen species, hydroxyl ions and superoxide anions. Finally DNA can also be altered due to spontaneous

disintegration of some chemical bonds in DNA itself. Abasic sites generated by spontaneous hydrolysis of nucleotide bases and deamination of cytosine, adenine and guanine can result in bases, which mispair and alter DNA structure [reviewed in (Lindahl 1993, Cadet, Berger et al. 1997, Finkel and Holbrook 2000)]. The checkpoint proteins that detect various lesions are distinct but may overlap in different phases of the cell cycle.

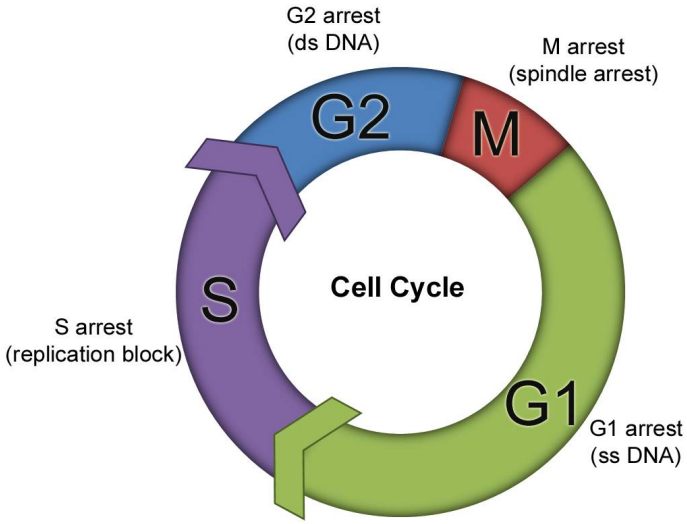

Fig 1.1: Schematic representation of cell cycle. The checkpoints are activated in response to different kinds of DNA damage encountered during different phases of the cell cycle.

The pattern of DNA damage response in every phase of cell cycle remains the same. In terms of space and time, DNA damage response pathway is defined as a signal transduction cascade, which consists of sensors, mediators and effectors proteins. Sensor proteins detect DNA lesions and the signal is then transferred to the mediator proteins, which amplify the signal and recruit other proteins onto the damaged sites. The mediator proteins also pass on the information to the effector kinases, which result in cell cycle arrest, DNA repair or apoptosis in case the damage is irreparable [reviewed in (Zhou and

Elledge 2000, Bartek and Lukas 2001, Nyberg, Michelson et al. 2002) [fig 1.2].

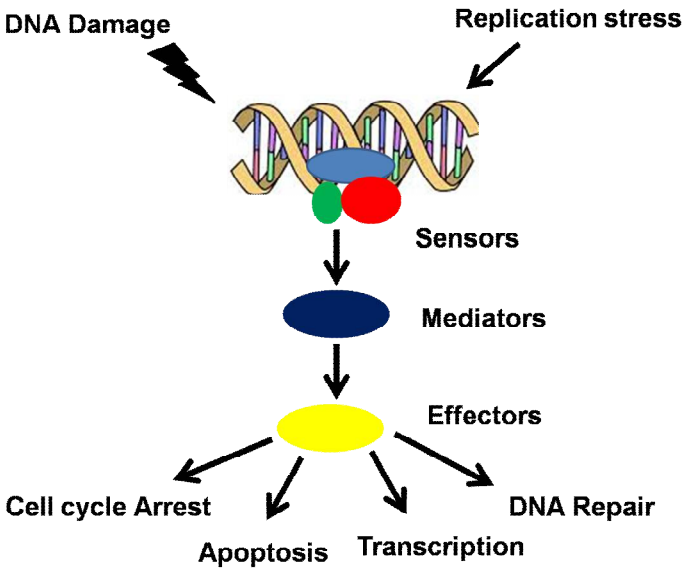

Fig 1.2: Organization of DNA damage response pathway in mammals. The general organization of DNA damage response pathway is depicted schematically showing sensors, transducers and effectors and the functions carried out by them in response to DNA damage. (Adapted from (Zhou and Elledge 2000)

1.1.1 DNA damage sensors and signal transducers

The recognition of DNA damage is the first and most important step in the DNA damage signaling cascade. DNA damage sensors have a gigantic task where they need to detect various types of DNA lesions and sometimes very low levels of DNA damage. Largely the identity of DNA damage sensors is not well understood. Initially Poly ADP ribose and DNA-PK were considered to be the sensors of DNA damage mainly owing to their capacity of binding to DNA breaks.

With advances in scientific knowledge, a group of four proteins have been identified that could possibly fall into the category of DNA damage sensing

proteins based on their ability to bind nucleic acids and activate DNA damage signaling. The trimeric complex of Rad9, Hus1 and Rad1 (9-1-1 complex) was first identified as sensor proteins in *Schizosaccharomyces pombe*. In both human and yeast, these proteins form a ring like structure which resemble the proliferating cell nuclear antigen (PCNA)-like sliding clamp (Volkmer and Karnitz 1999) [fig 1.4].

During DNA damage, the 9-1-1 complex is loaded onto the damaged site with the help of another conserved protein, Rad17. Rad17 was first identified in *Schizosaccharomyces pombe* and it shares homology with all five subunits of replication factor C (RFC). As PCNA is loaded on to the DNA by RFC_{1-5} during DNA replication, 9-1-1 complex is loaded on to the damaged site by a complex of proteins consisting of four subunits of RFC (2-5) and Rad 17 (O'Connell, Walworth et al. 2000). The 9-1-1 complex bound to the damaged site act as a scaffold for other proteins to be recruited on to the damaged site and may function in maintaining the checkpoint signaling. The DNA damage sensor proteins once bound to the chromatin relay the signal to the next set of proteins called the transducers. The two most important players acting as the signal transducers in the DNA damage signaling cascade are ATM and ATR. Ataxia Telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 related (ATR) are primarily serine threonine kinases belonging to the phosphoinositide -3 kinase-related family of kinases (PIKKs) [fig 1.3]. Homologues of ATM and ATR are present in all eukaryotic organisms including budding and fission yeast [reviewed in(Hunter 1995, Zakian 1995)].

ATM derives its name from a neurological disorder Ataxia Telangiectasia where the patients have neuro-motor disabilities, defective response to irradiation including G1 arrest, defective DNA synthesis and G2 arrest which results in high frequency of cancer (Shiloh 1997). ATM is a large protein with a molecular weight of 350 kDa containing elongation factor 3, a subunit of phosphatase 2 A and Tor 1 (HEAT) motifs and a kinase domain (Bakkenist and Kastan 2003, Perry and Kleckner 2003). ATM is activated in response to DSB, which cause a conformational change in ATM homodimer leading to the autophosphorylation of Serine 1981 present on ATM molecules. The complete activation of ATM occurs when the monomers of ATM are recruited to the

damaged DNA by Mre11-Rad50-Nbs1 (MRN complex) (Lee and Paull 2007). The ATM kinase once activated phosphorylates substrates in the close vicinity like γH2AX, MRN complex and the effector kinase Chk2 (Lukas, Falck et al. 2003, Kitagawa, Bakkenist et al. 2004).

ATR was identified as a molecule similar to ATM in sequence and function. ATR is a 300 kDa protein engaged in DNA damage signaling in response to stalled replication forks and single strand breaks caused by agents like ultraviolet light. Like ATM, ATR phosphorylates its substrates at serine and threonine positions followed by Glutamine (Banin, Moyal et al. 1998, Kim, Lim et al. 1999, Tibbetts, Brumbaugh et al. 1999, Abraham 2001). ATR with its obligate partner ATRIP is recruited to single stranded DNA coated with replication protein A (RPA) and undergoes autophosphorylation at threonine 1989 to launch the checkpoint response by phosphorylating Chk1 kinase (Cortez, Guntuku et al. 2001, Liu, Shiotani et al. 2011).

There are no known diseases caused by mutation of ATR (Cimprich, Shin et al. 1996). However, sometimes ATR mutation in humans is linked with a rare condition Seckel syndrome characterized by growth retardation and microcephaly (O'Driscoll, Ruiz-Perez et al. 2003). Mec1, the ATR homologue in *Sacharomyces cerevisiae* is also required for recovery from DNA replication stress. Thus far it has been suggested that ATR is required for normal progression of DNA replication (Desany, Alcasabas et al. 1998).

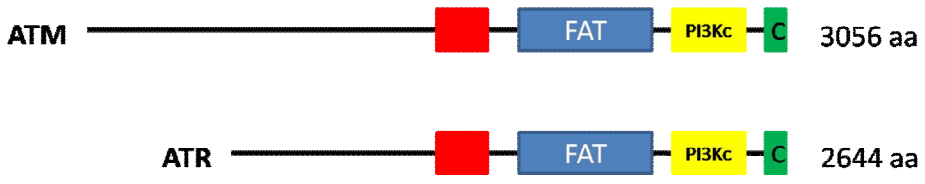

Fig 1.3: Schematic showing homology between ATM and ATR kinase. ATM and ATR kinase show homology in the domains found in both the proteins. The catalytic domain of the PIKK is located in the c-terminal region. Adapted from (Abraham 2001).

1.1.2 Mediators

An additional class of proteins exists that amplify damage signal and maintain checkpoint activation. Mediator proteins as they are called are cell cycle specific and interact with different sensors and proximal kinases to bring about lesion and cell cycle specific checkpoint activation [fig1.4]. Mediator proteins are downstream of the apical kinases, ATM and ATR. These proteins aid in the recruitment and assembly of other complexes. p53 binding protein (53BP1), Mediator of Chk1 (MDC1), Topoisomerase IIβ binding protein (TopBP1) and BRCA1 are some of the well known mediators working downstream of ATM and ATR. An important feature of mediator proteins is the presence of multiple BRCA1 C- terminal (BRCT) domains which facilitate multiple protein-protein interactions to recruit other proteins on the damaged site [reviewed in (Coster and Goldberg 2010)]. MDC1 is a downstream protein of ATM functioning in DNA damage signaling following double strand break. MDC1 is important for ATM signaling and is the first protein to bind to phosphorylated YH2AX which facilitates the recruitment of other checkpoint proteins- Mre11,Nbs1, Rad50 to mediate checkpoint activities (Paull, Rogakou et al. 2000, Goldberg, Stucki et al. 2003, Stewart, Wang et al. 2003).

p53 binding protein (53BP1), considered to be the orthologue of scRad9/spCrb2 is required in DNA damage signaling. 53BP1 contains tandem BRCT domains, a common feature among mediator class of proteins in checkpoint signaling. The two most important functions of 53BP1 are to recruit DSB responsive proteins on the damaged sites and amplify ATM signals in response to low levels of DSB (DiTullio, Mochan et al. 2002, Shibata, Barton et al. 2010).

Topoisomerase IIβ binding protein (TopBP1) was first identified as binding partner to Topoisomerase IIβ in a yeast two hybrid screen (Yamane, Kawabata et al. 1997). Human TopBP1 is essential for ATR mediated checkpoint activation as the absence of TopBP1 abrogates Chk1 phosphorylation and cells show defective G2/M checkpoint (Yamane, Chen et al. 2003).

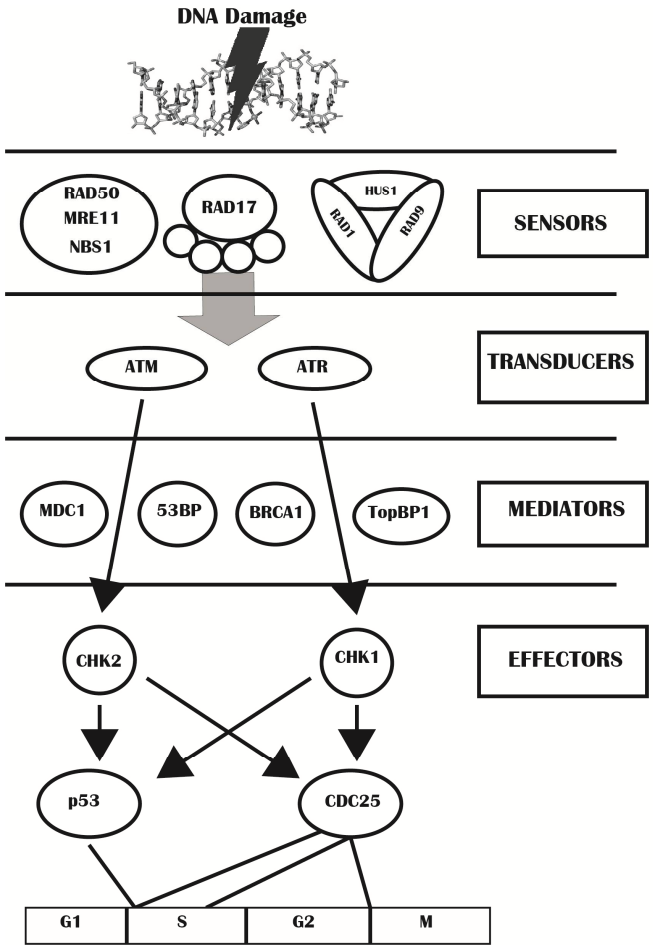

Fig 1.4: Components of DNA damage response pathways in Humans. Schematic of two canonical pathways of checkpoint activation showing sensors, mediators and effector kinases. Adapted from (Houtgraaf, Versmissen et al. 2006).

1.1.3 Effector kinases

The effector kinases Chk1 and Chk2 are downstream of ATR and ATM respectively, which relay the DNA damage signal in the cell through phosphorylation [fig1.4]. Human Chk1 and Chk2 (Rad53/Cds1) kinases were first discovered in yeast, all of which are required for DNA damage response (Walworth, Davey et al. 1993, Murakami and Okayama 1995). Chk1 and

Chk2 kinases are structurally unrelated serine/threonine kinases with overlapping functions in DNA damage signaling cascade (Bartek, Falck et al. 2001, McGowan 2002). In both, yeasts and mammals Chk1 is phosphorylated by ATR in response to damage caused by ultraviolet light or stalled replication forks, while Chk2 is activated by ATM kinase in response to double strand breaks formed due to IR radiation.

In humans, cancers associated with defects in Chk1 are extremely rare and the few known are limited to the carcinomas of colon, endometrium and stomach (Bertoni, Codegoni et al. 1999, Menoyo, Alazzouzi et al. 2001). Chk2 mutations in humans resulting in reduced or no kinase activity is associated with high incidence in familial as well as sporadic cancers of breast, prostate, ovarian, kidney, thyroid and bladder (Wu, Webster et al. 2001).

Chk1 is activated largely in S and G2 phases of cell cycle in response to single strand breaks and replication blocks. Upon DNA damage Chk1 is phosphorylated on multiple residues including Ser 345 and 317. The phosphorylated Chk1 is released from the chromatin and gets accumulated at the centrosomes, where it prevents CDK1 activation and entry into mitosis (Löffler, Lukas et al. 2006, Smits 2006). In contrast to Chk1, Chk2 is activated by double strand breaks throughout the cell cycle. Chk2 exists as monomers in an unperturbed cell cycle, upon DNA damage Chk2 is phosphorylated at the SQ/TQ motifs by ATM kinase.

The phosphorylated Chk2 (Thr 68) readily oligomerizes with another Chk2 molecule facilitating its trans autophosphorylation and kinase activation (Lee and Chung 2001, Ahn, Li et al. 2002, Xu, Tsvetkov et al. 2002).

Chk2 and Chk1 phosphorylates a number of nuclear proteins involved in various aspects of DNA damage response. The main targets for Chk1 include the Cdc25 phosphatases which when phosphorylated arrest the cell cycle progression. Another target known for Chk1 is p53 a transcription factor which promotes apoptosis in response to DNA damage occurring in cells undergoing transition from G1 to S phase (Chehab, Malikzay et al. 2000, Shieh, Ahn et al. 2000). A number of substrates are known for Chk2, many of which fall in four major functional categories performing DNA repair, cell

cycle regulation, apoptosis and p53 signaling (Seo, Kim et al. 2003). Some of the targets are shared by Chk2 and Chk1 which include repair protein BRCA1, p53 which is involved in apoptosis, Cdc25A for cell cycle regulation (Niida, Katsuno et al. 2007).

The DNA damage response proteins described above are active throughout the cell cycle. The effector kinases are responsible for activating the different checkpoints depending on the phase of cell cycle.

1.2 ATR signaling at a glance

Ataxia Telangiectasia and Rad3 related (ATR) belongs to the family of phosphoinositide -3 kinase like protein kinases (PIKK). ATR is the major proximal kinase activated in response to DNA damage in S and G2 phase of the cell cycle. ATR responds to various kinds of DNA damaging agents, which result in formation of single strand DNA and stalling of the replication fork. ATR is an essential kinase playing a crucial role in DNA replication and it maintains genomic integrity in stressed conditions [reviewed in (Brown and Baltimore 2003)].

In an unperturbed cell cycle, there are sources of endogenous DNA damaging agents, which can stall replication forks and activate ATR signaling. The eukaryotic genome is large and replication is initiated from multiple sites to complete the replication of genome before the cell divides. During replication, DNA is sufficiently exposed to damage by chemicals, which could result in base modifications, nicks and crosslinks. On most occasions these modifications are repaired by repair enzymes before the replication fork reaches the damaged bases, but on some occasions when they are not repaired, the modifications result in uncoupling of DNA polymerase and helicase resulting in formation of single strand DNA, a state which needs to be rescued by checkpoint activation. ATR signaling post activation stabilizes the replication fork for accurate replication of DNA. In mammals, the frequency of fork stalling is pretty high due to the large size of the genome[reviewed in (Jossen and Bermejo 2013)].

1.2.1 ATR activation pathway

The activation of ATR is an orchestrated event involving various protein components [fig1.5]. In eukaryotes the single stranded DNA generated during replication stress is actively recognized by replication protein A (RPA) which immediately coats the single strand DNA. ATR and its obligate binding partner ATR interacting protein (ATRIP) is localized to the RPA coated single stranded DNA [reviewed in (Zou and Elledge 2003, Ünsal-Kaçmaz and Sancar 2004)]. ATR and ATRIP are associated with each other independent of DNA damage and ATRIP is required for stability, localization and activation of ATR. (Cortez, Guntuku et al. 2001). Meanwhile, independent of ATR-ATRIP complex, another complex of three proteins Rad9-Rad1-Hus1 (9-1-1) is loaded onto the damaged site. The RPA coated single strand DNA recruits 9-1-1 complex on to the 5' site of single strand/double strand (ss/ds) junction (Parrilla-Castellar, Arlander et al. 2004). The 9-1-1 complex forms a ring like structure that resembles the sliding clamp of a proliferating cell nuclear antigen (PCNA). Rad17- RFC_{2-5} acts like a clamp loading complex which helps in loading of 9-1-1 complex onto the chromatin (Shiomi, Shinozaki et al. 2002). Localization of ATR-ATRIP onto the damaged site and recruitment of 9-1-1 complex does not activate ATR. An essential component required for ATR activation is TopBP1 recruitment, which depends upon 9-1-1 complex. TopBP1 is recruited to the sites of damage by Rad 9 of 9-1-1 complex. The C-terminal region of Rad9 is phosphorylated at Ser 387 position which creates a binding site for BRCT I and II domains of TopBP1, thereby recruiting TopBP1 to ATR (Furuya, Poitelea et al. 2004, Delacroix, Wagner et al. 2007, Lee, Kumagai et al. 2007).

TopBP1 contains a putative ATR activating domain (AAD), which resides between BRCT VI and VII domain of TopBP1. Over expression of AAD can result in activation of ATR *in vitro* in absence of DNA damage (Kumagai, Lee et al. 2006). ATR undergoes autophosphorylation at Thr 1989 when it is recruited to RPA coated ssDNA. TopBP1, which is independently recruited to damaged site by Rad9 interacts with ATR through its BRCT VII and VIII

region enabling the AAD domain of TopBP1 to fully activate ATR (Liu, Shiotani et al. 2011).

ATR mediated activation of effector kinases results in cell cycle regulation and maintenance of origin of firing and fork progression during replication stress. Chk1 is the major target of phosphorylation by ATR in response to DNA damage. Phosphorylation of Chk1 at multiple sites including Ser 317 and 345 is a trademark of ATR activation. Phosphorylated Chk1 spreads in the nucleus and relays the signal to other proteins involved in cell cycle regulation (Lukas, Falck et al. 2003, Smits, Reaper et al. 2006, Smith, Tho et al. 2010).

Recent studies have identified novel interactors of ATR kinase, which are required for ATR localization to the chromatin and stimulation of its kinase activities. ATRIP has long been known as the obligate partner of ATR, which is extremely important for localization of ATR to the RPA coated ssDNA following DNA damage. RPA-coated ssDNA is recognized by the checkpoint recruitment domain (CRD) of ATRIP. CRD domain binds to the basic cleft in the 70 subunit of RPA protein bound to the single strand DNA. Mutations in any of the domains of ATRIP or RPA 70 prevents recognition of single strand DNA but does not affect checkpoint signaling (Ball, Ehrhardt et al. 2007). These observations led to the discovery of novel interactors of ATR-ATRIP complex, which aids in their recruitment on to the damaged sites.

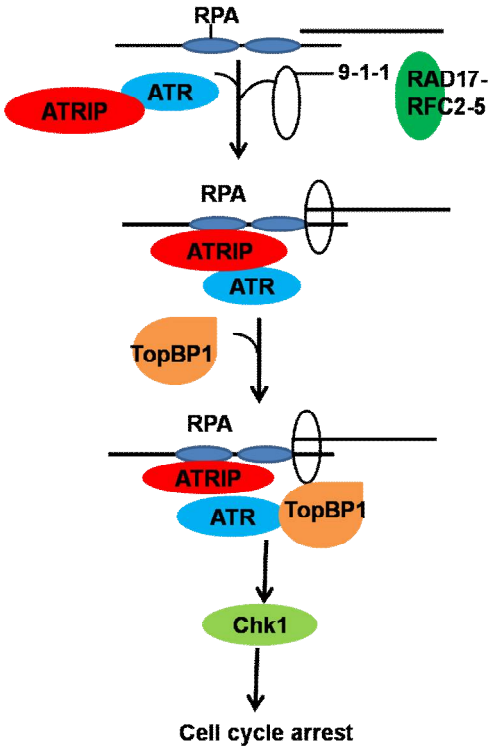

Fig 1.5: ATR signaling in humans. ATR-ATRIP and 9-1-1 complexes are independently recruited to single strand breaks or sites of replication blocks. Rad9 binds TopBP1 through its BRCT I+II domain and is recruited to DNA that brings TopBP1 closer to ATR leading to activation of ATR kinase. ATR kinase then phosphorylates the effector kinase Chk1 leading to cell cycle arrest. Adapted from (Cimprich and Cortez 2008)

Depending on the DNA damaging agents and lesions caused, ATR can interact with multiple proteins and mediate checkpoint signaling. In response to DNA damage caused by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) mismatch repair proteins MutS and MutL pair up with ATR and mediate checkpoint signaling (Stojic, Mojas et al. 2004). DNA base adducts generated by MNNG undergo processing by mismatch repair proteins resulting in RPA-coated ssDNA formation which may recruit ATR. Another possibility exists where mismatch repair proteins could interact with ATR by direct protein-protein interaction and mediate checkpoint signaling. Msh2 can bind ATR and recruit on to the chromatin when damaged by cisplatin. MutSα component of mismatch repair is known to bind multiple proteins in ATR-Chk1 pathway

including TopBP1 and Chk1 (Wang and Qin 2003) (Yoshioka, Yoshioka et al. 2006).

1.3 TopBP1 is a scaffold protein in checkpoint signaling

The BRCA1 C-terminal (BRCT) domain containing Topoisomerase IIβ binding protein (TopBP1) is evolutionary conserved with its homologues present across animal kingdom. Studies with mutants of TopBP1 in lower eukaryotes have helped in understanding the role of TopBP1 in DNA replication, DNA repair and checkpoint signaling.

1.3.1 TopBP1 and its homologues

TopBP1 was first identified in *Schizosaccharomyces pombe* in a screen to identify mutants that were sensitive to ultraviolet radiation. Rad 4^{TopBP1} mutants in *S. pombe* showed temperature sensitive lethal phenotype, decreased mutation frequency and unlike the wild type were not sensitive to DNA damage caused by caffeine (Hirano, Funahashi et al. 1986). $Rad4^{TopBP1}$ was also identified independently in a cut mutant screen and was known as Cut5. The cell untimely torn (cut) mutants are known to block the nuclear division but not the cytokinesis, which causes septum to cut through the nucleus, hence the name. Both the mutants were shown to play an important role in initiation of DNA replication and S-M checkpoint (Gómez, Angeles et al. 2005).

$DPB11^{TopBP1}$ in *Saccharomyces cerevisiae* was identified as a multicopy suppressor of the temperature sensitive mutation in Polε subunit, Dpb2 (Garcia, Furuya et al. 2005). DPB11 mutants in budding yeast showed premature mitotic entry of cells from when grown at the restrictive temperature. They were also sensitive to treatment with hydroxyurea, ultraviolet light and MMS. Dpb11 functions in initiation of DNA replication in budding yeast. It was observed in DPB11 mutants, the pre-replicative complex (MCM and RPA) binds to the chromosome but Polα-primase are not loaded

on the origins. Therefore DNA synthesis does not begin in such mutants (Masumoto, Sugino et al. 2000).

Mus101, the *Drosophila* counterpart of TopBP1, was first identified 25 years ago in a screen, identifying mutants sensitive to DNA damaging agents. Genetic analysis of Mus101 mutants has shown it to be essential for survival. These mutants exhibited defects in S phase checkpoint activation and initiation of DNA replication (Yamamoto, Axton et al. 2000). TopBP1 depletion studies done in *Xenopus* egg extracts showed that *Xenopus* TopBP1 is required for loading of pre-initiation complex (Cdc45 and RPA) but not the pre-replication components (Cdc6, ORC, Cdt1 etc) (Van Hatten, Tutter et al. 2002). Human TopBP1 was later identified in a yeast two-hybrid system as an interactor of Topoisomerase IIβ, however the functional relevance of the interaction is yet to be proven (Yamane, Kawabata et al. 1997). Human TopBP1 is required for loading of Cdc45 to start DNA synthesis during S phase of the cell cycle (Kim, McAvoy et al. 2005). Depletion of mouse TopBP1 results in early embryonic lethality. All these studies suggest that functions of TopBP1 are conserved in all the eukaryotic organisms.

1.3.2 Domain architecture of Human TopBP1

Human TopBP1 shares sequence homology with Dpb11 (*Sacharomyces cerevisiae*), Rad4 (*Schizosacharomyces pombe*), Mus101 (*Drosophila melanogaster*) and Cut5 (*Xenopus*) [fig 1.6]. Human TopBP1 and its homologues are scaffold proteins involved in various cellular processes through multiple protein-protein interactions made possible by their BRCT domains.

However the structure and organization of the BRCT domains in each of the proteins is different conferring them with versatility required to perform a variety of functions.

Human TopBP1 contains nine BRCT domains, which is about twice the size of its counterparts found in yeasts. Both Rad4 ^{TopBP1} and Dpb11 ^{TopBP1} have

four BRCT domains arranged in pairs of two domains (BRCT I+II and BRCT III+IV) (Garcia, Furuya et al. 2005). However in hTopBP1 BRCT domains III and VI occur as singletons while BRCT domains 0, I and II form a non canonical pair and IV+V domains and VII and VIII constitute the other canonical pairs of BRCT domains (Garcia, Furuya et al. 2005) [fig 1.5].

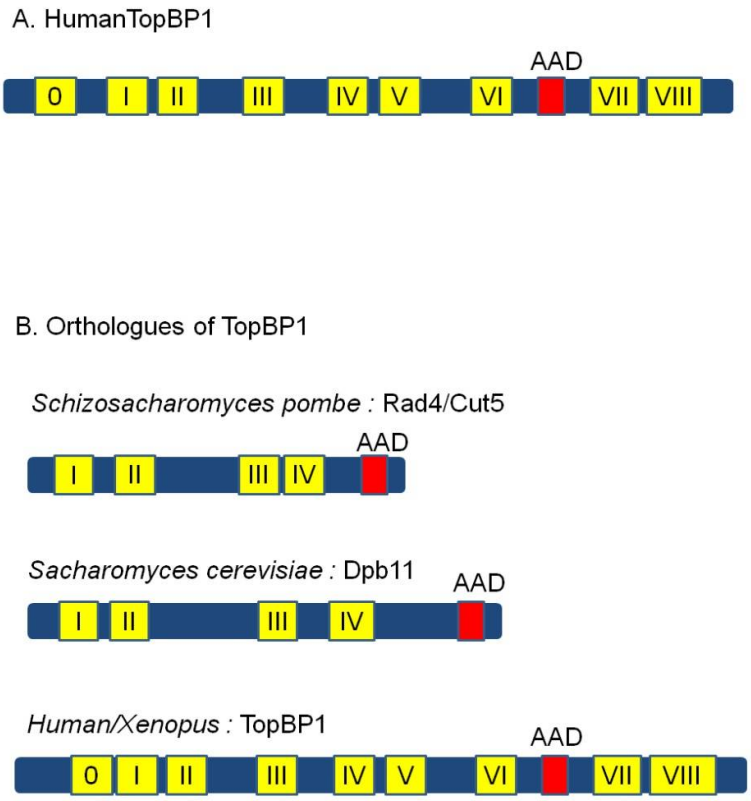

Fig 1.6: Schematic representation of TopBP1 and its homologues in other organisms. TopBP1 is a scaffold protein containing BRCA1 c-terminal (BRCT) domains. These domains are conserved among other homologues of TopBP1 found in yeast and *Xenopus*. Adapted from (Wardlaw, Carr et al. 2014)

In humans, the arrangement of β sheets of the BRCT I and II pair is perpendicular to each other which is also found in its yeast orthologue (Rad4/Dpb11) providing perfect docking site for phospho-protein binding (Rappas, Oliver et al. 2011). BRCT domain 0 closely associates with BRCT I and II domains to form a unique array of three BRCT domains not found in

any other checkpoint protein. BRCT 0 is predicted to stabilize the unusual pairing of BRCT I and II domains.

Human TopBP1 BRCT IV and V pair is homologous in structure to BRCT III and IV of its yeast orthologue (Rad4/Dpb11). The BRCT IV and V domain pair adopts a structure in a way to keep the central β sheets antiparallel with respect to each other (Qu, Rappas et al. 2013). BRCT IV domains lack the signature amino acids required for phospho-protein binding (Leung, Sun et al. 2013). The BRCT VII and VIII pair has a canonical structure that of BRCT domains as seen in *Schizosacharomyces pombe* Crb2^{53BP1}, mammalian MDC1 and BRCA1. The sequence of polar and charged residues in BRCT VII domain forms a docking site for phosphorylated serine and threonine in the interacting protein (Kilkenny, Dore et al. 2008, Rappas, Oliver et al. 2011). On the other hand the VIII domain has β sheets that are arranged parallel to each other and provide a separate binding groove for sequence specificity. Such an arrangement is useful in binding various proteins required for checkpoint function.

The singletons BRCT III and VI of human TopBP1 are unique and do not carry the signature amino acids present in other BRCT domains (Leung, Kellogg et al. 2010). BRCT VI is known to bind to the transcription factor E2F1 and also Poly (ADP Ribose) Polymerase 1 (PARP-1) (Liu, Paik et al. 2006) (Wollmann, Schmidt et al. 2007).

In addition to regular BRCT domains TopBP1 and its homologues also contain an ATR activating domain (AAD). In humans and *Xenopus* the putative AAD is situated between BRCT VI and VII domain while in fission and budding yeast it is found in the unstructured C-terminal region The AAD domain regulates the activation of ATR kinase in response to DNA damage and provides a new layer to various functions that TopBP1 can perform (Mordes, Glick et al. 2008).

1.3.3 TopBP1 - the interaction hub in DNA damage checkpoint

The various BRCT domains in TopBP1 protein provide a scaffold for different proteins to interact with TopBP1 and regulate various processes like DNA replication, checkpoint activation and DNA repair. TopBP1 interacts with

different proteins and is recruited to the damaged site, which can be visualized as foci. Most scaffold protein like TopBP1 remain bound to the chromatin and interact with different proteins till the damaged site is repaired. When a cell is under replication stress, TopBP1 is recruited to the damaged site through an interaction with c-terminus of Rad9.

In *S pombe*, the BRCT III and IV pair of Rad4 ^{TopBP1} interacts with Rad9 phosphorylated at Ser 423 and Thr 412 which recruits a mediator protein Crb ^{53BP1} and induces checkpoint activation (Furuya, Poitelea et al. 2004) (Taricani and Wang 2006). Similarly in *S. cerevisiae*, Dpb11^{TopBP1} is recruited to damaged sites and form foci in G1, S and G2 phases of cell cycle. In budding yeast, the complete checkpoint activation is achieved through a series of events involving phosphorylation of Ddc1^{Rad9} at Thr 602 by Mec1^{ATR}, followed by interaction of Dpb11^{TopBP1} with the phosphorylated Ddc1^{Rad9} which then helps in recruiting *Sc*Rad9^{53BP1} to complete the process (Germann, Oestergaard et al. 2011) (Pfander and Diffley 2011) (Puddu, Granata et al. 2008). In humans, the interaction between TopBP1 and Rad9 of 9-1-1 complex is mediated by BRCT I and II pair of TopBP1 and phosphorylated C-terminus of Rad9 (Rappas, Oliver et al. 2011). Rad9 is constitutively phosphorylated by casein kinase II at Ser 487 in C-terminus of Rad 9. Additionally BRCT IV and V domains of TopBP1 are also required for its recruitment to the damaged sites. The BRCT V domain interacts with MDC1 protein and amplifies the damage signal generated during double strand break formation. TopBP1 specifically binds to 53BP1 through the BRCT IV and V domain and the interaction is required for complete G1 checkpoint response (Cescutti, Negrini et al. 2010).

The BRCT VII and VIII domains in the higher eukaryotes are capable of protein –protein interaction. In human TopBP1, BRCT domains VII and VIII are capable of binding to phosphorylated BACH1 and triggering ATR mediated checkpoint activation [reviewed in (Wardlaw, Carr et al. 2014)]. The complexity and plasticity to bind different proteins through BRCT domains still needs to be explored. Under different kinds of DNA damage, TopBP1 binds to various proteins through its BRCT domains. Interestingly, in *Xenopus* egg extracts, TopBP1 is recruited to ss/ds junctions independent of 9-1-1

complex but dependent on its interaction with MRN complex (Duursma, Driscoll et al. 2013). BRCT domains I and II of TopBP1 were found to interact with Nbs1 of MRN complex in response to double strand breaks (Lee and Dunphy 2013). It is intriguing to understand the various roles that TopBP1 can perform by regulating interaction with its partner proteins.

1.3.4 TopBP1 mediates ATR activation

In addition to binding DNA damage sensors, mediators in checkpoint signaling, TopBP1 performs the most crucial step in ATR mediated signaling. It was first described using *Xenopus* egg extracts, over expression of the AAD of TopBP1 in humans or in *Xenopus* egg extracts can lead to activation of ATR independent of DNA damage (Kumagai, Lee et al. 2006).

The sequence of AAD is not conserved in yeasts and humans and the region following the BRCT III and IV domain of $Dpb11^{TopBP1}$ and $Rad4^{TopBP1}$ in budding and fission yeast is required for $Rad3^{ATR}$ and $Mec1^{ATR}$ activation respectively. Two additional AAD domains present in $Ddc1^{Rad9}$ and Dna2 in *S. cerevisiae* can also activate $Mec1^{ATR}$ (Navadgi-Patil, Kumar et al. 2011) (Navadgi-Patil and Burgers 2008) (Majka, Niedziela-Majka et al. 2006). In contrast in *S. pombe,* the AAD of $Rad4^{TopBP1}$ is required for activation of $Rad3^{ATR}$. Currently there is no evidence of existence of AAD in homologues of Dna2 or Rad9 in *S. pombe.* The sequence alignment of *S. cerevisiae* and *S. pombe* does not reveal a similar AAD in Dna2 or Rad9 of *S. pombe*. (Lin, Wardlaw et al. 2012).

A speculative model for TopBP1 mediated ATR activation can be put forth as below: ssDNA coated RPA recruits ATR-ATRIP on the chromatin. 9-1-1 and MRN complex can both recruit TopBP1 on to the damaged site. ATR is autophosphorylated at Thr 1989 and makes contact with BRCT VII and VIII of TopBP1. The AAD of TopBP1 and PIKK kinase regulatory domain (PRD) of ATR are brought closer by the interaction between N-terminal regions of TopBP1 with ATRIP, which stimulates the kinase activity of ATR. ATR phosphorylates TopBP1 at Ser 1131, which in turn amplifies the signal and further stimulates ATR kinase [fig 1.7].

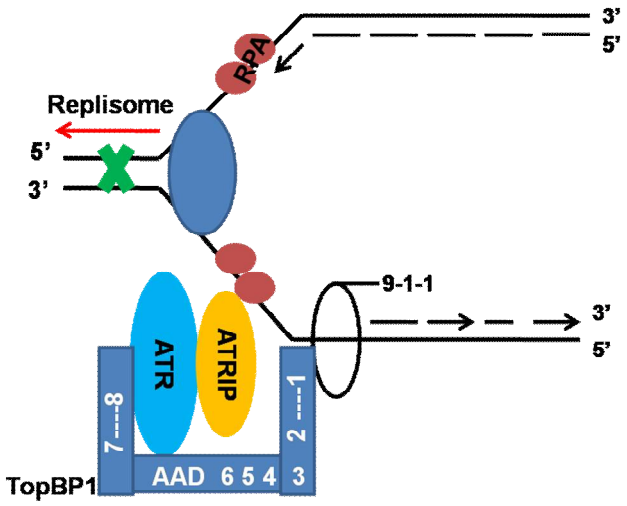

Fig 1.7: ATR activation in response to replication block. A stalled replication block creates single strand/double strand junction which are coated by replication protein A (RPA). ATR-ATRIP and 9-1-1 complex along with TopBP1 are recruited to the damaged sites. The ATR activating domain (AAD) of TopBP1 directly activates the ATR kinase. Adapted from (Wardlaw, Carr et al. 2014)

1.4 DNA damage response to alkylation damage

Eukaryotic genome is under constant threat of being damaged by various agents present in the environment and within the cell itself. Alkylating agents modify DNA bases by transferring alkyl group thereby altering the structure and function of the DNA. Alkylating agents are ubiquitous in the environment as well as within the cell itself. External sources of alkylating agents include food, air, water, biological byproducts such as abiotic plant material and pollutants such as from tobacco smoke, fuel combustion products [reviewed in (Ballschmiter 2003) (Hecht 1999)]. Alkylating agents are present inside the cells in the form of S-adenosyl methionine, which is a common co factor in biochemical reactions; byproducts of oxidative damage can also generate alkylating agents, which are toxic for the cells. In spite of being cytotoxic and carcinogenic, alkylating agents have been used as chemotherapeutic agents in order to kill cancer cells. The complex biological process activated in response to variety of DNA lesions caused by alkylating agents is not clearly

understood. Therefore it is important to understand the DNA damage response pathways being activated in order to improve cancer therapy. Various DNA repair mechanisms and DNA damage response factors are activated in response to alkylating agents which together define the sensitivity and resistance towards alkylating agents.

1.4.1 Molecular DNA damage caused by SN_1 Alkylating agent

Alkylating agents transfer alkyl groups to oxygen and nitrogen of the DNA bases forming adducts which can be either mutagenic or cytotoxic. The pattern of alkylation depends on the number of active group in the agent (mono or bifunctional), its chemical reactivity (SN_1 or SN_2 mechanism of nucleophilic substitution) and the kind of alkyl group being added and the DNA substrate itself (DNA/RNA) (Engelward, Allan et al. 1998). SN_1 alkylating agents target the nitrogen atoms in the bases and the extracyclic oxygen atoms to cause DNA damage while SN_2 alkylating agents target only the nitrogen atoms in the bases.

Most anticancer agents used are SN_1 alkylating agents, which can be either mono or bifunctional. Monofunctional agents have one active moiety that can be transferred on the nucleobases while bifunctional agents have two active groups available for modifying the bases. Methylating agents like N-methyl-N-nitrosourea (MNU) undergo non-enzymatic degradation to form carbonium ions which follow SN_1 mechanism to form 12 base adducts and phosphotriester bond. The carbonium ion is electrophillic which covalently binds to nucleophillic sites on DNA forming base adducts [reviewed in (Fu, Calvo et al. 2012)].

SN_1 type monofunctional agents like MNU add methyl group on the N7 of guanine (N7meG) , N3 of adenine (N3meA), N3 of guanine (N3meG) or O6 of guanine (O6meG) forming base adducts with different stabilities [reviewed in (Beranek 1990)] [fig 1.8]. The N7 position of the guanine is highly nucleophilic, resulting in the formation N7 meG base adduct. N7meG adducts accounts for around 80 % of the DNA lesions formed by SN_1 methylating

agent. N3meA accounts for 10-20 % of the base adducts and highly cytotoxic owing to their property of blocking DNA polymerases thereby inhibiting DNA synthesis. O6meG are the most cytotoxic base adducts formed though they only account for 0.3 % of the total base adducts formed following methylation [reviewed in (Kondo, Takahashi et al. 2010)].

1.4.2 DNA repair of lesions formed by SN_1 alkylating agent

The SN_1 alkylating agents like N-methyl-N-nitrosourea (MNU) cause a variety of DNA lesions, which lead to a complex cellular response. In order to repair the variety of lesions caused by alkylating agents, different repair pathways are recruited to the site of damage. The repair pathways and DNA damage response pathways collectively modulate cellular response towards alkylating agent.

1.4.2.1 Base excision repair for N-methylations

Base excision repair (BER) is activated in response to the N7meG, N3meA and N3meG adducts formed following methylation. Alkyladenine DNA glycosylase (Aag) specific for the N-methylation removes the N methylated base adducts resulting in formation of abasic sites (Wyatt, Allan et al. 1999). These sites are recognized by apurinic/apyrimidinic endonuclease (APE1) that excises the damaged strand. This leaves behind a 3'OH and 5'deoxyribosephosphate (5'dRP) groups at the margins. The gap generated by excising the damaged strand is refilled by DNA synthesis by DNA pol-β, which also removes the cytotoxic 5'dRP (Sobol and Wilson 2001). The cytotoxic 5'dRP can also be removed by lyase activity of DNA pol-λ or DNA pol-ι. Finally the nick is sealed by DNA ligase I or a complex of DNA ligase III and XRCC1 (Wilson, Sobol et al. 2000). Absence of any of the components of BER pathways makes cells more sensitive to alkylating agents (Sobol, Prasad et al. 2000) [fig1.9 (A)]. For example, absence of polβ makes the cells hypersensitive to methylmethanesulfonate and mouse fibroblast cells deficient in pol β are sensitive to monofunctional alkylating agents (Bebenek, Tissier et al. 2001).

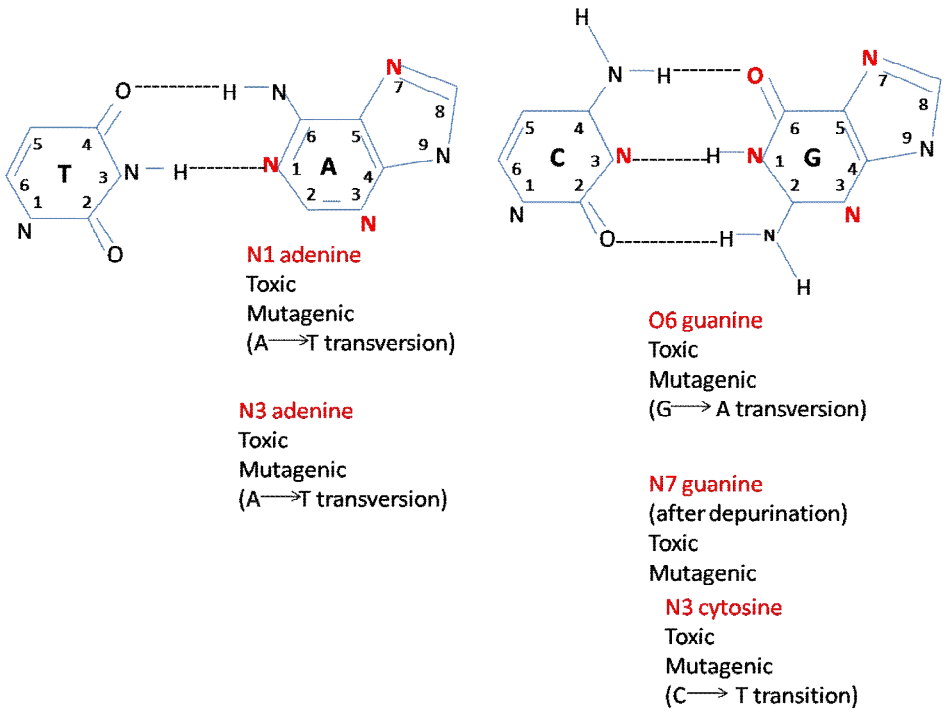

Fig 1.8: Sites of alkylation. The nitrogen and the exocyclic oxygen atoms in DNA bases are alkylated by SN_1 alkylating agents. Adapted from (Fu, Calvo et al. 2012)

1.4.2.2 Removal of O6 methylations by Methylguanine methyltransferase (MGMT)

MGMT removes O6 alkylation adducts in a single step reaction by transferring the alkyl group from the oxygen atom in the DNA to the cysteine residue in the catalytic pocket of MGMT. This reversal of alkyl group not only leads to restoration of DNA but it also inactivates the enzyme, hence MGMT is known as the suicide enzyme [fig 1.9 (B)]. In absence of DNA damage, MGMT is localized in the cytoplasm and is translocated in to nucleus upon alkylation damage. MGMT is more efficient in removing methyl groups than the ethyl or isopropyl group from the oxygen molecules. The inactivated MGMT is then ubiquitinated and undergo proteasomal degradation (Kaina, Christmann et al. 2007). MGMT status of the cell determines the sensitivity towards methylating agent As the MGMT proficient cells are efficient in removal of O6 methylations as compared to MGMT deficient cells (Hampson,

Humbert et al. 1997). O6meG can mispair with thymine if not repaired by MGMT. O6meG is not a bulky lesion hence it does not block replication. It is subjected to translesion synthesis, which can lead to mispairing of O6meG to thymine.

1.4.2.3 Mismatch repair (MMR)

MGMT does not discriminate between the O6meG or O6meG:T therefore the O6methyl adduct repair can occur post replication. The mispair left behind by MGMT can be subjected to MutS α mediated mismatch repair. Mismatch repair mediated removal of O6 methylation is essential in cells, which have low levels of MGMT or are deficient in MGMT due to silencing of MGMT promoter.

Mismatch repair as the name suggests removes base mismatches caused during DNA replication or induced by deamination, oxidation and methylation of bases. MMR is directed exclusively to the newly synthesized strand containing the mismatches. The base mismatches are recognized by MutSα complex comprising of Muts homolog 2 (Msh2) and Muts homolog 6 (Msh6). MutSα complex bound to the mismatch undergoes ATP driven conformational change thereby converting it into a sliding clamp that can move along the DNA [reviewed in (Jiricny 2006, Li 2008)].

Another complex MutLα comprising of Mlh1 and Pms2 binds to the MutSα-DNA-ATP complex in an ATP dependent manner. This complex translocates on the DNA in search for strand discontinuity, as MMR will excise the newly synthesized strand. The newly synthesized strand will be discontinued on some stretches due to formation of okazaki fragments during synthesis of lagging strands. MutSα complex bound to the DNA recruits an exonuclease, which can degrade a stretch of nucleotides starting from 5' end of a mismatch. MutSα complex can also bind proliferating cell nuclear antigen (PCNA) and direct degradation of nucleotides in 3' direction from the mismatch. When the stretch of DNA including the mismatch is degraded by the exonuclease, a new

strand devoid of mismatch is synthesized by DNA polymerase δ and the DNA ligase finally seals the nick completing the mismatch repair [reviewed in (Kunkel and Erie 2005) (Jiricny 2006) [fig 1.9 (C)].

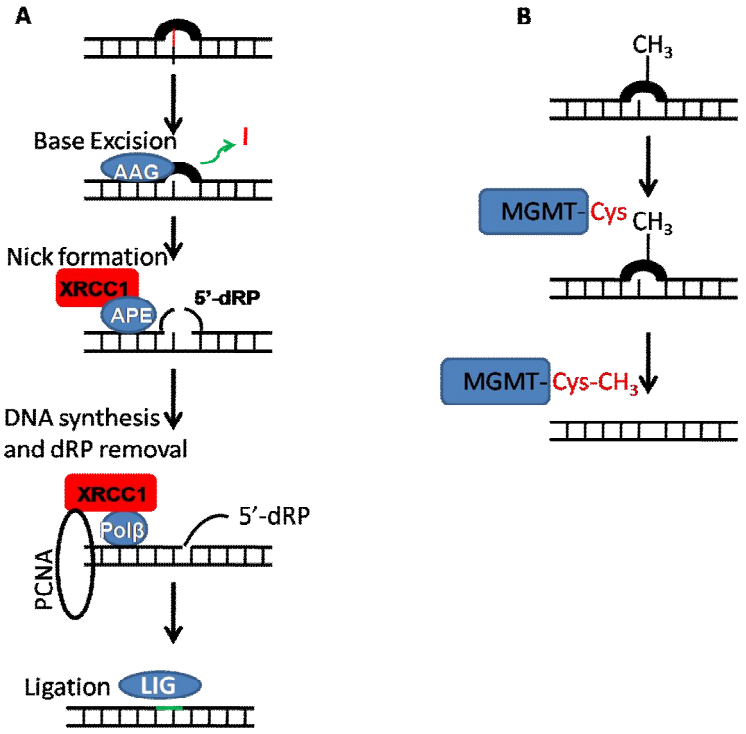

Fig 1.9: Various repair mechanisms activated in response to alkylation damage. A) Base excision repair (BER) removes alkylated bases by employing DNA glycosylase and re-synthesizing the new strand using DNA polymerases generating a repaired DNA strand and B) Methylguanine transferase (MGMT) directly removes alkylated bases by transferring the alkyl group on to the enzyme itself. Adapted from (Fu, Calvo et al. 2012)

In case of methylating agents, MNU or MNNG cause O6 methylations, which if not repaired by MGMT, can lead to mispairing of O6 meG with thymine. The O6meG:T mispairs are recognized by MutSα and it tries to correct the mismatch by carrying of the excision of nucleotides and resynthesis of new strand. As the mismatch is in the new strand by the methylated base is in the parental strand, it leads to a futile cycle by MMR leading to checkpoint activation, which is discussed in section 1.5.3.

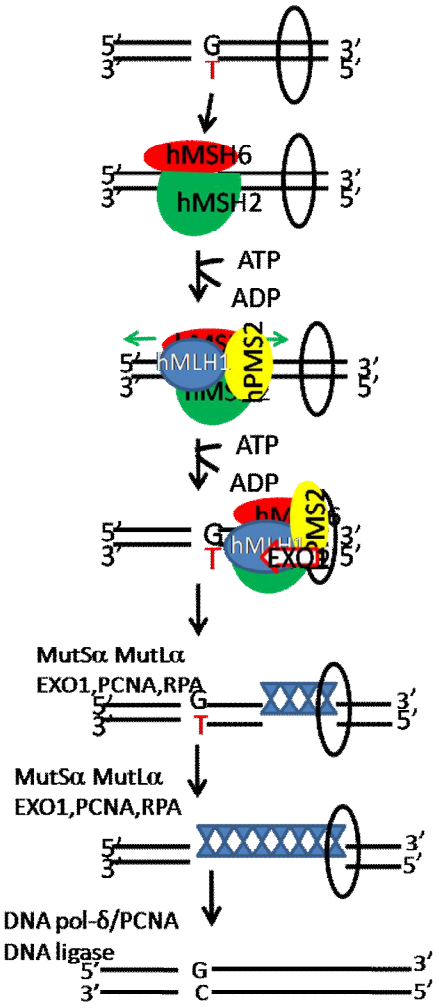

Fig 1.9: Various DNA mechanisms activated in response to alkylation damage. C) MutSα complex recognizes O6meG:T/C mispairs and mediates recruitment of Exo1 which removes the strand containing the wrong nucleotide. A new strand is synthesized by DNA polymerase δ and the final sealing of the nick is done by DNA ligases. Adapted from (Stojic, Brun et al. 2004)

1.4.3 Role of MMR in checkpoint signaling

In multicellular organisms, the DNA damage response is well coordinated with DNA repair pathways. In the last decade, a number of studies have provided evidence of role of MMR in checkpoint signaling following DNA damage caused by SN_1 alkylating agents like MNNG and Temozolamide (TMZ) [reviewed in (Fishel 1999)]. SN_1 alkylating agents like TMZ cause cell death when used as anti cancer agents and also causes DNA damage making

them a double-edged sword. Therefore it is important to understand mechanism and different players involved in processing of DNA damage caused by alkylation damage.

MMR repair mismatches caused by O6 methylation of guanine and induce G2 DNA damage checkpoint and apoptosis. Two disparate models have been put forward to explain the role of mismatch repair proteins in signaling cell cycle arrest and apoptosis – the futile cycle and the direct signaling model [fig 1.10].

In the futile cycle, MMR attempts to repair the damaged strand which leads to formation of gapped DNA resulting in G2/M checkpoint following alkylation damage. All SN_1 methylating agents methylate O6 position of guanine (O6meG) which can either pair with thymine (T) or cytosine (C), which are recognized by MutSα. Binding of MutSα initiates the repair of O6MEG/T mismatches. Since the methylation is in the parental strand, the O6meG mispair again with T or C in the second round of replication and MMR repeatedly attempts to clear the mismatch which leads to formation of breaks in the DNA signaling G2/M arrest and apoptosis (Wang and Edelmann 2006).

The MMR deficient cells are highly tolerant of the O6meG/T or O6meG/C which in absence of MMR is not repaired by the cells; thereby the cells survive and acquire large number of mutations. MMR processing of methylated bases has been shown to activate checkpoint response, which is dependent on ATR activation. Inhibition of ATR or Chk1 kinase by RNA interference abolishes the MMR dependent accumulation of cells in G2 phase following MNNG damage. ATR- Chk1 was found to be phosphorylated after 24 hours of MNNG damage indicating the MMR dependent processing of methylated bases leads to formation of gapped DNA which leads to ATR – Chk1 pathway activation. It is believed that the cells arrested in G2 phase have accumulated double strand breaks due to repeated processing of O6meG impairs which leads to collapse of replication forks ultimately leading to cell cycle arrest and cell death (Mojas, Lopes et al. 2007) .

The direct signaling model implicates MMR proteins in detecting and activating checkpoint signaling following alkylation damage. MutSα

recognizes the O6meG/T or O6meG/C and acts as sensor for alkylation damage (Yoshioka, Yoshioka et al. 2006). MutSα binding to the methylated DNA bases acts as signal for recruitment of other checkpoint proteins like ATR kinase leading to a checkpoint cascade which signals cell cycle arrest. Genetic evidence in mice has shown that MMR has dual functions in MMR as well as in checkpoint signaling. A point mutation in the ATPase domain of mouse Msh6 and Msh2 genes conferred a separation of function mutation, which enables MutSα complex to bind and recognize the mismatches but fails to carry out the repair function. The cellular extracts showed a normal checkpoint response, cell cycle arrest and apoptosis in response to MNNG, cisplatin and other alkylating agents. The study provides strong evidence that repair function of MMR is not required for checkpoint signaling following alkylation damage (Geng, Sakato et al. 2012) (Lin, Wang et al. 2004).

1.4.4 MutSα complex in checkpoint signaling

DNA mismatch repair system and the proteins involved are conserved from bacteria to humans. MutS homolog 2(Msh2) forms a heterodimer with Msh6 or Msh3 similar to its yeast counterparts. Msh2-Msh6 heterodimer (MutSα) binds to mismatches and the Msh2-Msh3 heteroduplex (MutSβ) binds insertion deletion loops (IDLs) (Acharya, Wilson et al. 1996, Marsischky, Filosi et al. 1996). Msh2 and Msh6 are divided into four conserved domains, MutSI, MutSII, MutSd and MutSac (SMART). MutSI (1-124) domain with a mixed alpha/beta structure of Msh2 binds mismatches (Guerrette, Wilson et al. 1998). The Phe-X-Glu motif known to bind mismatches in bacterial MutS proteins is conserved in eukaryotic Msh6 but not in Msh2. The region spanning from 329-645 amino acids of Msh2 (MutSII and MutSd) binds to Msh6/Msh3 (Guerrette, Wilson et al. 1998). Similar to its other homologues Msh2 has an ATPase domain (MutSac) conferring ATP binding and hydrolysis which is essential for MMR activity.

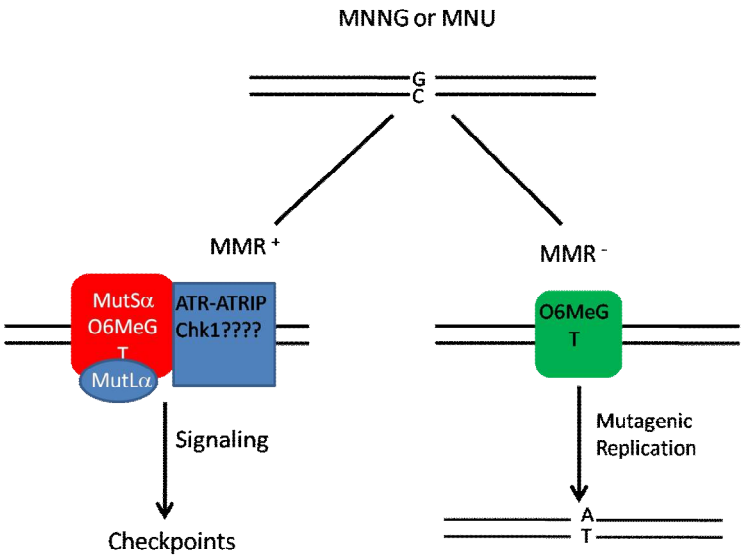

Fig: 1.10: Checkpoint signaling functions of MMR proteins. The replication errors (RER) and mismatches caused by methylating agents like MNNG or MNU are also repaired by MMR. Apart from its repair activities MutSα can directly recognize the O6meG:T/C mispairs and signals activation of checkpoint pathways. Adapted from (Wang and Edelmann 2006)

Recognition of DNA damage lesions by MutSα recruits other factors to repair the error in the strand. Alternately it also recruits proteins that signal checkpoint activation and apoptosis [reviewed in (Kunkel and Erie 2005, Iyer, Pluciennik et al. 2006, Jiricny 2006)]. MutSα has varying binding and repair affinities depending on the lesion and the sequence in context. It can specifically recognize O6meG mispairs, pyrimidne dimmers, cisplatin adducts and other bulky adducts like benzo [c] phenanthrene dihydrodiol epoxide. Msh2 and ATR form a separate signaling module, which is activated in response to MNNG and activate two different effector substrates-Chk1 and Smc1. The two branches of checkpoint signaling activated by Msh2 and ATR leads to phosphorylation of either Chk1, which causes S phase checkpoint or Smc1 phosphorylation required for cell survival (Wang and Qin 2003). 9-1-1 interaction with Msh6 is essential for nuclear localization of Msh6 in response to MNNG. Following methylation damage by MNNG, hyperphosphorylated Msh6, RPA, 9-1-1 complex remain chromatin bound till the second phase of cell cycle and it is believed that ATR mediated checkpoint activation occurs

during that time (Bai, Madabushi et al. 2010) (Schroering and Williams 2008) (Kaliyaperumal, Patrick et al. 2011). MutSα can pair up with various checkpoint proteins like ATR, TopBP1 and Chk1 in response to MNNG damage. The functional relevance of the interactions and the mechanism involved in mediating checkpoint signaling is still not well understood. The multifaceted functions of MutSα could be consequences of its distinct ways of binding to base anomalies as well as protein complexes. It is possible that various other proteins along with of MutSα could be recruited depending on the kind of DNA lesion, which results in distinct biological outcomes.

Aims and objectives of the thesis

A GST pulldown with TopBP1 in HeLa nuclear extracts in the presence and absence of DNA damage helped in identifying novel interacting partners of TopBP1. MutSα (Msh2-Msh6) complex was one of the proteins that were enriched in the DNA damage fraction when analyzed by mass spectrometry.

Cellular process such as DNA damage signaling cascade is a well orchestrated event which requires a number of protein –protein interactions. Therefore it is important to understand the nature of interaction between TopBP1 and Msh2 as well as its role in checkpoint function. The aim of the thesis is to characterize the structural aspects of interaction between TopBP1 and Msh2 and also characterize the function of this interaction in ATR-Chk1 pathway activated following methylation damage.

Objectives of the study

1. *In vitro* interaction studies to understand the nature of interaction between TopBP1 and Msh2.

2. To investigate the functional relevance of Msh2-TopBP1 interaction.

Experimental Plan

In order to investigate the nature of interaction between TopBP1 and Msh2 *in vitro*, the following experiment plan was followed.

1. Recombinant TopBP1 as well as the deletion BRCT mutant proteins will be prepared by using GST tagged constructs which will be expressed in bacteria

2. Recombinant Msh2 will be expressed in Sf9 cells and purified using chromatography. Deletion mutants of Msh2 protein will be prepared by expressing FLAG-tagged Msh2 constructs in bacteria.

3. In order to check if the two proteins could bind each other *in vitro*, far western analysis will be used.

The second objective is to understand the role of Msh2 and TopBP1 interaction in ATR-Chk1 pathway. The following experimental plan will be

followed to elucidate the role of TopBP1-Msh2 interaction in ATR-Chk1 pathway

1. To study the role of Msh2-TopBP1 interaction in ATR-Chk1 pathway, N-methyl-N-nitrososurea (MNU) will be used as a source of DNA damage.

2. The dose and time required for checkpoint activation following MNU exposure will be established using MTT based cytotoxicity assay and immunoblot methods.

3. *In vivo* interactions between TopBP1, Msh2-Ms6 complex will be examined using co-immunoprecipitation.

4. To understand if Msh2 or TopBP1 are essential for ATR-mediated Chk1 phosphorylation, TopBP1 and Msh2 will be knocked down using siRNA approach.

Chapter 2: Materials and Methods

2.1 Reagents

2.1.1 Chemicals

DNA damaging agent-N-methyl-N-nitrosourea (MNU) was purchased from Sigma (catalogue no: N1517). For co-immunoprecipitation magnetic Protein A/G beads were used which were purchased from Ademtech (catalogue no: 4631). Glutathione agarose resin (catalogue no: 16101) and Anti FLAG M2 affinity gel (catalogue no: A2220) were obtained from Pierce and Sigma respectively.

Polyvinyl diflouride (PVDF) membrane (catalogue no: IPVH00010) and Immobilon western reagent (catalogue no: WBKL S0500) for immunoblotting were purchased from Millipore. siRNAs against LacZ, TopBP1 and Msh2 were purchased from Dharmacon. All other chemicals were purchased from Sigma. Restriction endonucleases were purchased from New England Biolabs. Pfu polymerase was procured from Stratagene.

All cell culture media and supplements were purchased from Invitrogen.

2.1.2 Antibodies

Primary antibodies against Chk1 (catalogue no: sc8408), Msh2-N20 (catalogue no: 494, Msh6 (catalogue no: sc-1243) were obtained from Santacruz. Antibodies specific to phospho-Chk1 [Ser 345] (catalogue no.: 2348S), phospho-Chk2 [Th68] (catalogue no.: 2661S), 53BP1 (catalogue no.: 4937), Chk2 (catalogue no.: 344S), RPA 32 (catalogue no.: 2208S) were obtained from Cell Signaling Technology (CST, USA). Primary antibodies specific for TopBP1 (catalogue no.: A300-111A), phospho Chk1 [Ser 317] (catalogue no.: A 300-163A), BRCA1 (catalogue no: A300-000A) were

procured from Bethyl Laboratories. phospho RPA [T21] (catalogue no.: ab61065), Msh6 (catalogue no: ab92471) were bought from Abcam. Antibody against αTubulin (catalogue no: T6199) was bought from Sigma and antibody for Msh2 (catalogue no: NA27) was obtained from Millipore. Anti GST antibody was kindly provided by Dr. Girish Ratnaparkhi (IISER Pune).

Rabbit IgG fractions used for co-immunoprecipitation was obtained from Bethyl laboratories (catalogue no: P120-201).

Secondary antibody anti rabbit HRP (catalogue no: 111-035-033,), anti mouse HRP (catalogue no: 115-035-033,) for immunoblotting were purchased from Jackson immune research:.

For immunofluorescence goat anti-mouse alexa fluor 488 (catalogue no: A11-029) and goat anti-rabbit alexa fluor 568 (catalogue no: A11-036) were obtained from Invitrogen.

The dilutions for each antibody as used for immunoblotting and immunofluorescence assays are mentioned in the Appendix.

2.2 Cell culture and cell lines

HeLa cells (a generous gift from Dr. Sorab N Dalal, ACTREC, India) and HeLa S3 cells (kind gift from Dr. Jomon Joseph, NCCS, India) were cultured and maintained in DMEM media supplemented with fetal bovine serum (10 %), penicillin-streptomycin (1 X) and L-glutamine (1 X). The cells were grown in monolayers at 37 $^{\circ}$C in a humidified incubator with 5 % CO2 and 95 % air at 37 $^{\circ}$C. All cell culture products including fetal bovine serum, media were obtained from Invitrogen.

2.3 Plasmids

GST - tagged full-length TopBP1 and its BRCT deletion constructs were a kind gift from Dr. Lee Zou (MGH, Boston). Msh2 and its deletion constructs were cloned into pT7FLAG vector (Sigma). First, Msh2 was PCR amplified from pCMVtet GFP Msh2 (gift from Prof. Josef Jirciny, IMCR, Zurich) using primers with Not I and Kpn I restriction sites as overhangs (sequences mentioned in the Appendix). Then the digested PCR products were inserted into the expression vector pT7FLAG. For over-expression of full-length Msh2 in *Spodoptera frugiperda*9 (Sf9) cells, Msh2 cloned in pFASTbac vector was a kind gift from Prof. Josef Jiricny (IMCR, Zurich).

2.4 Over expression of Recombinant proteins

2.4.1 Over expression of GST-tagged proteins

GST-tagged constructs of TopBP1 were transformed into BL21 DE3 competent cells. A 2 mL over-night culture was transferred into 50 mL of LB medium containing 100 μg/mL ampicillin and grown till the optical density (OD) was around 0.5-0.6. IPTG was added to a final concentration of 0.4 mM and induction was carried out for 14 hours at 25 ºC. Cells were collected by centrifugation and resuspended in 4 mL of extraction buffer containing 20 mM HEPES (pH-7.6), 0.5 M NaCl, 0.5 mM EDTA, 10 % glycerol, 0.5 % NP40 and 1 X protease inhibitor cocktail. The cells were then lysed by sonicating (VCX 130, Vibracel Sonics) at 70% amplitude using an on-off cycle of 30 seconds for 15 minutes. The lysate was centrifuged at 12000 RPM for 30 minutes and the supernatant was added to Glutathione sepharose beads (GE Healthcare) for 2 hours at 4 ºC. Following incubation, the beads were collected and washed 3 times with wash buffer I containing 20 mM Tris (pH8.0), 150 mM NaCl, 1 % Triton X-100 and 1 X protease inhibitor cocktail and then washed once with wash buffer II containing 100 mM Tris-Cl (pH7.6), 100 mM NaCl and 5 % glycerol).

GST-tagged proteins bound to beads were incubated with 20 mM reduced Glutathione in 50 mM Tris-Cl pH8.0 buffer for 2 hours at room temperature on a rocker (Biosan). The beads were centrifuged at 1000 RPM for 2 minutes at 4ºC [swing bucket rotor, 5810 R (Eppendorf)]. The supernatant containing the eluted proteins was stored at -80 ºC for further use.

2.4.2 Over expression of FLAG-tagged proteins

FLAG- tagged constructs of Msh2 were transformed into BL21 DE3 competent cells. A 2 mL over-night culture was transferred into 50 mL of LB medium containing 100 µg/mL ampicillin and grown till the optical density (OD) was around 0.5-0.6. IPTG was added to a final concentration of 0.4 mM and induction was carried out for 10 hours at 25 ºC. Cells were collected by centrifugation and resuspended in 4 mL of FLAG-extraction buffer containing (50 mM Tris-Cl pH-7.5, 1 mM EDTA pH8.0, 1 mM DTT, 150 mM NaCl, 0.5 % NP40, 1 X protease inhibitor cocktail and 10 % glycerol). The cells were then lysed in a sonicator (VCX 130, Vibracel Sonics) at 70 % amplitude for 15 minutes using an on-off cycle of 30 seconds. The cell lysate was centrifuged at 12000 RPM for 30 minutes in refrigerated centrifuge [fixed angle rotor, 5810 R (Eppendorf)] and the supernatant was added to anti-FLAG M2 affinity beads (Sigma) for 4 hours at 4ºC. Following incubation, the beads were collected and washed 5 times with FLAG-rinsing buffer containing (50 mM Tris-Cl pH-7.5, 500 mM NaCl, 0.5 % NP40, 1 mM EDTA pH8, 1 mM DTT, 5 % glycerol, 1 X protease inhibitor cocktail).

FLAG-bound Msh2 deletion proteins were incubated with 100 µg/mL of FLAG peptide reconstituted in tris buffer saline (pH7.6) for 4 hours at 4 ºC on a nutating mixer (VWR). The beads were then centrifuged at 1400 RPM for 2 minutes at 4 ºC in a refrigerated centrifuge [swing bucket rotor, 5810 R (Eppendorf)]. The eluted proteins in the supernatant were stored at -80 ºC till further use.

2.4.3 Overexpression and purification of full-length Msh2 from Sf9 cells

*Spodoptera frugiperda*9 (Sf9) cells were infected with Msh2 recombinant

baculovirus at a multiplicity of infection (MOI) of 10. After 48 hours of infection the cells were collected and lysed in lysis buffer (50 mM Tris-Cl pH8.0, 1 mM EDTA, 1 mM DTT, 1 X protease inhibitor cocktail, 0.1 % triton X-100, 150 mM NaCl) and sonicated with a probe sonicator (VCX 130, Vibracel Sonics) at an amplitude of 50 %, with a pulse of 30 seconds (on and off) for 2 minutes. The supernatant was collected after spinning at 20000 RPM for 30 minutes

The supernatant was diluted 10 times with 50 mM Tris-Cl (pH8.0) and loaded on to a 1 mL Mono Q column (GE healthcare). The protein was eluted by applying a gradient from 25 % to 100 % with elution buffer (50 mM Tris-Cl pH8.0 and 1 M NaCl). The eluted fractions were then dialysed against 50 mM Tris-Cl (pH8.0) for 3-4 hours at 4ºC. The dialysed protein fraction was resolved on a gel filtration column (Sephacryl S-200; XK10/300, 24). 500 µl of the protein solution was loaded on the column equilibrated with two column volumes resolving buffer (25 mM HEPES pH7.5, 0.5 mM EDTA, 0.5 M NaCl, 5 % glycerol and 2 mM 2-mercaptoethanol). The sample was resolved at a flow rate of 0.5 mL/minute and fractions were collected using an automated fraction collector (AKTA, GE Healthcare). Fractions corresponding to a peak of approximate molecular weight of 100 kDa were analyzed on SDS-PAGE and those devoid of contaminants were pooled.

2.5 Far Western protocol

Far western detects the interaction between the bait and prey proteins by means of immunoblotting. The bait proteins are detected using the antibodies at the site of immobilized prey protein according to its molecular weight.

A modified protocol for far western as described by Wu and colleagues (Wu, Li et al. 2007) was used to detect interaction between Msh2 and TopBP1.

2.5.1 TopBP1 and Msh2 interaction

Sf9 cell extracts expressing Msh2 was run on a SDS-PAGE and transferred to PVDF membrane. The proteins on the PVDF membrane were denatured and renatured with a series of guanidine hydrochloride containing buffer (AC

buffer having 0.1 M NaCl, 20 mM Tris-Cl pH7.6, 1mM EDTA, 0.1 % Tween-20, 1 mM DTT and 2 % non-fat dry). The membrane was blocked with 5 % non-fat dry milk and incubated with full-length GST-TopBP1 in protein binding buffer (100 mM NaCl, 20 mM Tris-Cl pH7.6, 10 % glycerol, 0.1 % Tween-20, 2 % skimmed milk, 0.5 mM EDTA pH8.0) at 4 ºC on a rocker for 16-18 hours.

2.5.2 Mapping domains of Msh2 binding to TopBP1

Purified FLAG-tagged Msh2 deletion constructs were used as prey proteins on the PVDF membrane. Following denaturation and renaturation by AC buffer, the membrane was incubated with the GST-TopBP1 (bait) for 16-18hours at 4 ºC on a rocker.

2.5.3 Mapping BRCT domains of TopBP1 binding to Msh2

GST-tagged BRCT deletions of TopBP1 expressed and purified from bacteria was used as prey protein. The prey protein was denatured and renatured following guanidine hydrochloride treatment and incubated with full-length Msh2 purified from Sf9 cells for 16-18 hours at 4 ºC on a rocker.

Following incubation with the bait protein, the membrane was washed with 1X TBS-T (0.1 % Tween-20) four times at an interval of 10 minutes. The membrane was then incubated with primary antibody against the bait protein [TopBP1 (Bethyl), Msh2 (Calbiochem) or GST (Millipore)] for 3 hours at room temperature. The membrane was washed with 1 X TBS-T (0.1 % Tween 20) four times and then probed with anti-mouse or anti-rabbit HRP for one hour at room temperature. The membrane was developed with Immobilon Western reagent (Millipore) on ImageQuant LAS4000 (GE healthcare).

2.6 MTT cytotoxicity assay

HeLa or HeLa S3 cells were seeded at a density of 10^5 cells/mL in 96 well plates (Corning, Sigma). Cells were grown for 14-16 hours at 37 ºC in a CO_2 incubator. Cells were then incubated with varying concentrations of MNU (0.2 to 20 mM) for 4 hours or 48 hours. Following MNU treatment, cells were

incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide (MTT) at a final concentration of 0.5 mg/mL prepared in DMEM for 4 hours in the dark at 37 ºC in a CO_2 incubator. After incubation with MTT, the media containing the MTT was aspirated out. 100 µl of DMSO was added and rocked for 10 minutes to dissolve the formazan crystals. The absorbance was recorded at 570 nm using a Varioskan multimode plate reader (Thermo Scientific). The absorbance obtained was then used to calculate the IC_{50} of MNU and the percent viability of cells after MNU damage.

2.7 Single Cell gel Electrophoresis

2.7.1 Alkaline single cell gel electrophoresis

To detect DNA damage, single cell gel electrophoresis or Comet assay was first described by Singh and McCoy (Singh, McCoy et al. 1988). The protocol has been modified for detection of single strand breaks. 10^5 cells/ mL were seeded in 12-well plates and grown overnight at 37 ºC in a humidified CO_2 incubator. Cells were treated with MNU (0.5 mM) for different time points and collected following trypsinisation with 0.005 % Trypsin-EDTA. 10,000 cells were mixed with 1 % low melting agarose (LMA) and spread on frosted glass slides previously coated with 1.5 % normal melting agarose (NMA). Another layer of 1 % LMA was coated on the slides and the slides were subjected to lysis at 4 ºC in alkaline lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base) for two hours. Following lysis the slides were then electrophoresed in freshly prepared alkaline buffer (300 mM NaOH/1 mM EDTA) for 20 minutes at 25 V, 300 mA. After electrophoresis the slides were neutralized with neutralization buffer (0.4 M Tris-Cl pH7.5) and stained with ethidium bromide (2 µg/mL) for 20 minutes. Distilled water was used to wash off any extra ethidium bromide stain. The slides were then imaged at a magnification of 20 X on an Axio Imager Z.1 ApoTome microscope (Zeiss).

2.7.2 Neutral single cell gel electrophoresis

A modified protocol from Olive and Banath lab (Olive and Banath 2006) was

used for detection of double strand breaks.

10^5 cells/ mL were seeded in 12-well plates and grown overnight at 37 ºC in a humidified CO_2 incubator. Cells were treated with 0.5 mM N-methyl-N-nitroso urea (MNU) for different time points and collected following trypsinization with 0.005 % Trypsin-EDTA. 10,000 cells were mixed with 1% low melting agarose (LMA) and spread on the frosted glass slide previously coated with 1.5 % normal melting agarose (NMA). Another layer of 1 % LMA was coated on the slides and the slides were subjected to lysis at 4 ºC in lysis buffer (2 % sarkosyl, 0.5 M Na_2EDTA). Following lysis the slides were subjected to electrophoresis in neutral buffer (90 mM Tris buffer, 90 mM boric acid, 2 mM Na_2EDTA pH 8.5) for 20 minutes at 25 V, 300 mA. After electrophoresis the slides were washed three times with chilled distilled water, followed by staining with ethidium bromide (2μg/mL). The cells were then imaged at 20 X using Axio Imager Z.1 ApoTome microscope (Zeiss).

2.8 Quantification of Comet Assay

Open Source plugin of Comet assay for image J was used to calculate the tail length and percent Tail DNA. The fluorescent images with a dark background were used. An oval was drawn over the head region of the comet and then an oval was drawn over the tail region of the comet. The plugin calculates the centroid and center of mass of the region identified by the oval drawn. The centroid is defined as the average of X and Y coordinates of all the pixels in the selection and the center of mass is the brightness weighted average of XY coordinates of a selection. Tail length is defined as the distance from the centroid of the head to the center of the mass of the tail. It is calculated as the pythogoran distance between these two points.

Percentage of DNA in the tail is calculated as the integrated density of the tail divided by the integrated density of tail plus integrated density of the head times 100.

2.9 Immunoblotting

The cell lysates were run on SDS-PAGE electrophoresis set-up (GE Healthcare) according to the manufacturer's protocol and transferred to a

PVDF membrane (Millipore). The membrane was blocked using 5 % fat free milk (Saco, USA) in 1X TBS-T (0.1 % Tween 20) for 1 hour or 1 X Block-ace (AbD Serotec) for phospho-specific antibodies. Primary antibody incubation was done for 3 hours at room temperature. The membrane was washed 4 times for 10 minutes in TBS-T (0.1% Tween) and then incubated in secondary antibody for 1 hour at room temperature. Thereafter, the membrane was washed again in 1 X TBS-T (0.1 % Tween). The blot was developed using Immobilon western reagent (Millipore) and visualized using ImageQuant LAS 4000 detection system (GE Healthcare). All the blots were quantified using minimum three independent experiments. All the values are fold difference over respective controls for each blot.

2.10 Cell Cycle analysis using Flow cytometry

Cells were plated at 60 % confluency and exposed to 0.5 mM MNU for various time periods. Following treatment cells were then trypsinized and centrifuged at 1000 RPM for 5 minutes. The cell pellet was washed twice with 1 X PBS and then fixed with 1 mL of chilled 70 % ethanol and stored at -20 ºC. The cells were then stained with propidium iodide (400 µg/mL) and RNAase was added at a concentration of 200 µg/mL for 1 hour at 37 ºC before acquiring the cells on BD FACS Calibur**.**

2.11 Immunofluorescence

Cells were grown on cover slips in 6 well plates. Following treatment with 0.5 mM MNU at different time points, cells were fixed for 20 minutes with 4 % paraformaldehyde in 1 X PBS. After three rinses with PBS, cells were incubated with 0.1 % glycine prepared in 1 X PBS for 5 minutes and then rinsed again with PBS. Cells were permeabilized using 0.1 % TritonX-100 in 1 X PBS for 10 minutes and rinsed again with 1 X PBS. Following permeabilization cells were blocked with 10 % fetal bovine serum for 1 hour at room temperature. Thereafter cells were incubated for 1 hour at room temperature with primary antibody against Msh2, TopBP1, BRCA1 and 53BP1. Cells were rinsed with 1 X PBS-T (0.1 % Triton-X-100) three times

for 5 minutes at room temperature. Cells were then incubated with alexa fluor secondary antibodies (mouse or rabbit alexa fluor 488/568). Cells were rinsed with 1 X PBS-T (0.1 %Triton-X-100) three times for 5 minutes at room temperature to wash off any extra secondary antibody sticking to the cells. Cells were incubated with Hoechst 33342 made in 1 X PBS to a final concentration of 1 μg/μl to stain the DNA and then mounted on glass slides in mounting media. Cells were analyzed for foci formation on Axio Imager Z.1 ApoTome microscope (Zeiss).

2.11.1 Fluorescence intensity measurement of Msh2

DMSO and MNU treated HeLa cells were stained with Msh2 and imaged at same intensity. The images were analyzed using Image J to calculate the total fluorescence of Msh2 in control as well as MNU treated samples. The fluorescent signal from the nucleus of a cell was identified by overlapping with its DAPI stained nucleus. The integrated density of the nucleus was normalized to its background intensity using the formula: Corrected total cell fluorescence: CTCF = Integrated density- (Area of selected cell X Mean fluorescence of background readings). Similarly CTCF was calculated for DAPI fluorescence and was used to normalize the total fluorescence obtained for Msh2 in both control and MNU treated samples. 30 cells were analyzed for each experiment and the normalized fluorescence intensity of control as well as MNU treated samples was plotted against time after MNU treatment. Data represented in the graphs are +/- standard error of mean for three independent experiments.

2.11.2 Scoring of TopBP1 and 53BP1 foci after immunofluorescence assay

After immunofluorescence assay, cells were imaged for scoring number of cells showing discrete TopBP1 foci. The images were taken at same intensity and exposure time for control and MNU treated samples. 150 cells in each experiment were scored for presence of TopBP1 foci in random fields. Total number of TopBP1 and 53BP1 foci was scored in a cell and the percentage of TopBP1 foci that co-localized with 53BP1 foci were calculated using the formula: Number of TopBP1 and 53BP1 foci that co-localized / Total number

of TopBP1 foci *100. 150 cells were scored in each experiment and the data represented is +/- mean error of three experiments.

2.12 siRNA transfections

Small-interfering RNA against LacZ, TopBP1 and Msh2 were synthesized (Dharmacon). The séquences have been mentioned in the Appendix. 0.25 X 10^5 cells/mL were seeded in 12-well tissue culture-treated plates (Corning, Sigma). siRNA transfection was performed twice at an interval of 24 hours using X-tremeGENE siRNA transfection reagent (Roche). Cell lysates were prepared after 48 hours of the second transfection by adding 2 X laemmli buffer. Immunoblotting was performed as mentioned earlier.

2.13 Co-immunoprecipitation

Whole cell extracts of HeLa S3 cells were prepared 6 hours post MNU (0.5 mM) damage in lysis buffer (25 mM HEPES pH 7.5, 450 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.15 % Triton-X 100). The lysate was diluted three fold by adding two volumes of dilution buffer (25 mM HEPES pH 7.5, 0.1 mM EDTA and 5 mM $MgCl_2$). The lysate was then incubated with 2μg of antibody [TopBP1 (Bethyl) or Msh6 (Abcam)] bound to magnetic beads (Ademtech) for 4 hours at 4ºC on a rocker. The beads were then washed twice with wash buffer (25 mM HEPES pH 7.5, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.05 % TritonX-100, and 150 mM NaCl). 40 μl of 2 X laemmli buffer was added to the beads and loaded on SDS-PAGE for immunoblotting.

2.14 Statistical analysis

Data represented in single cell gel electrophoresis (comet) assay is +/- standard error of mean of data analyzed for different parameters including tail length and % tail DNA from three independent experiments. One way Anova with Kruskal-Wallis non parametric tests was used to analyze the data sets of comet assay. Both % tail DNA and tail length were considered significantly

higher in the treatment groups than the control at $p < 0.001$. The data was analyzed using GraphPad Prism software (GraphPad software, La Jolla, CA, USA).

The cells showing TopBP1 foci as well as the percentage of co-localized TopBP1 and 53BP1 foci were significantly higher in the MNU treated samples than the controls at $p < 0.05$ when analyzed by using two way Anova with bonferroni post hoc test.

The normalized fluorescence intensity of Msh2 among the control and MNU treated cells was analyzed using Fisher's least square difference test in a two way Anova. Data represented in the graph is +/- standard error mean of three independent experiments. The data for foci scoring and fluorescence intensity measurements was analyzed using STATISTICA and $p < 0.05$ was considered significantly different.

The western blots were quantified using Image J and was represented as fold difference with respect to their controls. Data represented is +/- standard error of mean of three different experiments. For siRNA experiments, the control and treated samples were analyzed by student's t-test (unpaired, two tailed) and for time course assay blots were quantified using multigauge analysis software.

The fold difference between control and the different time points was considered significant at $p < 0.05$ when analyzed using one way Anova with tukey post hoc test GraphPAd Prism software (GraphPad software, La Jolla, CA, USA).

Chapter 3: *In Vitro* interaction studies between TopBP1 and Msh2

Background

All cellular processes – DNA replication, transcription, translation, splicing, cell cycle regulation, etc. are interwoven by protein networks that work round the clock for the survival of a cell. DNA damage response pathways are no exception and are carried out by various proteins, which have evolved modular structure to carry out all the processes. Detection of protein interactions within the cell or identifying interacting partners in a complex is the first step towards understanding their functions.

A combination of *in vitro and in vivo* approaches is used to detect protein – protein interactions. *In vitro* methods include mass spectrometry (Rigaut, Shevchenko et al. 1999), far western blotting (Wu, Li et al. 2007), pull down assays with GST or FLAG- tagged proteins (Vikis and Guan 2004) while *in vivo* approaches include co-immunoprecipitation and two hybrid systems using bacteria, mammalian cells or yeast (Fields and Song 1989, Vasavada, Ganguly et al. 1991, Dmitrova, Younes-Cauet et al. 1998) In cell cycle checkpoint signaling a number of proteins interact to recognize DNA damage and repair the damage. TopBP1 and Msh2 are two key components of the DNA repair complex. Human TopBP1 has nine BRCT domains, which forms canonical pairs and is homologous to TopBP1 found in budding and fission yeast. Human MutS homolog 2 (Msh2) consists of four domains, which are conserved all along the prokaryotic and eukaryotic evolutionary border. The modular structure of these proteins is advantageous in performing multiple functions in various processes like DNA replication, checkpoint activation and mismatch repair.

In this chapter we have characterized the interaction between TopBP1 and Msh2 using far western blotting. We have used deletion mutants of TopBP1

and Msh2, to understand the regions or domains essential for binding of the two proteins. Far-western blotting combines simple protein purification methods and western blotting to confirm interaction between two proteins. In this method the prey protein is resolved on a SDS PAGE gel according to its molecular weight and transferred to a PVDF membrane. The interaction is visualized on the membrane at the prey protein band when probed with antibodies against the interacting bait protein.

Results

3.1 Generation of plasmid constructs of Msh2 and TopBP1

Full length TopBP1 and its BRCT deletion constructs (cloned in pGEX-2TKcs) were a kind gift from Dr. Lee Zou (MGH, Boston) [fig3.1 (A)]. The four domains of Msh2 as mentioned in [fig 3.1 (B)] were amplified from pCMVtetGFP-Msh2 vector (gift from Prof Josef Jiricny) using PCR. The amplified products as well as the empty vector pT7FLAG were then digested with Not I and Kpn. The digested amplified PCR products were ligated with empty vector pT7FLAG using T4 DNA ligase (New England Biolabs). In order to verify all the Msh2 constructs in pT7FLAG vector, restriction digestion was carried out with Not I and Kpn I restriction enzymes. The digestion resulted in release of the following fragment sizes for the respective constructs indicated in the parentheses: 2.3 kb (Msh2Δ1), 1.8 kb (Msh2Δ1-2), 0.8 kb (Msh2Δ1-3) and 1.9 kb (Msh2Δ4). Msh2 full-length (2.8 kb) [fig 3.1 (C)] cloning into pT7FLAG was unsuccessful. Therefore, to over express full-length Msh2, the cDNA was cloned into pFASTbac vector (a kind gift from Prof. Josef, Jiricny, Zurich).

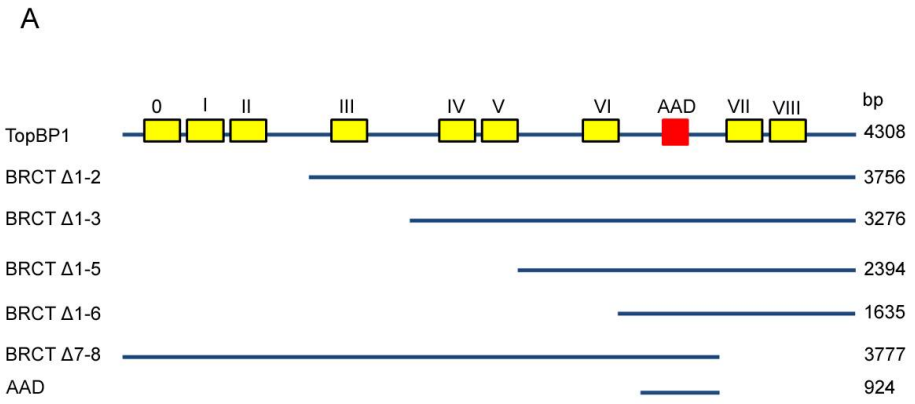

Fig 3.1: TopBP1 deletion constructs. (A) Schematic of BRCT deletions of TopBP1 cloned into pGEX-2tKcs vector.

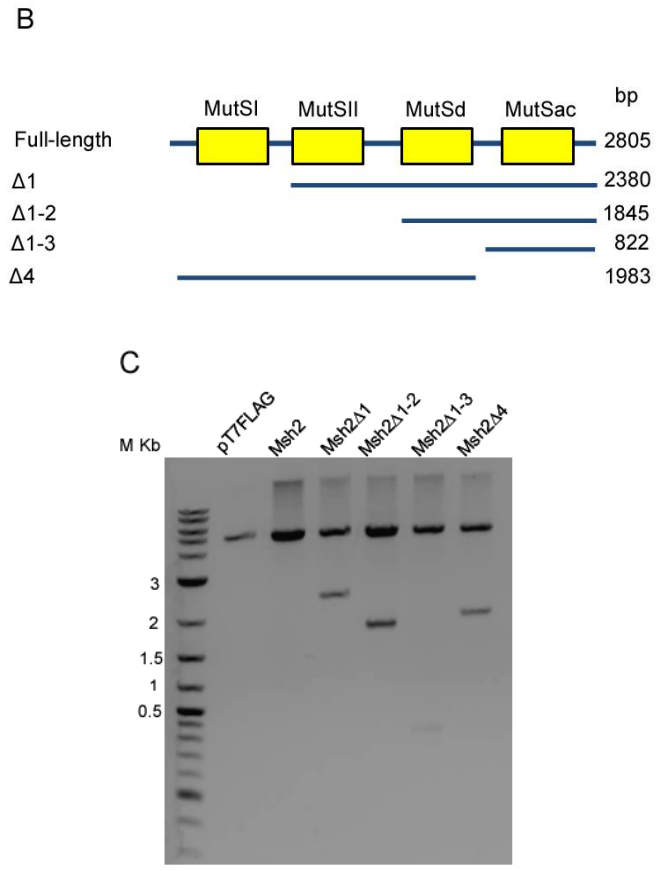

Fig 3.1: Mutant Msh2 constructs cloned in pT7FLAG vector. B) Schematic showing deletion constructs of Msh2. Each MutS domain is deleted sequentially and cloned into pT7FLAG vector to generate the Msh2 deletion mutants and C): Restriction digestion verification of Msh2 full-length and deletion mutants cloned in pT7FLAG vector on agarose 0.8 % gel. From left to right: lane 1: Bangalore Genei 1 kb plus DNA ladder. Lane2: linearized pT7FLAG vector, lane3: linearized pT7FLAG without insertion of full-length Msh2, lane 4: pT7FLAG vector (5 kb) and excised Msh2Δ1 (2.3 kb), lane5: pT7FLAG vector (5 kb) and excised Msh2Δ1-2 (1.8 kb), lane 6: pT7FLAG vector (5 kb) and excised Msh2Δ1-3 (0.8 kb) and lane 6: pT7FLAG vector (5 kb) and excised Msh2Δ4 (1.9 kb). Ethidium bromide stained agarose gels were imaged by Syngene® Gel Doc analyzer using UV source.

3.2.1 Over expression and purification of GST- tagged TopBP1 protein and its deletion mutants

TopBP1 and its BRCT deletion mutants were expressed in *E. coli* BL21 DE3 cells. Following purification using glutathione beads, the purified samples were run on SDS PAGE and stained with Coomassie Brilliant Blue R-250 for

visualization. The BRCT domains of TopBP1 were deleted in pairs to generate the following deletion mutants [fig 3.2.1 (A)]

i) BRCT Δ1-2 - TopBP1 without BRCT domains 0, I and II

ii) BRCT Δ1-3 – TopBP1 without BRCT domains I to III

iii) BRCT Δ1-5 -TopBP1 without BRCT domains I to V

iv) BRCT Δ1-6 - TopBP1 without BRCT domains I to VI.

v) BRCT Δ7-8 - TopBP1 without BRCT domains VII and VIII

ATR activating domain of TopBP1 was expressed solely as GST-tagged recombinant protein [fig 3.2.1 (A)]. The GST protein tag is 26 kDa in size that adds up to the total molecular weight of the protein being expressed. Hence all the proteins expressed in *E. coli* BL21 DE3 cells showed up at higher molecular weight than the expected size on the SDS PAGE gel. The resultant GST- tagged TopBP1 protein was of molecular weight ~200 kDa (160+26 kDa) which was over expressed and purified in native conditions [fig 3.2.1 (B)]. All the BRCT deletion mutants – BRCTΔ1-2 (~165 kDa), BRCTΔ1-3 (~147 kDa), BRCTΔ1-5 (˜115 kDa), BRCTΔ1-6 (˜86 kDa), BRCTΔ7-8 (~166 kDa) and the ATR activating domain-AAD (˜56 kDa) were found to be expressed in soluble fraction showing expected molecular weight on SDS PAGE [fig 3.2.1 (B)]. Following purification we observed certain amount of degradation in protein preps of all the recombinant proteins, which were found due to the high molecular weight of recombinant proteins being expressed. Different expression or purification conditions were tested; however they did not result in the reduction of the degradation products.

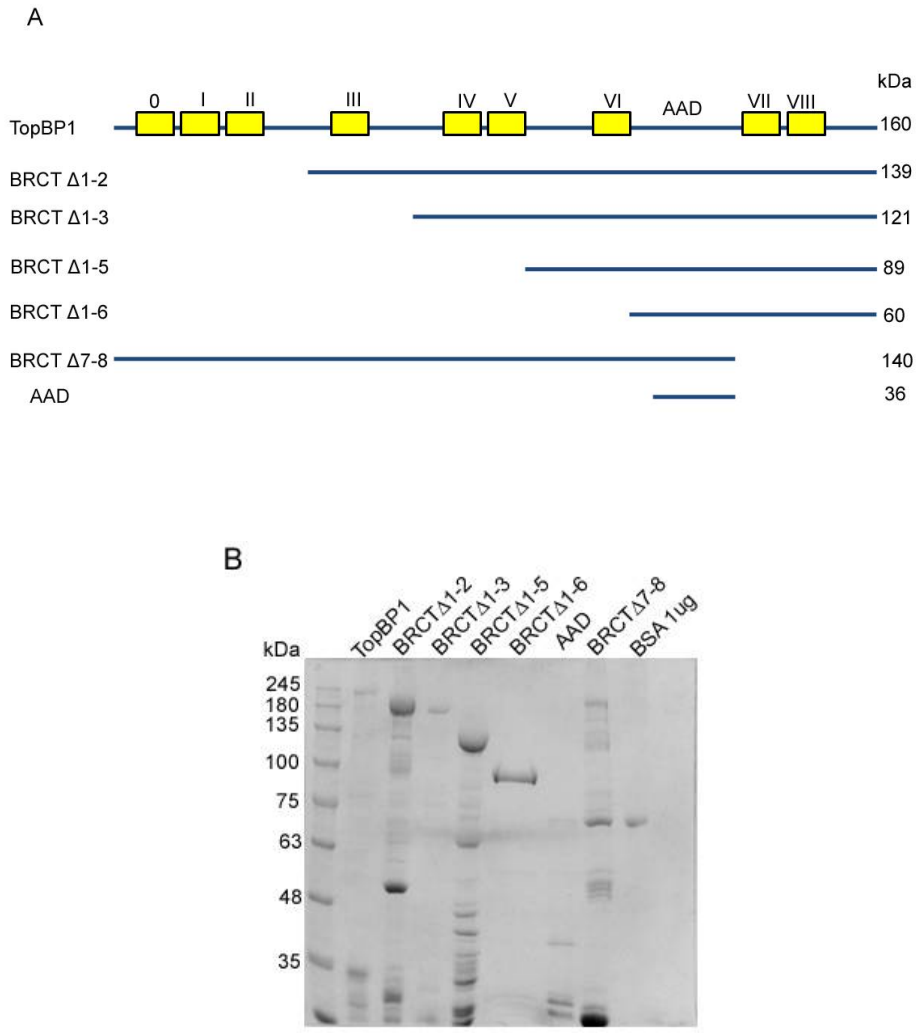

Fig 3.2.1: Over expression and purification of TopBP1 deletion proteins. A) Schematic of TopBP1 deletion constructs that were cloned in pGEX-2TKcs vector and B) Over expression of TopBP1 and its BRCT deletions was done at 25ºC for 14 hours with 0.4 mM IPTG induction. The purified proteins bound to glutathione beads were denatured and run on SDS PAGE. Coomassie Brilliant Blue R-250 was used to stain the proteins on the gel. Lane 1 is high molecular range protein ladder (Black biotech), lanes (2-7) Full-length TopBP1 (186 kDa), BRCTΔ1-2 (165 kDa), BRCTΔ1-3 (146 kDa), BRCTΔ1-5 (115 kDa), BRCTΔ1-6 (86 kDa), BRCTΔ7-8 (166 kDa), AAD (56 kDa), lane 8 is BSA. Images were taken with ImageQuant LAS4000 using the epi-illumination source (GE Healthcare)

3.2.2 Over expression and purification of FLAG tagged Msh2 deletion mutant proteins

The MutS domains of Msh2 were deleted sequentially to generate the deletion mutant clones. The first 147 amino acids corresponding to the MutSI domain were deleted to generate the Msh2 Δ1 construct. In Msh2 Δ1-2 the region spanning from 1-325 amino acids corresponding to MutSI and MutSII was deleted. Msh2 Δ1-3 was created so that it did not include the first three domains MutSI, MutSII and MutSd (1-661amino acids). The Msh2 Δ4 includes all the domains except the MutSac domain [fig 3.2.2 (A)]. Over expression of proteins of all the deletion mutant constructs was observed at their expected molecular weight except Msh2 Δ1 which did not express in the soluble fraction. Repeated attempts with varying IPTG and temperature conditions did not result in a soluble protein [fig 3.2.2 (B)]. The Msh2 Δ4 protein was found to be expressed in higher amounts than the other deletion mutants. Small amounts of Msh2 Δ1-3 and Msh2 Δ1-2 were expressed in soluble fractions [fig 3.2.2 (B)].

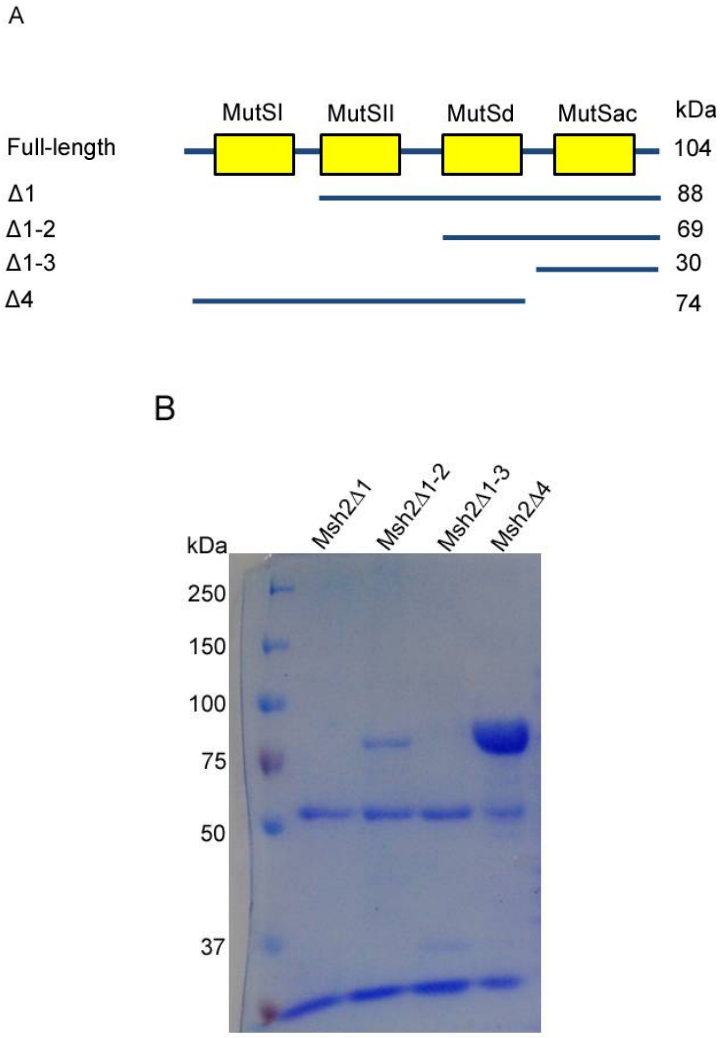

Fig 3.2.2: Over expression and purification of Msh2 deletion protein. A) Schematic of Msh2 and deletion mutants which was cloned in pT7FLAG and B) SDS PAGE (10 %) stained with coomassie brilliant blue R 250 Msh2 deletion mutant proteins expressed in *E. coli* BL 21 DE3 cells by IPTG induction (0.4 mM) for 10 hours at 25 ºC. The proteins were purified using anti FLAG M2 affinity gel and run on SDS PAGE. Lane1: Biorad® protein ladder, lanes (2-5): Msh2Δ1 (88 kDa), Msh2Δ1-2 (70 kDa), Msh2Δ1-3 (30 kDa), Msh2Δ4 (74 kDa).

3.2.3 Expression and purification of the full length Msh2 protein from Sf9 cells

Msh2 expressed in large amounts in Sf9 cells was found to be expressed mostly in the soluble fraction. The isoelectric point of Msh2 is 5.4, which has a negative charge at pH 8.0. We used anion exchange matrix for purification of Msh2 using FPLC. The Mono Q column is a strong anion exchange matrix, which was able to retain most of the Msh2 protein molecules while removing all the endogenous Sf9 proteins. The proteins bound to the matrix were then eluted using a buffer with high salt that disrupted the interaction between proteins and the matrix. The eluted fractions were then analyzed by western blotting for presence of Msh2 protein [fig 3.2.3 (A)]. The anion exchange based purification left some impurities, which were then removed by using gel filtration column (Sephacryl 200) with a fractionation range of 5000-60,000 da. The gel filtration column excluded the higher molecular weight Msh2 (104 kDa) protein, which were collected in fractions using automated fraction collector. The fractions were visualized on SDS PAGE by CBB staining [fig 3.2.3(B)].

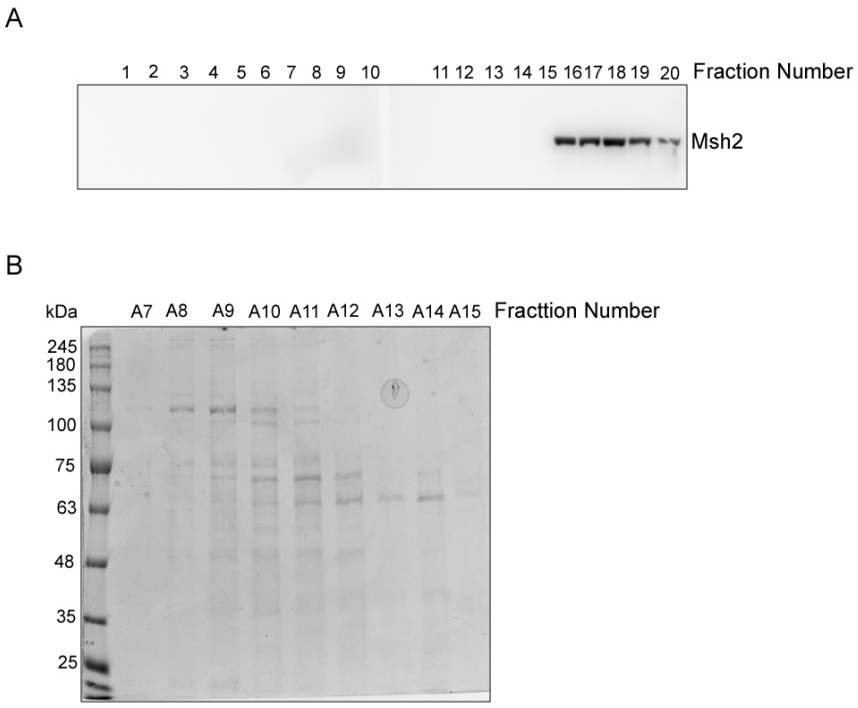

Fig 3.2.3: Purification of Msh2 full-length proteins by two step chromatography. A) The fractions collected following anion exchange chromatography were run on a 10 % SDS PAGE and detected using antibody against Msh2. The fraction number 15 to 20 contains the protein. B) The Msh2 protein was the obtained in a pure form using gel filtration chromatography. The fractions were collected and run on 10 % SDS PAGE gel and visualized by CBB staining. The fraction number A8 and A9 contain the protein. The fractions were then pooled and stored in -80 ºC. The images were taken using the white light source in ImageQuant LAS 4000 system (GE Healthcare)

3.3.1 Msh2 and TopBP1 interact *in vitro*

Far western blotting is useful to study interaction between two proteins when one of them is purified and the other protein is present in cell extracts. The prey protein Msh2 was expressed in Sf9 cells. The cell extract was run on SDS PAGE and immobilized on PVDF membrane. GST-tagged TopBP1 purified from bacterial cells was used as the bait protein. We used anti TopBP1 antibody to probe the interaction between the two proteins; the interaction was

observed at the molecular weight of Msh2 (104 kDa) on the membrane [fig 3.3.1 (A)]. When GST alone was incubated with the prey protein it did not show any interaction on probing with anti GST antibody [fig 3.3.1 (B)].

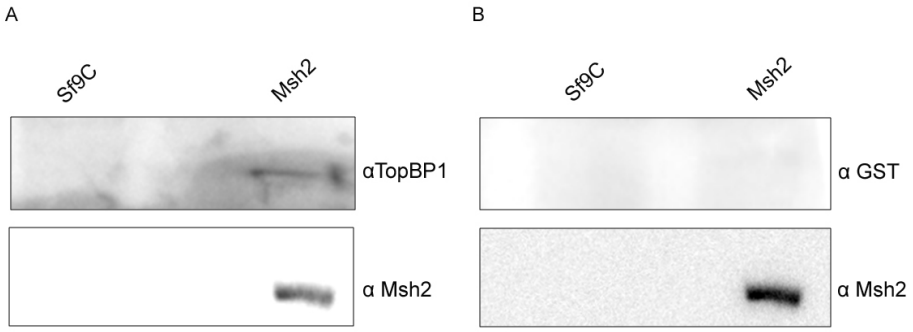

Fig 3.3.1: Far western analysis to confirm interaction between TopBP1 and Msh2. Left to right panel A: lane 1 is control Sf9 cell extract (without Msh2) and lane 2 is extract of Sf9 cells expressing Msh2. Both the lanes were probed with GST-TopBP1. Panel B: lane1 extract of Sf9 cells (without Msh2) and lane 2 is Sf9 cell extract expressing Msh2. Both the lanes were probed with GST alone. Right side of the panel indicates the antibodies used for western blotting. 10 μg of cell extracts and 1ug of purified GST-TopBP1 or GST alone were used for the assay.

3.3.2 Mapping of Msh2 domains essential for binding to TopBP1

For mapping the region in Msh2 protein that is responsible for its interaction with TopBP1, deletion mutants were constructed [fig 3.3.2 (A)]. Purified Msh2 deletion mutants were used as prey proteins bound on the membrane. Full-length purified GST-TopBP1 was incubated with the prey protein and the interaction was visualized using antibody against TopBP1. We observed that only Msh2 Δ1-2 deletion mutant showed interaction with TopBP1 amongst all the other Msh2 deletion mutants (Msh2 Δ1-3 and Msh2 Δ4) [fig 3.3.2 (B)]. When GST alone was incubated with all the deletion mutants, no interaction was observed [fig 3.3.2. (C)]. This concludes that MutSd and MutSac domains together are essential for its binding to TopBP1, as the presence of either of the domain in cases of Msh2 Δ1-3 or Msh2 Δ4 did not result in interaction between the two proteins.

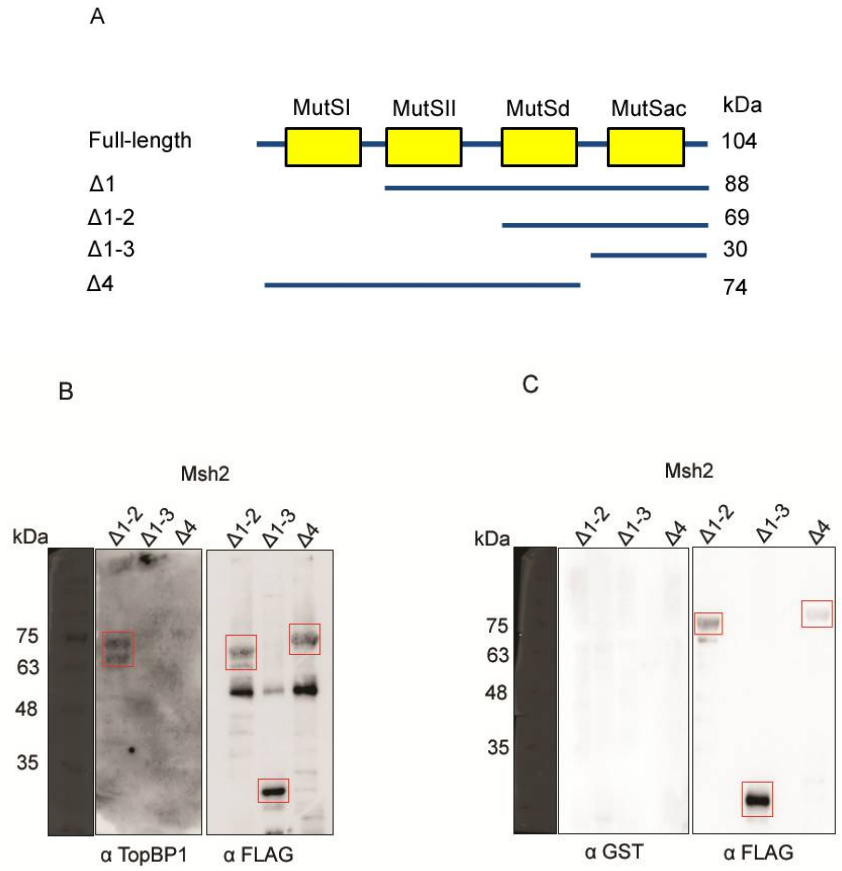

Fig 3.3.2: Far western assay to map Msh2 domains required for binding to TopBP1. (A) Schematic of Msh2 deletion mutant proteins used, (B and C) Msh2 deletion mutant proteins were immobilized on PVDF membrane and probed with GST-TopBP1 and GST alone respectively. Western blotting was done with anti TopBP1 and anti GST antibody to detect the interaction. The images were taken by ImageQuant LAS 4000 detection system (GE Healthcare).

3.3.3 Mapping BRCT domains in TopBP1 required for its interaction with Msh2

Full-length TopBP1, various BRCT deletion mutants and GST alone was immobilized on PVDF membrane and used as prey protein. Following incubation with Msh2, the interaction was visualized with antibody against Msh2. The interaction was observed at TopBP1 molecular weight on the membrane [fig 3.3.3 (A)]. Among the various BRCT deletion mutants used,

only BRCT Δ7-8 lacking the C- terminal region of TopBP1 abrogated the interaction between the two proteins [fig 3.3.3 (A)]. GST alone when probed with Msh2 did not show interaction [fig 3.3.3 (B)]. For far western assay, 1 µg of the protein was used which was quantified visually on the SDS PAGE using BSA as standard [fig (3.3.3 (C, D)]. When Sf9 cell extracts expressing Msh2 (prey protein) was used and purified GST-TopBP1 along with its deletion mutants were used as bait proteins, we obtained similar results. Full-length TopBP1 and all other BRCT deletion mutants except BRCTΔ7-8 showed interaction with Msh2 [fig 3.3.3 (E)]. The C-terminal region after BRCT domain VI of TopBP1 includes the ATR activating domain (AAD) and the BRCT domains VII and VIII. The AAD is an important domain of TopBP1 as it is sufficient for ATR activation and possibly the only way to activate ATR kinase in higher eukaryotes. Though in our assay we did not see any direct interaction between AAD and Msh2 [fig 3.3.3 (A)]. However loss of VII and VIII domains of TopBP1 abolished the interaction with Msh2, which was observed in the case of all other deletion mutants with intact C-terminal region.

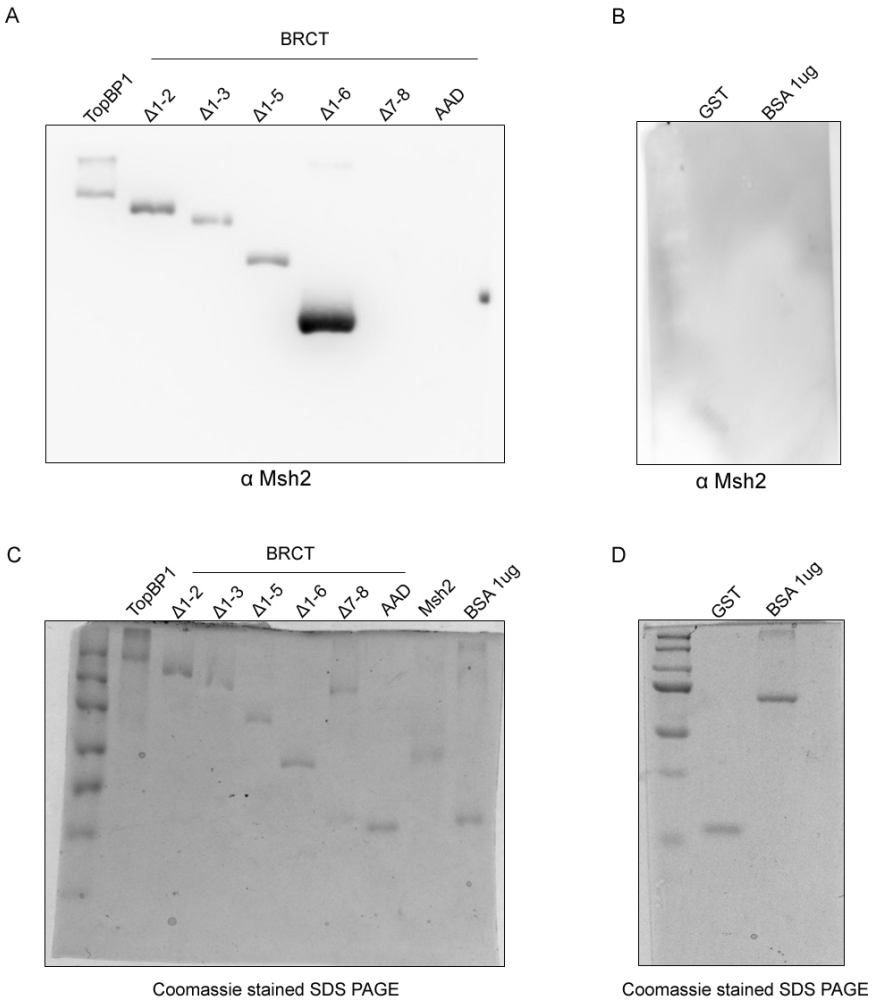

Fig 3.3.3: Far western blotting to map BRCT domains of TopBP1 essential for binding to Msh2. (A) GST-tagged full-length TopBP1 and its deletion mutant proteins were immobilized on the membrane and probed with full-length Msh2 as a bait protein. (B) GST alone was also immobilized on the membrane and probed with Msh2. The interaction was confirmed using antibodies against Msh2. (C and D) 1μg of purified protein was used for far western analysis as can be seen in this coomassie stained SDS PAGE

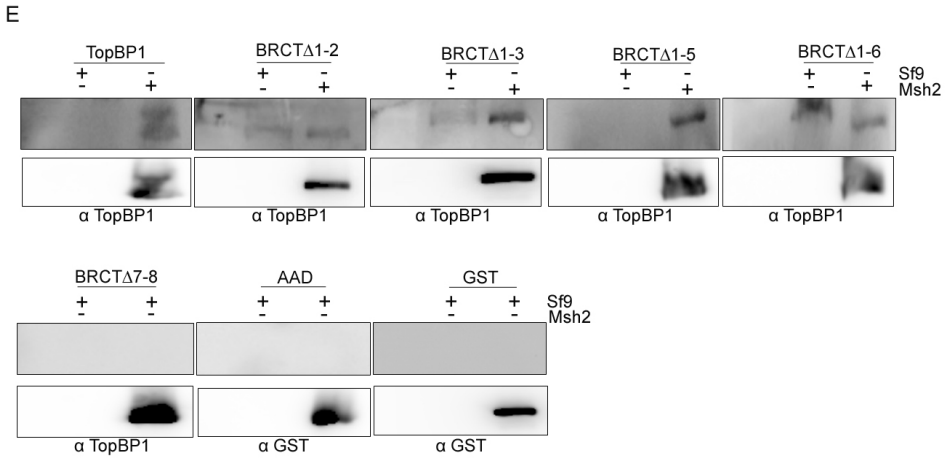

Fig 3.3.3: Reverse Far western assay to confirm interaction between TopBP1 deletion proteins and full-length Msh2. (E) Sf9 cell extracts with (+) or without Msh2 (-) was immobilized on the membrane. Purified full-length GST - tagged TopBP1, BRCT deletion mutant proteins and GST alone were used as bait proteins. The interaction was confirmed using antibodies against TopBP1 or GST.

Discussion

There are numerous studies implicating MMR proteins to be involved in functions other than mismatch repair. There are a number of studies that have identified components of ATR-Chk1 pathway to be novel interactors of MMR proteins. Interplay between these two pathways will shed some light on the DNA damage signaling activated by SN_1 type methylators. Msh2 component of MutSα complex is known to bind directly to ATR (Wang and Qin 2003); another study also demonstrated that the functional MutSα (Msh2-Msh6) can bind to multiple components of ATR-Chk1 pathway (Liu, Fang et al. 2010). MutSα was shown to bind Chk1 and TopBP1 directly upon MNNG damage (Liu, Fang et al. 2010). Msh2 was shown to bind to TopBP1 in nuclear extracts of HeLa cells upon MNNG damage. We argue that TopBP1 and Msh2 can bind different proteins owing to their modular structure; understanding the nature of interaction between TopBP1 and Msh2 will give insights into the activation of a checkpoint response following methylation damage. In our studies we showed that TopBP1 could directly bind to Msh2 using recombinant proteins [fig 3.3.1 (A)]. Far western analysis gave us the advantage of using standard western blotting techniques and purification methods for obtaining proteins for assay. With far western analysis we could use Sf9 cell extracts expressing Msh2 and GST-TopBP1 expressed in bacteria. We could detect the interaction with antibodies against TopBP1 or Msh2 making it a convenient method to use.

TopBP1 and its homologues are scaffold proteins that comprise of BRCA1 C-terminal (BRCT) domains. Owing to their BRCT domains they can bind multiple proteins forming temporal complexes, which regulate various cellular processes like DNA replication and checkpoint activation. The nine BRCT domains found in TopBP1 exhibits diverse architecture in terms of their packing and sequence of amino acids thereby extending diversity to the functions carried out by TopBP1. In our studies we found that the C-terminal region after BRCT VI domain of TopBP1 is essential for binding to Msh2 [fig 3.3.3 (A, E)]. The C-terminal region following the BRCT VI domain in

TopBP1 contains a putative ATR activation domain and pair of BRCT VII+VIII domain which resembles the canonical pair of BRCT domains found in homologues of TopBP1 in yeast. ATR activating domain of along with other BRCT deletion mutant of TopBP1 was used as prey protein and full-length Msh2 as bait protein in far western analysis. The far western analysis showed that AAD alone did not interact with Msh2 and absence of BRCT VII+VIII domain also abolished the interaction between TopBP1 and Msh2. Of the three BRCT pairs in TopBP1 only BRCTVII+ VIII pair shows a canonical arrangement as seen in other BRCT pairs in mammalian MDC1 and BRCA1or Crb2^{53BP1} of *S. pombe*. In case of a canonical BRCT pair the first BRCT domain of the pair contains charged amino acids and is required for binding to the phosphopeptide or phosphoproteins while the second BRCT domain provides sequence specificity for binding partners (Wardlaw, Carr et al. 2014). Other studies have also shown the tandem pair of BRCT VII and VIII to bind to proteins like ATR or RecQ4 (Liu, Shiotani et al. 2011, Ohlenschlager, Kuhnert et al. 2012) mediating ATR activation and initiation of DNA replication respectively.

Mammalian MutS homolog 2 (Msh2) heterodimerises with Msh6 (MutSα) or Msh3 (MutSβ) that binds to mismatches or insertion deletion loops respectively. Msh2 too has a modular structure thereby promoting various DNA recognition and protein interaction required for mismatch activity and DNA damage signaling. In our studies we identified the C-terminal region of Msh2 to be essential for binding to TopBP1. The MutSd and MutSac domains comprise the C-terminal region of Msh2. MutSac domain has the conserved walker A type motif essential for ATPase activity required for mismatch repair. Mutations in ATPase domain can act as dominant negative inhibitor of MMR activity (Drotschmann, Topping et al. 2004, Lin, Wang et al. 2004).

The *in vitro* studies with recombinant proteins provide a platform to understand the nature of interaction between two proteins. Many times two proteins could be binding to each other but may or may not have any functional relevance. It was important for us to understand interplay between

TopBP1 and Msh2, which would give us insight into the complex network of DNA damage signaling.

Summary

To summarize, we prepared recombinant proteins for *in vitro* protein - protein interaction studies. TopBP1 was GST tagged and expressed in BL21 E. *coli* cells. Msh2 was expressed in Sf9 cells and purified by two-step chromatography (ion exchange and gel filtration) to obtain protein in pure form. Far western blotting was used to determine physical interaction between TopBP1 and Msh2. Msh2 was immobilized on the membrane and probed with full-length GST–TopBP1. The western blot showed interaction at the same spot where Msh2 was immobilized on the membrane confirming the direct interaction between two proteins. We also identified the regions essential for binding of TopBP1 and Msh2. FLAG-tagged deletion mutants of MutS domains of Msh2 were generated. These mutants were used as prey protein and the full–length GST-TopBP1 as bait protein in far western blotting. We found MutSd and MutSac domains were essential for binding between Msh2 and TopBP1. Similarly GST-tagged deletion mutants of TopBP1 were generated so as to use as prey protein in far western blotting. In this assay Msh2 purified from Sf9 cells was used as a bait protein. Our assay concluded that the C-terminal region containing the VII and VIII domain of TopBP1 is essential for binding to Msh2.

Chapter 4 : DNA damage response to methylating agent

Background

Eukaryotic genomic DNA is under constant threat of being damaged by various sources. Exogenous sources including the ultraviolet and ionizing rays from sunlight can cause single and double stranded breaks. Various chemicals like alkylation agents or chemotherapeutic agents can also cause variety of lesions in the DNA. DNA lesions can also result from erroneous DNA replication or cellular metabolism. To ensure genomic integrity cells have evolved a well-defined and intricate mechanism called DNA damage response (DDR) pathway. DDR consists of cell cycle checkpoint proteins that delay cell cycle transition, allowing time for recruitment of DNA repair proteins to repair the lesion and in case of irreparable damage it signals to the apoptotic machinery for the induction of cell death (Lukas, Lukas et al. 2004).

SN_1 type methylating agents like Temozolamide, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or N-methyl-N-nitrosourea (MNU) are a class of alkylating agents that modify DNA bases by adding methyl group to the oxygen and nitrogen atoms forming base adducts like O6MeG or N7MeG (Fu, Calvo et al. 2012). The response to damage from alkylating agents varies according to the dose, time of exposure and the MMR status of the cell. The coordinated response to modified bases of DNA is a combination of direct recruitment of checkpoint proteins and MMR dependent processing of mispairs caused by O6MeG (Wang and Edelmann 2006). MNU was first tested as a chemotherapeutic agent but it is also a known mutagen and carcinogen (TSUBURA, LAI et al. 2011).

The checkpoint response to MNU-induced lesions in DNA has not been characterized till now. The cellular response of HeLa cells following exposure to MNU was characterized using methods like single cell gel electrophoresis

(Comet assay) which estimates the extent of DNA damage in terms of single strand or double strand breaks formed, western blotting to assess the checkpoint proteins being activated and flow cytometry to assess the impact of MNU in causing arrest in cell cycle.

We established that MNU induced DNA lesions could be used as a model system to understand the interplay between TopBP1 and Msh2.

Results

4.1 Determination of lethality of MNU (N-methyl-N-nitrosourea) by MTT cytotoxicity assay

3- (4, 5-Dimethylthiazol-2-yl)-2, 5 Diphenyltetrazolium Bromide (MTT) based assay was used to determine the number of viable cells following exposure to MNU. The colorimetric assay is based on the reduction of yellow colored MTT by the mitochondrial succinate hydrogenase into deep purple colored formazan crystals which are solubilized by DMSO. Since only the live cells can actively reduce MTT, the absorbance of the purple color read at 570 nm is directly proportional to the number of viable cells.

4.1.1 Dose response curve of HeLa cells following treatment with MNU

To determine the effective dose for MNU which would not kill the cells but cause enough damage for checkpoint activation, we used a colorimetric based cytotoxicity assay (MTT) as explained in materials and methods. HeLa cells were treated with varying concentrations of MNU (0.2-2 mM) for 4 or 48 hours. MNU had a growth inhibitory effect on HeLa cells in a dose dependent manner. The IC_{50} of MNU for HeLa cells was about 2 mM; that is, about 50 % of cells were dead at 2 mM concentration of MNU. The time of exposure did not affect the IC_{50} of MNU [fig 4.1.1 (A and B)]. Based on these results MNU concentration of 0.5 mM, which is lower than the IC_{50} value, was chosen for *in vivo* experiments.

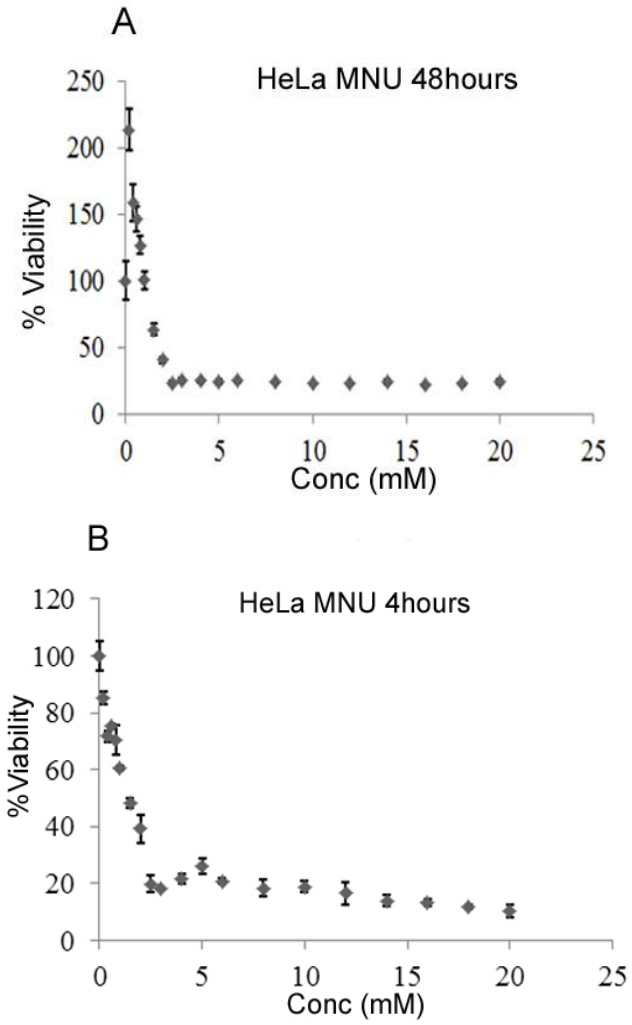

Fig 4.1.1: Dose response curve for MNU in HeLa cells for different exposure times. HeLa cells were treated with varying concentrations of MNU (0.2-20 mM) for 48 hours (A) and 4 hours (B). The number of viable cells was calculated and plotted as % viability on the y-axis and MNU concentration on the x-axis. 50 % cell death was observed at 2 mM MNU on exposure for 4 hours and 48 hours.

4.1.2 Dose response curve of HeLa S3 cells following treatment with MNU

MNU causes methylation at various sites in DNA like O6 position of guanine, N7 of guanine and N3 of adenine. Methyl guanine methyltransferase (MGMT) is a suicide enzyme, which removes the O6 alkyl product in a one step reaction following which the enzyme becomes inactive. HeLa S3 cells are MGMT proficient hence while determining the IC_{50} for HeLa S3 cells; we

used O6 benzylguanine (O6 BG) which is an inhibitor of MGMT. HeLa S3 cells treated with 20 μm of O6 benzylguanine were subjected to varying concentrations of MNU (0.2-2 mM) and DMSO (vehicle control) for 4 hours. MNU had an inhibitory effect on HeLa S3 cells in a dose dependent manner. The IC_{50} of MNU for HeLa S3 cells was about 2 mM in the presence or absence of MGMT (fig 4.1.2 (A and B). Based on these results a concentration 0.5 mM MNU was used for co-immunoprecipitations done with HeLa S3 cells as explained in chapter 6.

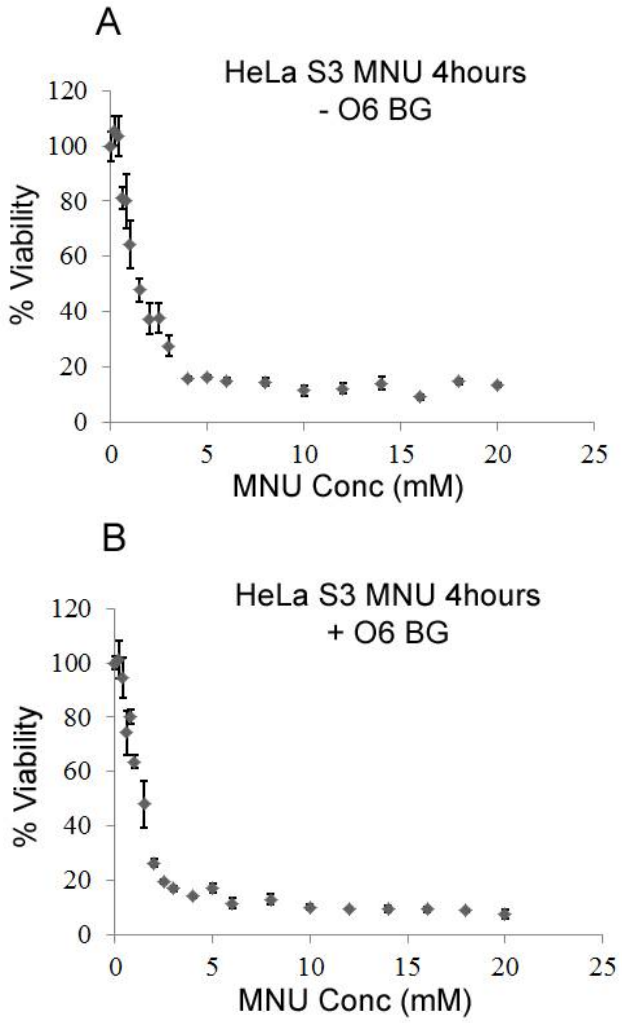

Fig 4.1.2: Dose response curve for varying concentrations of MNU in HeLa S3 cells in presence and absence of MGMT. A) HeLa S3 cells were treated with varying concentrations of MNU (0.2-2 mM) for 4 hours. B) HeLa S3 cells were pretreated with 20 µm O6 benzylguanine (O6 BG) for 1 hour before treatment with MNU. The cells were incubated with MTT for 4 hours following which DMSO was added to solubilize the formazan crystals. The absorbance was read at 570 nm. The % viability was calculated and plotted on the y-axis with MNU concentrations on the x-axis. From the plots we found that the IC_{50} of MNU for HeLa S3 cells was about 2 mM in presence or absence of MGMT.

4.2 Analysis of DNA break formation resulting from MNU damage by single cell gel electrophoresis (also called Comet assay)

Single cell gel electrophoresis (SCGE) combines DNA electrophoresis with fluorescence microscopy to detect DNA breaks depending on the migration of DNA embedded on agarose slides.

In SCGE, the cells embedded in agarose coated on the slides are subjected to lysis which removes cell membrane and cellular proteins. The DNA is then exposed which when subjected to either alkaline (pH>13) or neutral (pH7 to10) helps in identifying single strand breaks, double strand breaks, crosslinks, unrepaired excision repair sites [reviewed in (Anderson, Yu et al. 1998)].

Double strand breaks results in formation of DNA fragments which migrates upon electrophoresis in neutral pH. On the otherhand, single strand breaks, nicks in the DNA or unrepaired excision sites in the DNA do not result in DNA fragments. Therefore it is important to unwind the two strands of DNA in alkaline pH at which single strand breaks and apurinic sites are labile and results in unwinding of DNA strands which migrate upon electrophoresis in alkaline pH. Double strand breaks can also migrate under alkaline pH conditions, therefore the Alkaline comet assay protocol do not differentiate between single or double strand breaks while the neutral pH comet assay protocol helps in identifying only the double strand breaks [reviewed in(Fairbairn, Olive et al. 1995, Tice, Agurell et al. 2000)].

4.2.1 MNU causes formation of single strand breaks

The cell suspension layered on agarose slides were subjected to lysis and electrophoresis under alkaline pH-13 conditions. The apurinic sites are alkali labile which relaxes the supercoiled DNA; hence the broken DNA lags behind in the form of balloon shaped tails while the undamaged DNA is supercoiled which migrates faster on electrophoresis in agarose slides. The extent of damage is quantified using a comet assay macro tool of Image J. It calculates

the percentage of DNA in the tail and head of the comet and also the tail length. We used a concentration/titre of 0.5 mM MNU, which allows cell viability greater than 80% to understand the DNA damage response in HeLa cells. HeLa cells were either treated with 0.5 mM MNU for 0.5, 6, 12, 24 and 48 hours or with DMSO (vehicle control) for 48 hours. The comet tails representing the single strand breaks appear as balloon shaped within 30 minutes of MNU treatment [fig 4.2.1 (A)]. MNU treated cells show high percentage of tail DNA and longer tail length than the cells treated with DMSO indicating single strand break formation [fig 4.2.1 (A)]. We also observed that MNU exposure caused breaks to form within the first 30 minutes as higher tail length and % tail DNA was observed than control. We also observed that the damage caused by MNU was persistent over time indicating unrepaired DNA [fig 4.2.1 (B and C)].

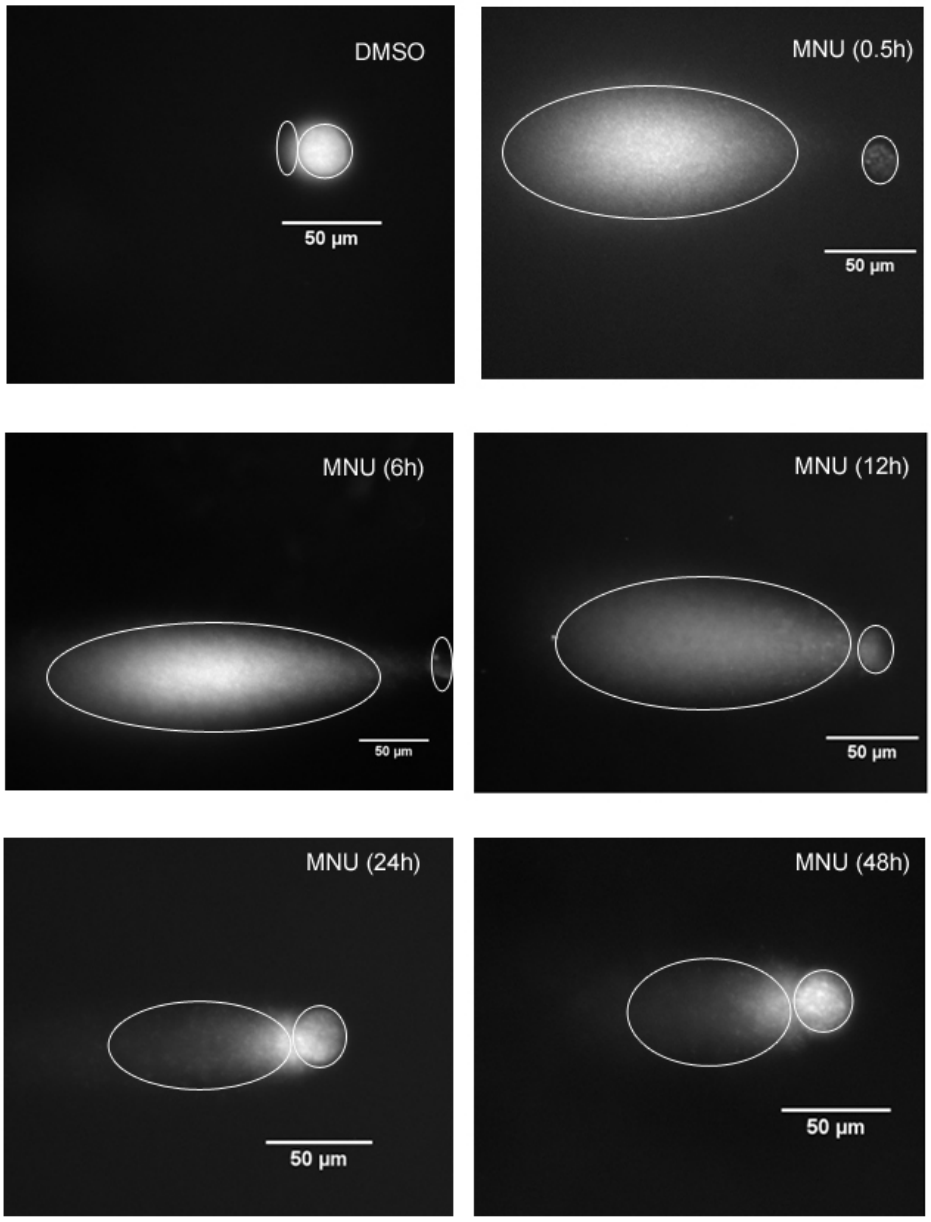

Fig 4.2.1: Comet assay showing single strand breaks caused by MNU damage in HeLa cells over a time course. (A) HeLa cells show an increase in DNA strand breakage with time of exposure to MNU. The comet tails persist at 48 hours post DNA damage suggesting unrepaired breaks. HeLa cells were treated with MNU for time duration as indicated in the top right corner of image. The cell suspension was layered on agarose slides. The agarose slides were stained with ethidium bromide and imaged on Axio Imager Z.1 ApoTome microscope (Zeiss). The regions which are quantified are marked in white in the first two images. Same region of interest was chosen for all the images. The images were taken at 20 X magnification.

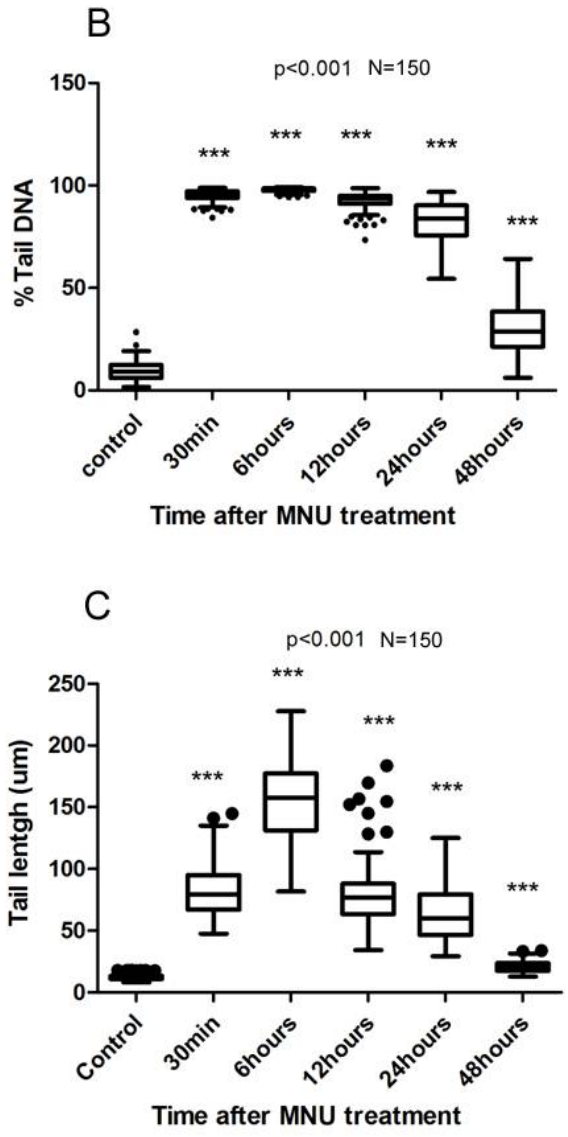

Fig 4.2.1: MNU causes single strand break formation in HeLa cells. (B, C) The % tail DNA (B) and tail length (C) indicates high amount of DNA in the comet tail due to breakage of DNA following MNU exposure. Both % tail DNA and tail length were significantly higher in the treatment groups than the control at $p < 0.001$ in one way Anova with Kruskal-Wallis non parametric tests.

4.2.2 MNU causes double strand break formation

HeLa cells treated with DMSO for 48 hours and with 0.5 mM MNU for various time points (0.5, 6, 12, 24, 48 hours) were layered on agarose slides.

The slides with cell suspension were then lysed and electrophoresed at pH-8, which would expose the broken ends of DNA resulting in comet formation. The comets were visualized with ethidium bromide staining and imaged on a fluorescence microscope. The comets showing double strand breaks appeared within 30 minutes of exposure to MNU [fig 4.2.2 (A)] and they seem to persist till 48 hours post damage. The extent of damage was quantified in terms of % tail DNA and tail length using image J. Tail length and % tail DNA showed an increase within 30 minutes of exposure to MNU indicating the presence of double strand breaks [fig 4.2.2 (B and C)].

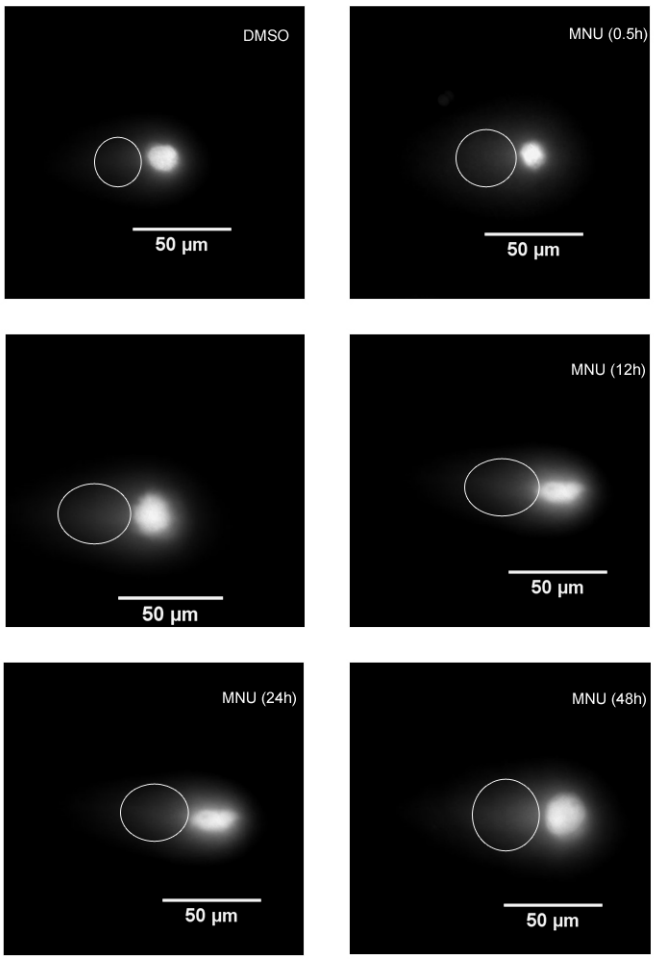

Fig 4.2.2: Comet assay showing double strand breaks caused by MNU damage in HeLa cells over a time course. (A) HeLa cells were exposed to 0.5 mM MNU for indicated times given on the right hand corner of the images. HeLa cells show tail formation indicating double strand breaks. The tails were observed with 30 minutes of exposure and they persist till 48 hours post damage suggesting unrepaired breaks. The cell suspension was layered on agarose slides. The agarose slides were then stained with ethidium bromide and imaged on Axio Imager Z.1 ApoTome microscope (Zeiss). The images were taken at 20 X magnification.

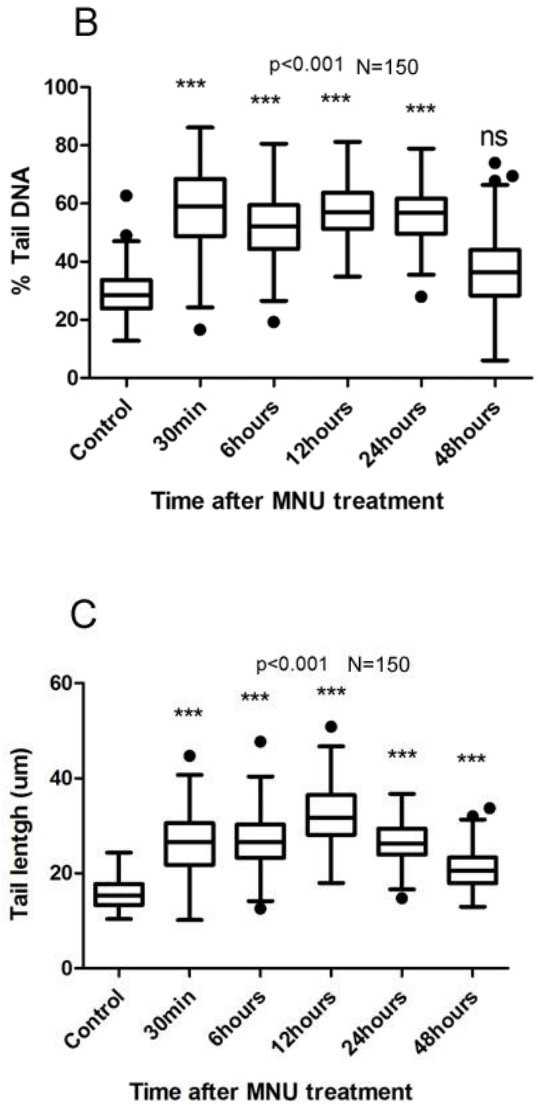

Fig 4.2.2: MNU causes double strand break formation in HeLa cells. (B, C) Tail length (B) and % tail DNA (C) show an increase within 30 minutes of exposure to MNU and the high values over time show the damage persists. The % tail DNA and tail length were significantly different in the treatment groups than the control at $p < 0.001$ in one way Anova with Kruskal-Wallis non parametric tests.

4.3 MNU causes checkpoint activation

Since we observed both double and single strand break formation following MNU treatment, we checked if those breaks could signal the checkpoint protein kinases for checkpoint activation. Chk1 and Chk2 are effector kinases which are phosphorylated at Ser 345 and Thr 68 by ATR and ATM kinase respectively. In our studies we observed phosphorylation of Chk1 at Ser 345 (fig 4.3), which corroborates with the occurrence of tails in alkaline comet assay following MNU treatment [fig 4.2.2 (A, B and C)]. In parallel we also observed phosphoryltaion of Chk2 at Thr 68 (fig 4.3) corresponding to the double strand break formation seen in the neutral comet assay [fig 4.2.3 (A, B and C)]. Both Chk1 and Chk2 phosphorylation persists till 48 hours of damage. Replication protein A (RPA) is hyper-phosphorylated at multiple sites following DNA damage and one of the sites is Thr 21. We observed RPA phosphoryltaion at Thr 21 to appear only at 24 hours post MNU damage even though Chk1 and Chk2 phosphorylations were observed at earlier time points (fig4.3).

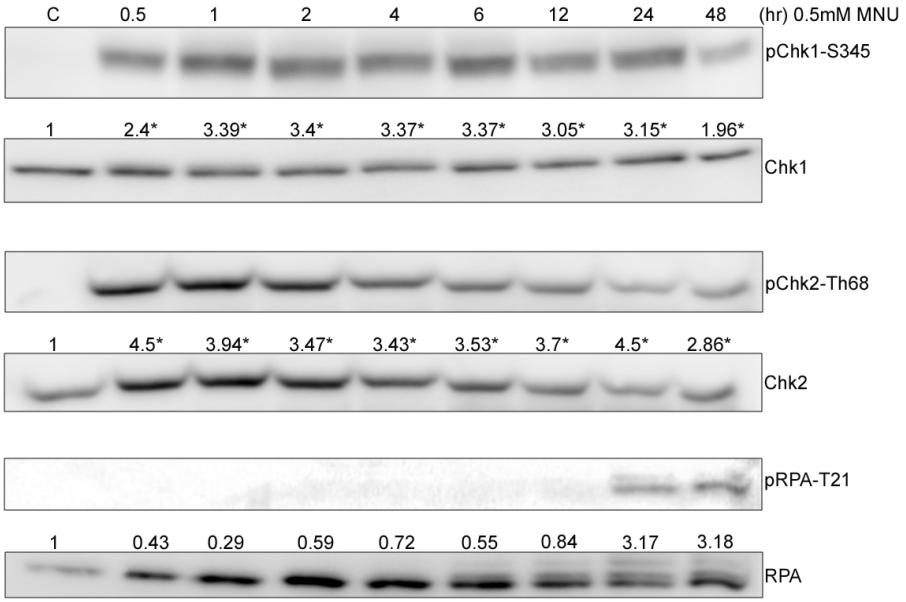

Fig 4.3: MNU causes early checkpoint activation in HeLa cells post MNU treatment. The ATM and ATR signaling was active following MNU exposure. Early Chk1 and Chk2 phosphorylation was observed following MNU exposure. RPA phosphoryltaion peaked at 24 hours post damage. The cell lysates were prepared at 0.5, 2, 4, 6, 12, 24 and 48 hours post treatment and checked for checkpoint activation by immunoblotting. The immunoblots showing activation of Chk1 and Chk2 were done at least three times while blot showing RPA phosphorylation was only done once. The fold difference between control and different time points was significant at $p < 0.05$ when analyzed by one way Anova with Tukey post hoc test. The images were taken using ImageQuant LAS 4000 detection system (GE Healthcare).

4.4 MNU causes G/2 arrest in HeLa cells following MNU damage.

We analyzed cell cycle progression of HeLa cells at 0, 0.5, 1, 2, 4, 6, 12, 24 and 48 hours post MNU treatment using propidium iodide staining and flow cytometry. Propidium iodide intercalates within DNA and fluoresces on excitation at 535 nm wavelength. The cells with varying DNA content will emit out different fluorescence intensity indicating their cell cycle phase. HeLa cells were observed to progress normally until 48 hours post damage

where they accumulated with a 4N DNA content indicating a G2 arrest (fig 4.4).

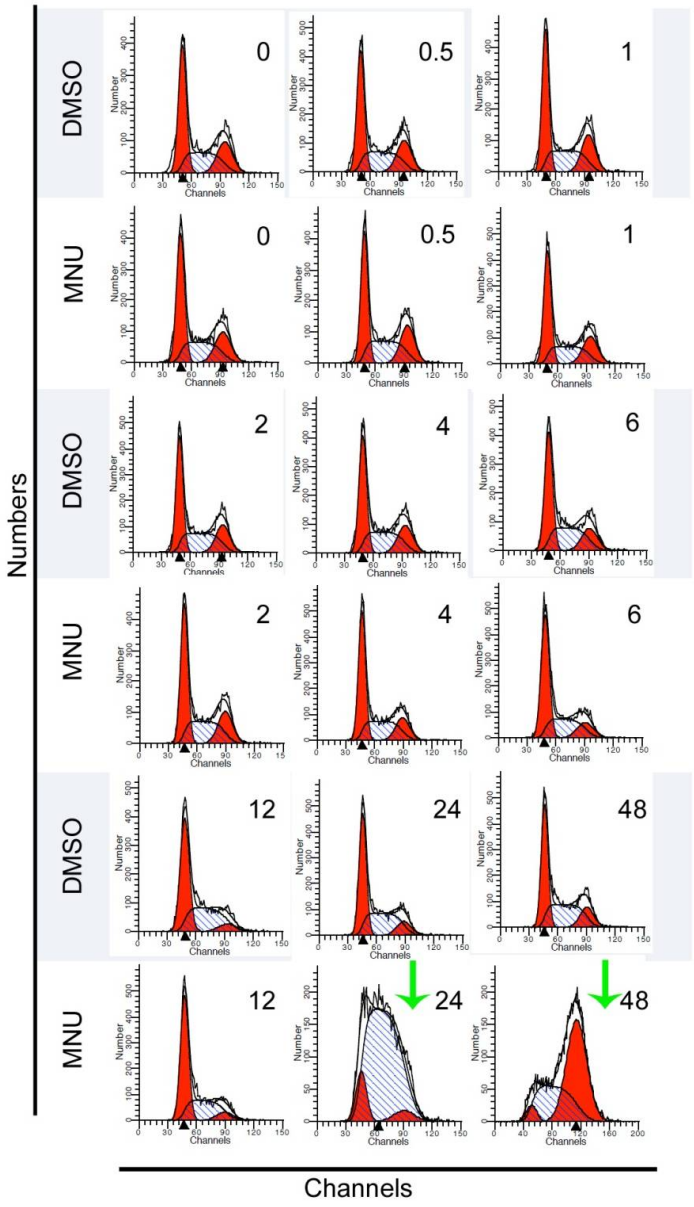

Fig 4.4: MNU results in accumulation of cells in G2 phase following MNU exposure G2/M arrest in HeLa cells. HeLa cells were either untreated or treated with 0.5 mM MNU. The cells were harvested at the indicated times to monitor cell cycle progression by staining with propidium iodide and flow cytometry.

Discussion

In this chapter we focused on the cellular response to the alkylating agent N-methyl-N-nitrosourea (MNU), which is to be used as the damaging agent in further experiments. We demonstrated that MNU is cytotoxic to both HeLa and HeLa S3 cells. MNU reacts with DNA and can form different kinds of base adduct. Alkyl DNA base adducts like N3 methyladenine (N3MeA), N7 methylguanine (N7MeG), O6 methylguanine (O6MeG) have different stabilities [reviewed in (Beranek 1990)]. In the absence of methylguanine methyltransferase (MGMT) which repairs O6MeG adducts, the cells become sensitive to methylating agents, (Day, Ziolkowski et al. 1980, Yarosh, Foote et al. 1983). In our studies we showed that the variants of HeLa cells - HeLa (MGMT negative) and HeLa S3 (MGMT positive) were equally sensitive to MNU (fig 4.1.1). MNU was also found to be equally toxic to HeLa S3 cells independent of its MGMT status (fig 4.1.2). MGMT removes alkylation products in a one step reaction that inactivates the enzyme. As there is one MGMT molecule for one alkyl adduct; the rate of removal of alkyl groups depends on the size of the group, the number of MGMT molecules present per cell, the rate at which the cell can replenish its MGMT levels and also the amount of alkylating agent used [reviewed in (Kaina, Christmann et al. 2007)]. In our studies we used a range of concentration of MNU, which might be saturating for the cells suggesting that the first line of defense by MGMT is inefficient. MGMT deficient HeLa cells employ the nucleotide or base excision repair pathways to get rid of the alkylated DNA, on the other hand MGMT proficient HeLa S3 cells utilizes MGMT as well as other repair pathways to remove the O6 MeG. In our studies the concentration of MNU seemed to be saturating, therefore there no difference in the sensitivity towards MNU among the two cell lines was observed.

We showed that within 30 minutes of exposure to MNU, both double and single strand DNA breaks were generated as seen in the alkaline and neutral comet assays (fig 4.2.1 and fig 4.2.2). MNU like any other SN_1 type alkylating agent creates DNA adducts which includes O6MeG, N7MeG and 3MeA that

lead to distinct forms of DNA damage. DNA adducts like 3MeA and N7G are stable under alkaline conditions used in comet assay and do not result in artifactual DNA breakage. But they do however lead to apurinic sites and single strand breaks as a consequence of repair by base excision repair pathway (Singer and Brent 1981). During BER, DNA glycosylases remove the glycosidic bond between DNA backbone and nitrogen moiety, which creates an apurinic (AP) site. These AP sites undergo AP directed strand excision which then is refilled by new strand synthesis. *In vitro* studies have shown that new strand synthesis is a rate limiting factor which could also explain the generation of strand breaks in case of the early breaks that were observed to be formed following MNU exposure (Srivastava, Berg et al. 1998, Sung and Mosbaugh 2003).

MMR dependent G2 checkpoint activation in response to alkylating agents has long been established. It was shown (Stojic, Mojas et al. 2004) that low doses of MNNG trigger G2/M arrest in MMR proficient cells following second round of DNA replication. N7MeG or 3MeA lesion caused by alkylating agents do not show mispairing and undergo processing by mismatch repair (MMR) which would lead to formation of DNA double strand breaks (Wyatt, Allan et al. 1999, Boysen, Pachkowski et al. 2009). Only O6MeG lesions are known to mispair with thymine, which undergoes processing by MMR proteins. As the methylation is in the parental strand, MMR is unable to fix the lesion thereby leading to generation of gaps in the DNA and checkpoint activation (Stojic, Brun et al. 2004). In our study, HeLa cells which are MMR proficient and MGMT deficient were arrested in G2 phase following MNU damage.

Even though in case of HeLa cells exposed to 0.5 mM MNU, we observed double and single strand breaks early on, but did not observe an early cell cycle arrest (fig 4.4). The G2 arrest in HeLa cells following MNU exposure was preceded by posttranslational modification of proteins involved in DNA damage signaling (fig 4.3). Both Chk1 and Chk2 phosphorylation was observed within 30 minutes of MNU damage. The activation of ATM and ATR pathways coincided with the occurrence of DNA strand breaks.

Lesions formed due to MNU treatment that contribute to the activation of checkpoint signaling are not well understood. But it is known that 3MeA and N7meG can lead to phosphoryltaion of Chk1 (Garvik, Carson et al. 1995, Dai and Grant 2010). Assuming that Chk1 and Chk2 were phosphorylated by their sensor kinases ATR and ATM respectively, we can speculate that the breaks generated by processing of methylated DNA bases by BER were responsible for early checkpoint activation.

It was also observed that the Chk1 and Chk2 phosphorylations persisted till 48 hours post MNU damage (fig 4.3). It is quite possible that the O6 methylations are responsible for persistent checkpoint signaling following MNU exposure. It appears that the initial checkpoint signaling could be a result of processing of N3MeA and N7MeG lesions by BER and the O6MeG are processed slowly and unsuccessfully by thymine or guanine thymine glycosylases (Au, Welsh et al. 1992). The O6 methylations result in mispairing of guanine with thymine following first the replication cycle which are substrates for MMR proteins. MMR processing of mispaired O6MeG:T generates breaks to which ATR/RPA are recruited which could explain the occurrence of RPA phosphoryltaion only after 24 hours of MNU damage when most of the cells are in S phase (fig4.3 and fig4.4). Earlier studies done with alkylating agents like MNNG and Temozolamide have also shown Chk1 phosphorylation to occur within 3 hours of treatment in MMR proficient cells (Stojic, Cejka et al. 2005, Ito, Ohba et al. 2013). It is still not known whether the early Chk1 phosphorylation observed during MNU exposure is due to processing of lesions by BER or MMR or a combination of both.

Summary

In this chapter we describe the DNA damage response pathways that are activated when cells are exposed to N-methyl-N-nitrosourea (MNU). MNU is a potent methylating agent that is cytotoxic to HeLa and HeLa S3 cells as seen in the colorimetric assay (MTT). We also showed that the concentration of MNU in the millimolar range was saturating for the cells and hence presence or absence of methylguanine methyltransferase (MGMT) did not change the

sensitivity of cells towards MNU. HeLa cells undergo DNA strand breaks within 30 minutes of MNU exposure as observed by single cell gel electrophoresis. The DNA breaks persisted till 48 hours following damage. MNU like other SN1 alkylating agent caused a G2 arrest at 48 hours following damage. MNU causes methylations on DNA bases, which are processed by BER and MMR proteins resulting in strand breaks. Parallel to DNA breakage we also noticed checkpoint activation following MNU damage. We noticed that Chk1 and Chk2 were phosphorylated as early as 30 minutes but RPA phosphoryltaion peaked at 24 hours following damage. Due to unsuccessful processing of O6 methylations by MMR, the DNA strand breaks may persist which then could lead to sustained phosphoryltaion of Chk1 and Chk2 till 48 hours post damage.

Chapter 5: *In vivo* Msh2-TopBP1 interaction studies following methylation damage

Background

SN_1 type alkylating agents like N-methyl-N-nitrosourea (MNU) cause DNA base modifications by adding methyl group. The covalent DNA modifications when processed by BER and MMR activate ATR signaling. Ataxia Telangiectasia Rad3 (ATR) related sensor kinase is implicated in response to broad range of DNA damaging agents like UV, hydroxyurea and other chemical agents. ATR recognizes single strand DNA breaks generated during replication fork stalling or by other external agents. ATR activity is a part of a complex network with a number of mediator proteins like Topoisomerase IIβ binding protein (TopBP1) and Claspin. ATR works by activating a large number of downstream substrates like Chk1 and Smc1, which result in cell cycle arrest and apoptosis (Shechter, Costanzo et al. 2004). TopBP1 is an important scaffold protein that regulates multiple cellular processes like DNA replication and checkpoint activation [reviewed in (Zegerman and Diffley 2009, Branzei and Foiani 2010)].

DNA damage signaling in response to alkylating agents require MMR based processing of alkylated bases. The repeated attempts by MMR to repair the mismatches caused by O6MeG signals checkpoint proteins to halt the cell cycle or bring about apoptosis [reviewed in (Karran 2001)]. Even when MNNG causes a delayed cell cycle arrest post replication, the checkpoint proteins ATM, Chk2 and Chk1 are phosphorylated within 12 hours of damage (Stojic, Mojas et al. 2004). This fuelled a second line of thought of an alternate signaling pathway. Previous studies have proposed that MutSα could be directly involved in checkpoint signaling following methylation damage [reviewed in (Fishel 1999, Li 1999)]. The notion was later supported by the

fact that Msh2 was known to interact with a number of checkpoint proteins like Chk1 and Chk2 (Brown, Rathi et al. 2003, Adamson, Beardsley et al. 2005) in humans. Msh6p was found to interact with Mek1p, the ATR homolog in budding yeast (Gavin, Aloy et al. 2006). MutSα can also recognize O6MG adducts directly and recruit ATR-ATRIP to activate the checkpoint machinery in response to methylation damage (Yoshioka, Yoshioka et al. 2006). MutSα is also known to interact with a number of proteins in the ATR-Chk1 pathway including TopBP1 (Liu, Fang et al. 2010).

A fundamental question regarding the role of Msh2 and TopBP1 in methylation damage is yet to be answered. It is not known whether the early checkpoint signaling that is observed post methylation damage is dependent on the processing of base adducts by Msh2 and TopBP1.

In this chapter we investigated whether TopBP1 and Msh2 affected the early checkpoint response to MNU damage.

Results

5.1 TopBP1 localizes to damaged sites to form DNA damage repair foci

To study the recruitment of TopBP1 to damaged sites following MNU damage, immunofluorescence assay was used. The cellular relocalization of TopBP1 in HeLa cells was examined by staining the endogenous protein with anti-TopBP1 antibody following MNU damage. A time course assay was done wherein HeLa cells were exposed to 0.5 mM MNU for different time periods (0.5, 1, 2, 4, 6, 12, 24, 48 hours) before they were fixed and stained. Upon MNU damage TopBP1 protein relocalizes to damaged sites on DNA in the form of discrete and cytologically detectable foci [fig 5.1.1 (A)]. TopBP1 foci were observed within 30 minutes of exposure to MNU. The number of cells that showed TopBP1 foci increased post 48 hour MNU exposure [fig 5.1.1 (B)]. The representative image of HeLa cells showing TopBP1 foci at 6 hours post MNU damage s show in [fig 5.1.1 (B)], images for all the time points showing TopBP1 foci are shown in [fig 5.5] in appendix I. To confirm that the TopBP1 foci were indeed damage induced, cells exposed to MNU were scored for 53BP1 and TopBP1 foci. Asynchronously growing HeLa cells were exposed to MNU for different time periods (0.5, 1, 2, 4, 6, 12, 24 and 48 hours). The cells were then fixed and stained with antibodies against TopBP1 and 53BP1. TopBP1 and 53BP1 foci were found to co-localize following MNU damage [fig 5.1.1 (C)]. The cells were scored for TopBP1 and 53BP1 foci in random field of vision. The percentage of TopBP1 foci co-localizing with 53BP1 out of the total number of TopBP1 foci observed was represented in the micrograph. Around 30 % of the TopBP1 foci were found to co-localize with 53BP1 at any given time post MNU treatment [fig 5.1.1 (D)]. Interestingly at 48 hours post MNU damage, the number of TopBP1 foci increased but the percentage of TopBP1 foci that co-localized with 53BP1 decreased [fig 5.1.1 (B) and fig 5.1.1 (D)]. HeLa cells showing co-localized TopBP1 and 53BP1 foci at different time points following MNU damage are shown in [fig 5.6] in appendix I.

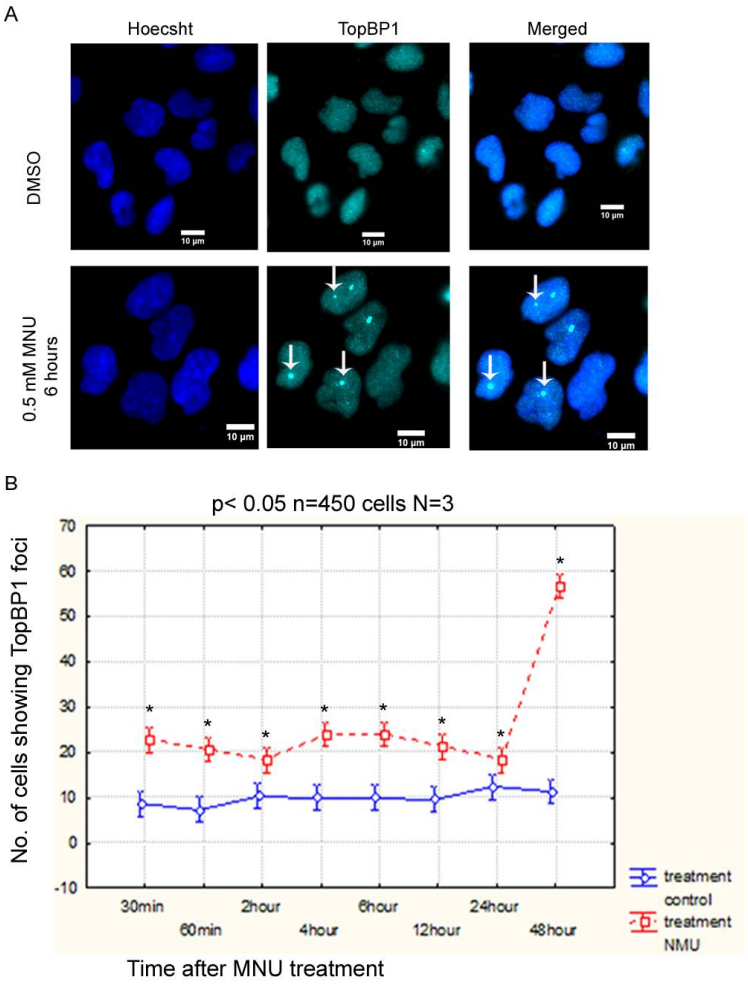

Fig 5.1.1: TopBP1 forms DDR foci following methylation damage. (A). TopBP1 forms distinct nuclear foci following methylation damage (as indicated by the white arrows). (B) Number of foci increases at 48 hours post damage when cells are arrested in G2 phase. HeLa cells treated with MNU were fixed at indicated time points and stained with antibodies against TopBP1. Representative image of TopBP1 foci at 6 hours post MNU damage as seen at 63 X magnification on Axio Imager Z.1 ApoTome microscope (Zeiss). Data represented are +/- standard error of mean of three independent experiments where 150 cells were scored for presence or absence of TopBP1 foci in each experiment. Two way Anova with Bonferroni post-test was used to analyze the data sets using STATISTICA. Bar = 10 µm

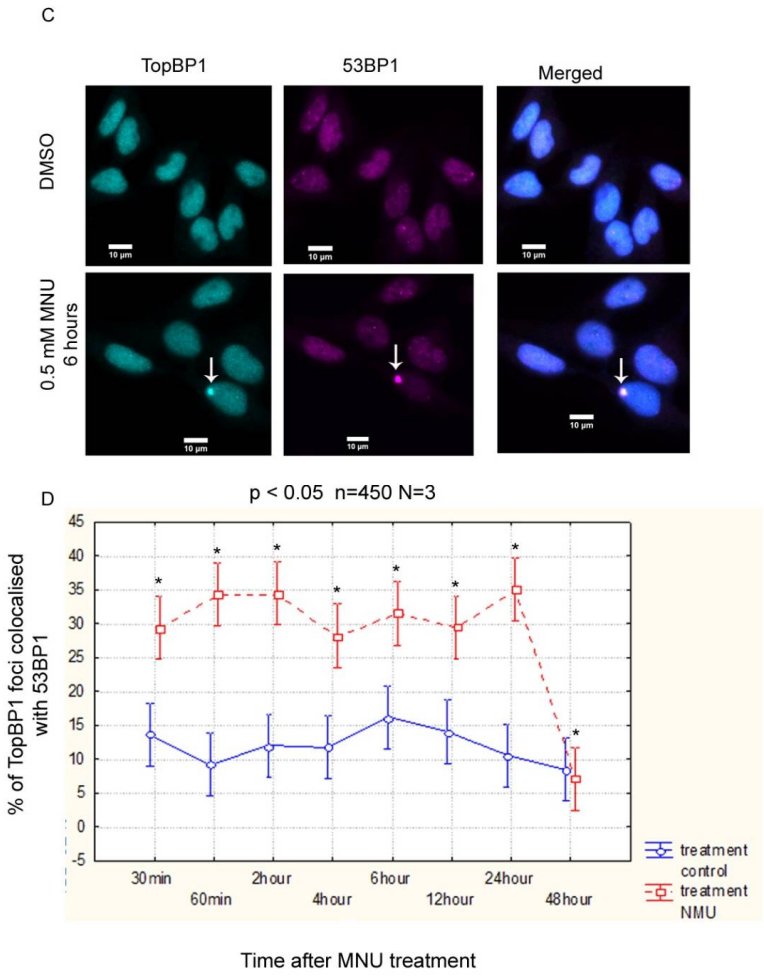

Fig 5.1.1: TopBP1 co-localizes with 53BP1 following MNU damage. (C) Co-localization of endogenous TopBP1 and 53BP1 following 0.5 mM MNU for 6 hours as shown by the white arrows. (D) The line plot shows the percentage of TopBP1 foci co localized with 53BP1 following MNU damage in a time course assay. Total number of TopBP1 foci and the number of TopBP1 foci that co-localized with 53BP1 were scored randomly and percentage of co-localized TopBP1 and 53BP1 foci was calculated. 150 cells were scored for each experiment and the data represented graph is +/- standard error of means for three independent experiments. Two way Anova with Bonferroni post-test was used to analyze the data sets using STATISTICA. Bar = 10 µm

5.2 Msh2 levels increase in nucleus post MNU damage

Msh2 is located both in the nucleus and cytoplasm and has been earlier reported to translocate into the nucleus upon MNNG damage (Christmann and Kaina 2000).

To investigate if MNU damage can induce accumulation of Msh2 in the nucleus, an immunofluorescence assay was used. HeLa cells treated with 0.5 mM MNU for different time periods (0, 0.5, 1, 2, 4, 6, 12, 24 and 48 hours) were fixed and stained with anti Msh2 antibody. The total fluorescence intensity of Msh2 after normalization in treated samples was found to be higher than the control (DMSO) [fig 5.2]. Msh2 starts accumulating in the nucleus within half an hour of treatment with MNU, but the levels are saturated after 6 hours of MNU treatment. Msh2 levels inside the nucleus remain unchanged till 48 hours post MNU damage. HeLa cells showing translocation of Msh2 into nucleus following MNU damage at different time points is shown in [fig 5.7] in appendix I.

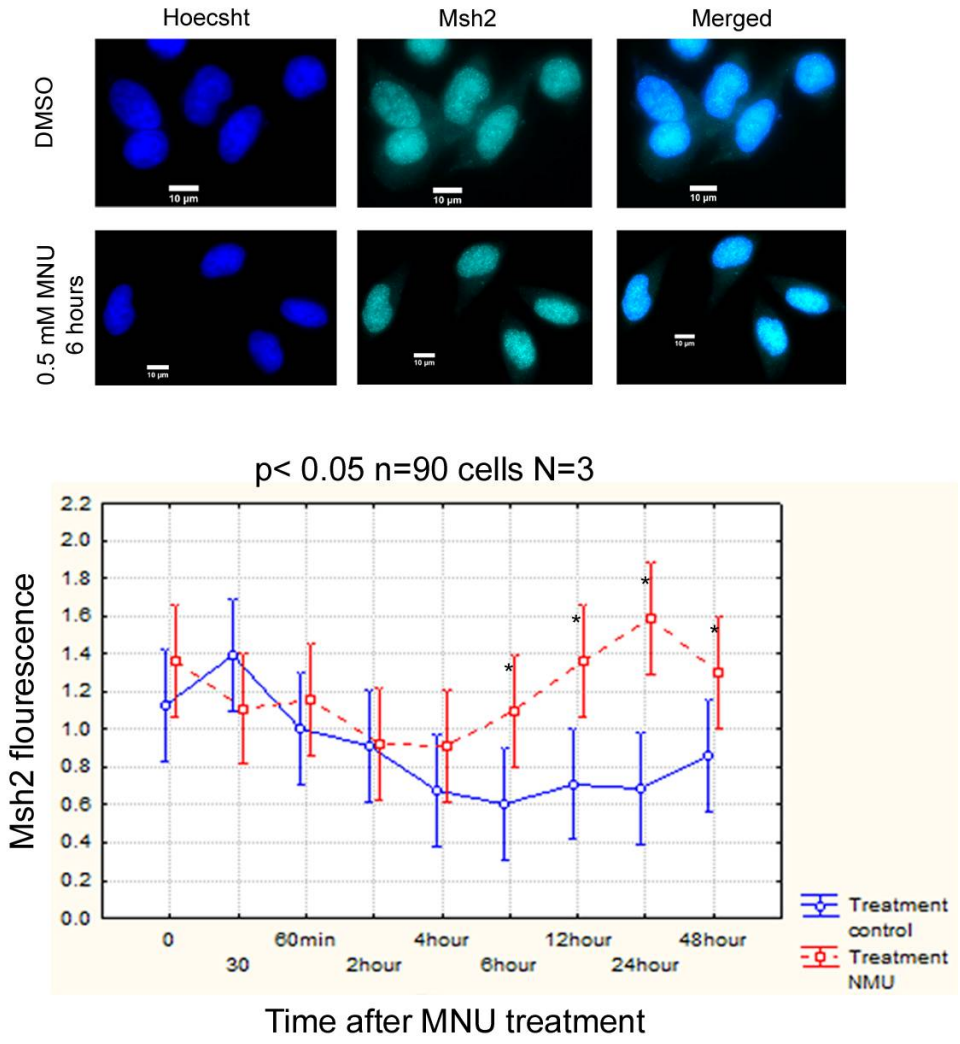

Fig 5.2: Msh2 accumulates in the nucleus following MNU damage. Fluorescence intensity of MNU treated samples is higher than the DMSO treated samples post 6 hours of damage. Total nuclear fluorescence of Msh2 normalized to Hoechst levels in each nucleus was plotted. Fluorescence intensity of 30 cells was measured in three independent experiments. Data represented in the graph is +/- standard error of means of three independent experiments. Two way Anova with LSD Fisher test was used to analyze the data sets using STATISTICA. Bar = 10 µm

5.3 TopBP1 co-precipitates with Msh2 independent of DNA damage and MGMT status of the cell line

In vitro studies with recombinant TopBP1 and Msh2 showed that the two proteins interact with each other in the absence of their auxiliary partners. *In vivo* studies suggest that both the proteins have many functions, which are mediated by virtue of their interaction with different proteins. Co-immunoprecipitation studies using HeLa S3 whole cell extracts was done in the presence and absence of DNA damage, to investigate if the two proteins associate with each other in presence of DNA damage and also if the interaction was enhanced following damage. HeLa S3 cells were chosen for co-immunoprecipitation as these cells are mismatch repair proficient and display checkpoint responses to SN_1 type methylators (Schroering and Williams 2008). HeLa S3 cells have high levels of methylguanine methyltranferase (MGMT), which is the primary repair mechanism against methylation damage. O(6) –benzylguanine (O6BG) is a small molecule inhibitor of O(6) alkyl guanine DNA alkyltransferase (AGT) which potentiates the effect of the methylating agent . Asynchronously growing HeLa S3 cells were treated with 20 μm of O6BG for 2 hours before addition of MNU or DMSO (6 hours). Msh6 antibody was used to immunoprecipitate MutSα complex to identify whether TopBP1 was a binding partner in response to MNU damage. We observed that the MGMT status of the cells did not matter since TopBP1 and Msh2 co-precipitated with Msh6 in the presence and absence of MGMT [fig 5.3 (A) and (B)]. Interestingly the three proteins were bound to each other even in undamaged cell extracts and the interaction was not enhanced upon MNU damage [fig 5.3 (A) and (B)]. Reciprocal immunoprecipitation using TopBP1 antibody also revealed that Msh2 and TopBP1 are constitutively bound to each other irrespective of DNA damage [fig 5.3 (C) and (D)]. Despite using different antibodies against Msh6, it was not detected in western blot in which TopBP1 was immunoprecipitated.

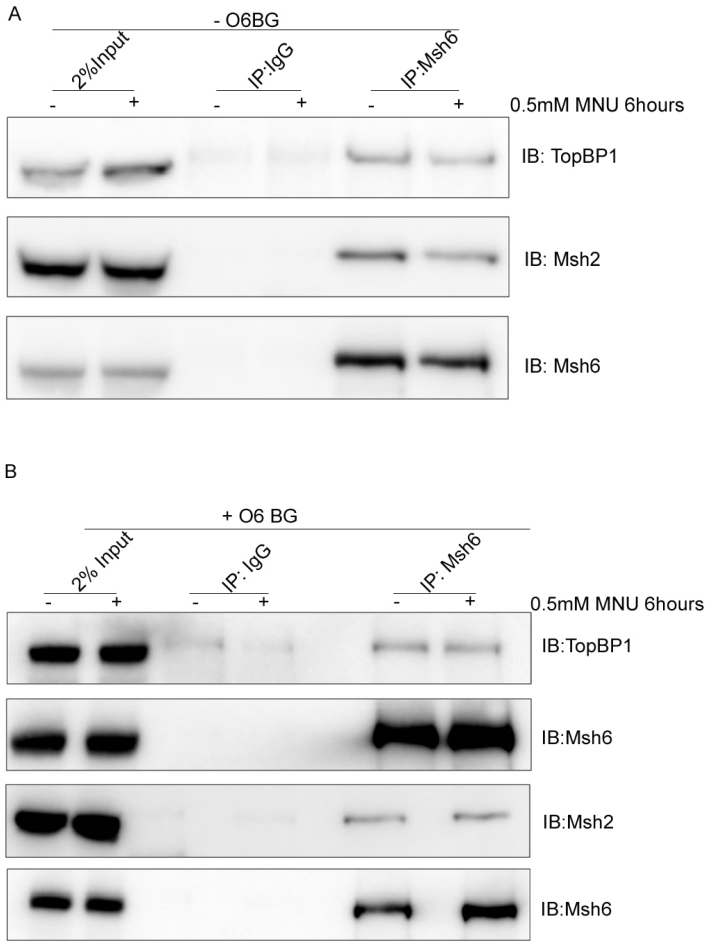

Fig 5.3 (A, B): Co-precipitation of Msh2 and TopBP1 from HeLa S3 cell extracts. TopBP1 was found to co-precipitate with Msh2 and Msh6. HeLa S3 cells were either pretreated or untreated with 20 μm O6BG before addition of MNU. Immunoprecipitation was done from HeLa S3 whole cell extracts using antibody against Msh6. 2 % of the total extract was loaded as input (lane marked as 2% input) on to the gel. Rabbit IgG was used as negative control for precipitation with Msh6 antibody. The labels on the right indicate proteins co-precipitating with Msh6 as determined by western blotting. The Msh6 in the immunoprecipitates was evaluated following western blot using antibodies against Msh6 (upper panel or left panel). The images were taken using ImageQuant LAS 4000 (GE Healthcare) system.

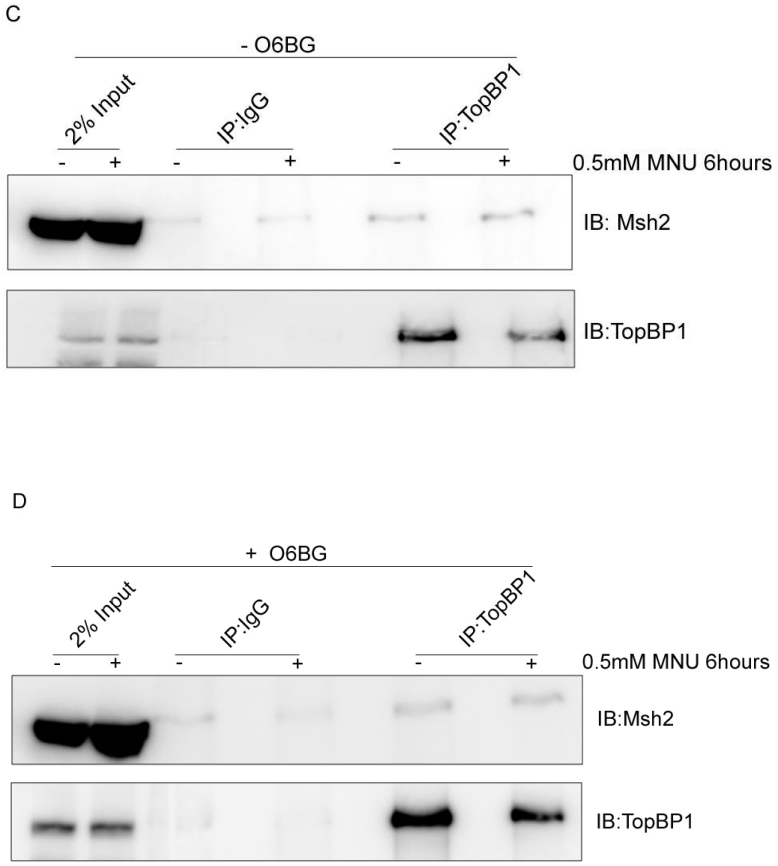

Fig 5.3: (C, D) Msh2 associates with TopBP1 in HeLa S3 whole cell extracts. TopBP1 and MSh2 co-precipitates in presence or absence of DNA damage. HeLa S3 cells were either pretreated or untreated with 20 μm O6 benzylguanine (O6BG) before addition of MNU. TopBP1 was immunoprecipitated from HeLa S3 whole cell extracts. A parallel IP using anti rabbit IgG antibody serves as a negative control. 2 % of the total cell extract used for immunoprecipitation was loaded on to the gel. TopBP1 in the immunoprecipitates was evaluated using antibodies against TopBP1 (upper or left panel). The images were taken using ImageQuant LAS 4000 (GE Healthcare) detection system.

5.4 Early Chk1 phosphorylation following MNU damage is dependent on TopBP1 and Msh2

To investigate whether checkpoint activation in response to MNU damage was dependent on TopBP1 and/or Msh2, both TopBP1 and Msh2 were transiently knocked down by RNA interference (RNAi) using small interfering RNA (siRNA) in HeLa cells. HeLa cells transfected with siRNA against mammalian TopBP1 or Msh2 were treated with 0.5 mM MNU for 6 hours. Knocking down of TopBP1 abrogated the checkpoint activation in response to MNU damage. Phosphorylation of Chk1 at Ser345 and Ser 317 was down-regulated in MNU-treated TopBP1 RNAi depleted cells (fig 5.4). When Msh2 was knocked down in cells using siRNA against the endogenous protein, checkpoint activation was partially abrogated in response to MNU treatment. Following MNU damage, Msh2 knocked down cells did not show downregulation of phosphorylation of Chk1 at Ser345 but Chk1 phosphorylation at Ser 317 was abolished (fig 5.4). Chk2 phosphorylation in response to MNU was also unperturbed in the absence of either Msh2 or TopBP1 (fig 5.4). The levels of phospho Chk2 in MNU-treated TopBP1 or Msh2 depleted cells were similar to that of Lac Z treated control (1.6 fold in case of TopBP1 depletion and 1.4 in case of Msh2 depletion) (fig 5.4).

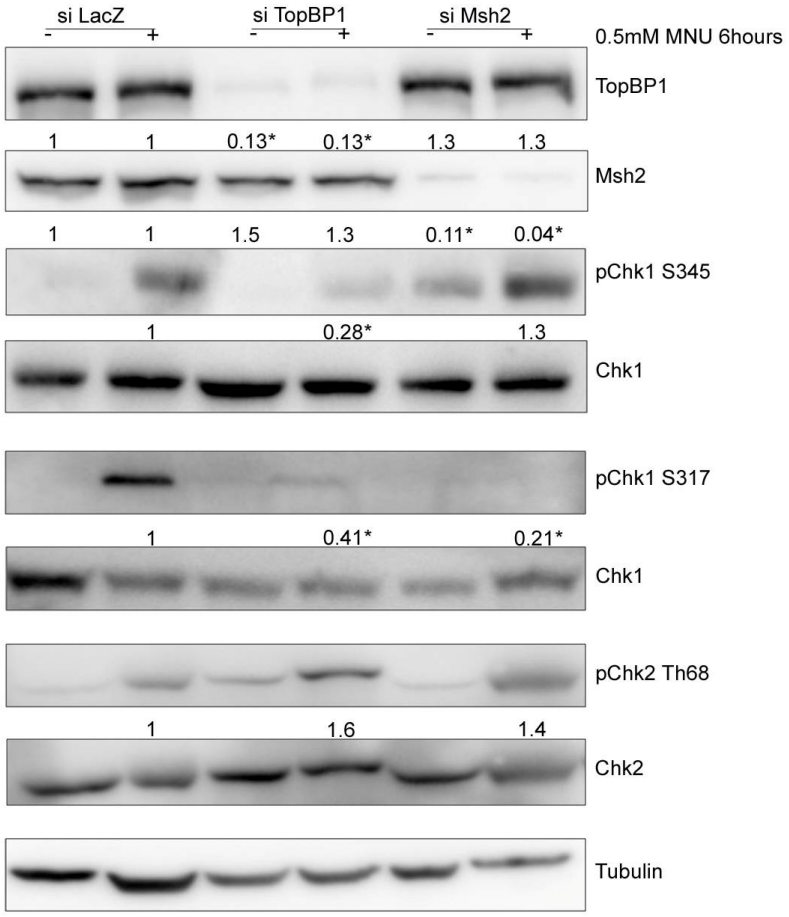

Fig 5.4: Dependency of phosphorylation of Chk1 Ser 345 or Ser 317 in response to MNU damage in TopBP1 or Msh2 siRNA knocked down cells. HeLa cells transfected with siRNA against TopBP1, Msh2 or LacZ were treated or untreated with 0.5 mM MNU for 6 hours. The cells were harvested and subjected to western blotting. Efficiency of RNA interference and fold difference for phosphorylation of Chk1 and Chk1 between control and treated samples was evaluated by quantifying the band intensities of the protein using Image J. Student't test (unpaired, two tailed) was used to analyze the significance in fold difference for pChk1 ,pChk2 and knockdown of TopBP1 and Msh2.Data represented are +/- of standard error of mean of three independent experiments.

Discussion

TopBP1 is a scaffold protein acting as a hub for different proteins to interact and mediate checkpoint signaling. TopBP1 is an important player in DNA replication checkpoint activation, which maintains genomic stability in eukaryotes (Bartek and Mailand 2006, Burrows and Elledge 2008). The role of TopBP1 in maintenance of genomic stability is emphasized by the fact that it is found to be over expressed in 60 % of breast cancer tissues and an increased risk of breast cancer relapse is seen in cases where TopBP1 mRNA levels are high (Liu, Bellam et al. 2009). A characteristic hallmark of DNA damage response is the accumulation of DNA damage response proteins to the damaged sites in the form of foci. In this study, we showed that TopBP1 forms foci following MNU exposure and the number of foci increases at 48 hours post MNU damage [fig 5.1.1 (A)]. TopBP1 is a protein that is also involved in DNA replication initiation and forms foci which are recognized as replication centers (Kim, McAvoy et al. 2005). Upon DNA damage TopBP1 dissociates itself from replication centers, is recruited to damaged DNA sites and forms DNA damage dependent foci. MNU, an alkylating agent causes single strand and double strand breaks in DNA to which different repair pathway proteins are recruited. TopBP1 is known to collaborate with 53BP1 in response to double strand breaks formed in G1 phase of the cell cycle (Cescutti, Negrini et al. 2010). 53BP1 is recruited to sites of double strand breaks to form foci that co-localize with TopBP1 foci. Around 30% of the TopBP1 foci formed post MNU damage co-localize with 53BP1 [fig 5.1.1(B)] indicating that the TopBP1 foci observed following MNU damage are DNA damage dependent.

MMR proteins are essential for maintaining DNA replication fidelity and genomic stability. Loss of Msh2 or Mlh1 results in a hereditary form of cancer-Hereditary nonpolyposis colorectal cancer (HNPCC) which affects the large intestine and rectum. The mechanism of action of each of the mismatch repair proteins involved in repair has been widely studied. Though there is scarcity of information on the regulation of MMR proteins. Previous studies have shown that Msh2 is a cell cycle regulated protein and the levels of Msh2

are higher in dividing cells than the resting cells (Marra, Chang et al. 1996). Another study showed that MutSα is regulated by exogenous stimuli like damage induced by alkylating agents like MNNG. Msh2-Msh6 (MutSα) complex is evenly distributed in cytoplasm and nucleus in an undamaged cells and DNA damage induces translocation of MutSα complex into the nucleus. (Christmann and Kaina 2000). In this study, we observed that Msh2 starts accumulating in the nucleus post 6 hours of MNU damage (fig 5.2). We speculate that following MNU damage both TopBP1 and Msh2 relocalize in the nucleus to act at the lesions. Both the proteins may interact with each other at the same site of damage. Immunoprecipitation studies with Msh6 antibody or TopBP1 antibody showed that Msh2 and TopBP1 constitutively associate with each other [fig 5.3 (A), (B), (C) and (D)]. MGMT levels are highly variable among different cell lines and tumors. In MGMT deficient cells, O6MeG:T mispairs are repaired by MutSα mediated mismatch repair activity. Among the variants of HeLa cells**,** HeLa S3 cells have high amount of the MGMT enzyme which makes them sensitive to methylating drugs (Ochs and Kaina 2000)**.** We inferred from the immunoprecipitation experiments that MGMT expression in HeLa S3 cells did not affect the association of Msh2 with TopBP1 [fig 5.3 (A), (B), (C) and (D)]. Interestingly it was also observed that the two proteins are constitutively bound to each other and DNA damage did not affect their interaction [fig 5.3 (A), (B), (C) and (D)]. It may be speculated that the two proteins remain bound to each other to and scan DNA for damage and are possibly recruited to the damaged sites on the chromatin. Since we used whole cell extracts for immunoprecipitation, only the soluble fraction was pulled down using the antibodies, therefore it is possible that the proteins bound to the chromatin did not measure up in the results obtained.

It is known that following MNNG damage, Msh2 forms a complex with PCNA on the chromatin in the first replication cycle. In the second cycle, the complex does not include PCNA indicating the complex containing Msh2-Msh6 is dynamic and may have different functions (Mastrocola and Heinen 2010). It is quite possible that the trimeric complex of Msh2-Msh6-TopBP1 as observed in this study might be part of a larger complex where TopBP1 and

MutSα complex be the master regulators directing the recruitment of other proteins.

In the co-immunoprecipitation studies, even 6 hours of MNU damage was sufficient to observe an interaction between TopBP1 and Msh2. The 6 hours time point is short for replication to occur, therefore we reasoned that the two proteins might be regulating the early checkpoint activation observed following methylation damage as discussed in chapter 4 results section 4.1.2. It is well known that TopBP1 can directly activate ATR kinase (Kumagai and Dunphy 2006). TopBP1 depleted HeLa cells did not show Chk1 phosphorylation following MNU damage, indicating that Chk1 phosphorylation upon MNU damage is TopBP1 mediated. Previous studies have also shown that Msh2 can also interact with ATR and recruit it to damaged sites following cisplatin damage (Pabla, Ma et al. 2011). Msh2 depleted cells showed partial activation of Chk1 by phosphorylating Chk1 at Ser 345 and not at Ser 317 suggesting there may exist substrate specificity of Msh2-ATR complex to phosphorylate Chk1 at Ser 317.

Summary

In this chapter, we described the response of TopBP1 and Msh2 when cells were inflicted with MNU damage. TopBP1 is recruited to sites of damage and forms DNA damage response foci. The foci formation was visible within 30 minutes of exposure of MNU and the number of foci increased at 48 hours post damage. On the other hand Msh2 did not form foci but the total nuclear levels of Msh2 increased upon methylation damage. The time course assay showed that Msh2 accumulated in nucleus at 6 hours of MNU damage. The immunoprecipitation studies using HeLa S3 whole cell extracts showed that the two proteins interact with each other. TopBP1 and Msh2 were found to be associated with each other independent of DNA damage. The knockdown studies were done to understand the role of Msh2 in early checkpoint activation. Knockdown of Msh2 revealed that the Chk1 phosphorylation is partially dependent on Msh2. Msh2 depleted cells did not show phosphoryltaion of Chk1 at Ser 317. TopBP1 is essential for activation of

ATR-Chk1 pathway following MNU damage as TopBP1 depleted cells did not show phosphoryltaion of Chk1 following MNU damage.

Chapter 6: Discussion

DNA damage response (DDR) pathway is an intricate network of proteins that carry out DNA damage recognition, signal transduction, DNA repair and cell cycle regulation. Faithful transmission of genetic material requires accurate replication of genome and detection and repair of any errors in the genome by components of the DDR pathway. Mutation in any of the components of DDR pathway genes results in malfunctioning of the pathway leading to genomic instability. Specific kinds of DNA lesions are targeted by different checkpoint and repair pathways protecting the genetic material in the face of a variety of external and internal sources of DNA damage. An intricate and complex level of regulation has been observed between DNA repair and DNA damage response pathways. It is important for the cell to optimize functioning of all the cellular processes being carried out at any given time (Nyberg, Michelson et al. 2002).

DDR is a signal transduction pathway that is dependent on protein-protein interactions, which enable detection and repair of various DNA lesions. The biochemical pathways are organized well in space and time to accommodate various DNA damage signals to achieve correct and efficient response of the cell towards a danger signal. Protein–protein interaction forms the basis of the signaling cascade that is activated in response to any kind of DNA damage [reviewed in (Canman 2003)].

Interaction among components of the DDR pathway allows regulation of different signaling pathways to protect the cell from different kinds of DNA damage that may lead to genome instability.

DDR kinases like ATR and ATM regulate the checkpoint signaling and DNA repair mechanisms. They recruit and modify a number of proteins, while altering the chromatin structure to facilitate repair. Another level of regulation can occur at the level of mediator proteins, which are recruited by the sensors in order to amplify the damage signal. The mediators are known to bind DNA

lesion directly or through interactions with other proteins. TopBP1 is a mediator protein, its function in DNA replication initiation and checkpoint activation is well known. TopBP1 is at a crucial node in many of the cellular processes such as inhibition of apoptosis, checkpoint function which are essential for maintaining genomic stability in eukaryotic cells. A recent study done by Weei-Chin-Lin showed TopBP1 could be used as a therapeutic target for anti cancer drugs as it functions at a converging point of multiple oncogenic pathways (Chowdhury, Lin et al. 2014). Most of the solid tumors show a deregulation in receptor tyrosine kinases like Ras/RTK/PI-3K and retinoblastoma (Rb) protein. Inactivation of Rb leads to over expression of TopBP1 which leads to repression of the pro-apoptotic function of E2F1 transcription factor leading to uncontrolled division of cells. On the other hand deregulation of Akt protein in most cancer cells leads to phosphorylation of TopBP1 at Ser 1159, which leads to oligomerization of TopBP1. Oligomerization of TopBP1 perturbs its ability to bind ATR thereby inhibiting checkpoint function and induces its binding to E2F1 inhibiting apoptotic function of E2F1 (Chowdhury, Lin et al. 2014). TopBP1 employs its BRCA1 C-terminal region as a scaffold for its interaction with different proteins to modify various cellular processes. There are a number of proteins, which have been identified as interactors of TopBP1 and other components of the ATR-Chk1 pathway. TopBP1 and its homologues are comprised of BRCA1 C-terminal (BRCT) domains that can bind multiple proteins. They form temporal complexes which regulate various cellular processes like DNA replication and checkpoint activation [reviewed in (Wardlaw, Carr et al. 2014)]. Components of mismatch repair were identified as novel interactors of TopBP1 following methylation damage. Msh2 component of MutSα complex can directly bind ATR (Wang and Qin 2003); another study implicated the functional MutSα (Msh2-Msh6) to bind to different components of ATR-Chk1 pathway (Liu, Fang et al. 2010). MutSα was also shown to bind Chk1 and TopBP1 directly upon MNNG damage (Liu, Fang et al. 2010). The current study characterizes the biochemical interaction between TopBP1 and Msh2, to help us understand the functional relevance of their interaction in checkpoint activation. Far western analysis using recombinant TopBP1 and Msh2 proved that the two

proteins could interact physically *in vitro* [fig 3.3.1]. Further analysis with deletion mutants of both TopBP1 and Msh2 revealed the regions essential for their interaction. Far western analysis with BRCT deletion mutants of TopBP1 and full-length Msh2 showed that absence of BRCT VII and VIII domain abrogated the interaction between the two proteins. On the other hand, the ATR activating domain alone was not sufficient for interaction with Msh2 [fig 3.3.3 (A and B)]. The BRCT domains are 95 residues long with four stranded parallel β sheets surrounded by three α helices. BRCT domains can exist as single module like BRCT III and VI domain of TopBP1 or C-terminal region of XRCC1 and tandem pairs of BRCT domains as observed in mediators proteins like MDC1, TopBP1. The BRCT domains can form complexes with other BRCT domains within the protein, which provides the protein specificity and helps in regulating various processes. The crystal structure of tandem BRCT domains of BRCA1 revealed a canonical BRCT – BRCT domain packing which occurs through the hydrophobic interface α_2 helix of N-terminal BRCT domain and α'_1 and α'_3 helices of C-terminal BRCT domain. This canonical packing between BRCT domains is also observed in BRCT VII and VIII domain of TopBP1. The crystal structure of BRCT VII and VIII domain of TopBP1 bound to BACH1 revealed conserved binding pockets, one in the N-terminal region of BRCT domain and the other at the BRCT-BRCT interface. It was also observed that there is a conserved arginine residue at position 1280 in BRCT VII and VIII domain of TopBP1 which recognizes the phosphate group in the phosphoprotein, which is binding to TopBP1 (Sheng, Zhao et al. 2011). It is not clear as to which residues are required for facilitating binding between TopBP1 and Msh2. Though the *in vitro* interaction between TopBP1 and Msh2 negated the requirement of phosphorylated state of Msh2 but it still needs to be determined if any of the earlier known conserved residues of TopBP1 BRCT VII and VIII take part in interaction with Msh2. Far western analysis with deletion mutants of Msh2 and full-length TopBP1 helped in determining the region of Msh2 required for binding to TopBP1. Our studies revealed that the C-terminal region of Msh2 comprising of MutSd and MutSac domains was essential for interaction with TopBP1 [fig 3.3.2 (A and B)].

Mammalian Msh2 contains additional domains as compared to its homologue in bacteria. The MutS domains in eukaryotes have duplicated from its bacterial counterparts and acquired additional functions like cell cycle checkpoint control, DNA repair and apoptosis (Bellacosa 2001). MutSac domain is the ATPase domain which harbors the walker A type motif GNNNNGKS/T required for binding to phosphate group of ATP and is involved in ATP hydrolysis (Gorbalenya and Koonin 1990) (Walker, Saraste et al. 1982, Pai, Krengel et al. 1990). The ATPase domain is required for normal mismatch repair activity and is required for class switching during somatic hypermutation and also plays an important role in MMR dependent apoptosis in response to DNA damage (Martin, Li et al. 2003). A point mutation in mice Msh2 in ATPase domain at 674^{th} amino acid revealed that the mutant Msh2 was capable of inducing DNA damage response and apoptosis but was deficient in DNA repair activity (Lin, Wang et al. 2004). As both TopBP1 and Msh2 have multiple domains which provides them with versatility and capacity to bind different proteins and perform various functions, we hypothesized that TopBP1-Msh2 interaction may orchestrate various aspects of DNA damage response which includes detection of DNA damage, binding to DNA lesions, recruiting other proteins to form a signaling complex for induction of checkpoint activation and apoptosis. We observed that upon alkylation damage by MNU HeLa cells showed checkpoint activation within 30 minutes of exposure and the cells underwent G2/M arrest following the second round of replication [fig 4.3 and 4.4]. The early checkpoint activation as observed in our system could be a consequence of detection of methylated bases by MutSα complex of MMR or proteins involved in BER or NER. Earlier studies in HeLa S3 cells treated with Temozolamide and other alkylating agents have proven that early checkpoint activation is not dependent on MMR based processing of methylated bases but is rather activated due to processing of methylated bases by NER and BER. During NER or BER the resynthesis of new strand lags behind which results in exposed single stranded DNA, which could be a source of checkpoint signaling (Garvik, Carson et al. 1995, Dai and Grant 2010). The knockdown of Msh2 in HeLa cells showed that the early activation of checkpoint signaling upon MNU damage partially

depends on Msh2. Absence of Msh2 abrogated Chk1 phosphorylation at Ser 317 but not at Ser 345, which suggests that Msh2 along with TopBP1 is essential for phosphorylation of Chk1 at Ser 317. As TopBP1 is an essential activator of ATR kinase, upon depletion of TopBP1, phosphorylation of Chk1 at Ser 345 and Ser 317 was completely lost [fig 5.4]. Earlier studies have shown that upon DNA damage, Chk1 phosphorylation at Ser 345 is dependent on initial phosphorylation of Ser 317 (Wilsker, Petermann et al. 2008). Our studies indicate independent role for Ser 317 and 345 phosphoryltaion upon DNA damage and we cannot rule out the possibility of requiring different interacting partners for ATR mediated phosphorylation of Chk1 at different sites. TopBP1 is a nuclear protein, which remains bound to the chromatin during DNA replication and dissociates in times of DNA damage to bind to the damaged sites which can be detected as foci. Msh2 on the other hand is distributed in both cytoplasm as well as nucleus in absence of DNA damage and is translocated to nucleus upon induction of DNA damage. The knockdown studies suggest that both Msh2 and TopBP1 are required for early checkpoint activation in response to methylation damage. However it still needs to be ascertained if the interaction between TopBP1 and Msh2 is essential for phosphorylation of Chk1 at Ser 317 following MNU damage. The co-immunoprecipitation studies showed that the two proteins, Msh2 and TopBP1 are bound to each other. Interestingly the immuno pull downs showed that there was no enhanced interaction between TopBP1 and Msh2 in presence of DNA damage [fig 5.3]. As the TopBP1-MutSα complex was preformed it is possible that the proteins are bound to each other and their recruitment on the chromatin is dependent on DNA damage. The TopBP1-MutSα preformed complex could be a regulatory complex which when recruited to the DNA scans for DNA damage. Upon induction of DNA damage, the complex stops and binds to the damaged bases and then recruits more molecules of TopBP1 on the chromatin which are observed as foci and in case of Msh2 detected as high levels of Msh2 fluorescence in nucleus [fig 5.1 and 5.2]. The Msh2 accumulation following DNA damage may be required for DNA repair activity of O6 methylations caused by MNU as MutSα only recognizes them. It is not uncommon with DNA damage response proteins to remain as

preformed complex before DNA damage. It has been shown that TopBP1 and Rad9 interaction is independent of DNA damage but the interaction is required for TopBP1 accumulation at the damaged sites following UV irradiation (Ohashi, Takeishi et al. 2014). It has been shown previously that low doses of alkylation damage recruits MutSα and PCNA on the chromatin. The extent of recruitment of MMR proteins on the chromatin is dependent on the interacting partners, extent of alkylation and the phase of cell cycle (Schroering and Williams 2008). On the basis of our studies we suggest that Msh2-TopBP1 interaction is orchestrating a sequence of events, which include recognition of methylated bases or the breaks generated by MNU damage, recruitment of checkpoint signaling complex and mediating checkpoint activation. The two proteins form a regulatory complex that scans DNA for damage and is recruited to the damaged sites and mediates ATR dependent phosphorylation of Chk1, which in turn induces cell cycle arrest [fig 6].

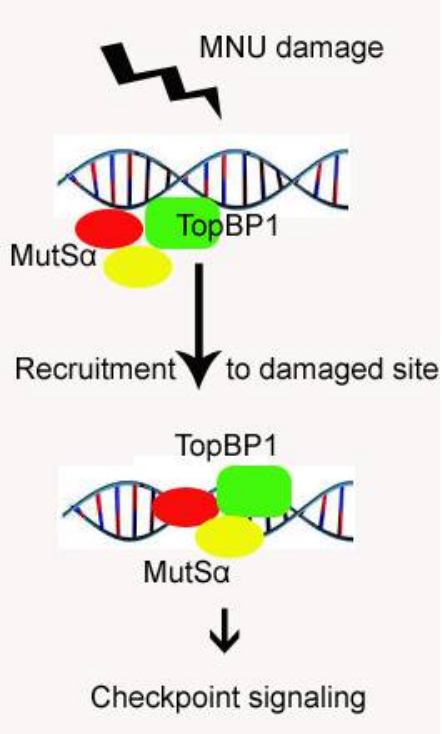

Fig 6: Schematic showing TopBP1-MutSα interaction model proposed for checkpoint activation following MNU damage. We propose that TopBP1-MutSα complex is preformed in the nucleus and is recruited to the chromatin upon methylation damage and may also recruit other checkpoint signaling machinery for checkpoint activation.

Future Perspectives

The current study using recombinant TopBP1 and Msh2 enabled us to identify regions in the proteins required for their interaction. In this study we also tried to understand the function of TopBP1 and Msh2 in checkpoint signaling following methylation damage. It is clear from the study that TopBP1 gets accumulated on the DNA following DNA damage in the form of foci but it is yet to be understood if Msh2 is required for TopBP1 accumulation on the chromatin. The co-immunoprecipitation studies done with whole cell extracts showed that TopBP1 and Msh2 are constitutively bound to each other. Further experiments using chromatin immunoprecipitation would help us to understand if the two proteins are enriched on the chromatin and whether their enrichment is DNA damage dependent. As the complex between TopBP1, Msh2-Msh6 seems to be pre-formed; it would be interesting to investigate if the same complex is recruited on the chromatin in response to other kinds of DNA damage like ultraviolet light, irradiation or other chemicals. The foci formed following DNA damage are sites of DNA repair where various proteins assemble and disassemble over time to carry out DNA repair. Based on our MSh2 knockdown results which suggest partial role of Msh2 in checkpoint activation, we suspect that the early checkpoint activation following MNU damage is dependent on NER or BER proteins along with MMR. Therefore it would be interesting to test whether absence of BER or NER proteins affects checkpoint activation and also if the proteins involved in NER or BER are recruited on the chromatin along with TopBP1 and Msh2. It is possible that TopBP1 being the scaffold protein could be regulating the assembly and disassembly of other proteins and mediating repair activities. It is still not clear if TopBP1 can directly detect the methylated bases and recruit Msh2 or other proteins onto the chromatin. Msh2 can detect O6meG:T or O6meG:C mispair and it is able to recruit ATR for checkpoint signaling (Yoshioka, Yoshioka et al. 2006). TopBP1 is known to bind single strand breaks, but it would be interesting to know if it can also detect methylated bases. Recombinant TopBP1 can be used to investigate the binding affinities

for TopBP1, Msh2 and TopBP1-Msh2 complex towards methylated bases using EMSA. We showed that knockdown of Msh2 causes abrogation of phosphorylation of Chk1 at Ser 317 suggesting a separate module being activated in response to methylation damage, which is dependent on Msh2. The investigation of downstream targets of Chk1 that are affected in Msh2 and TopBP1 depleted cells would help us narrow down on the exact function of dependency of Chk1 phosphorylation at Ser 317.

Complete Chk1 activation is dependent on phosphorylation of Ser 317 and 345 and in some studies it has been shown Ser 317 acts as a primer for phosphorylating Ser 345 (Wilsker, Petermann et al. 2008). It is not clear how cells are arrested in the G2 phase following 48 hours of MNU exposure even when checkpoints are activated within few minutes of treatment with MNU. We still do not know if Chk1 phosphorylation at Ser 317 acts as a signal for cell cycle arrest. Using Msh2 knockdown stable cell lines, we will be able to understand the complete mechanism of checkpoint activation as well as cell cycle arrest that occurs in G2 phase following MNU exposure.

Interaction studies with TopBP1 and Msh2 have identified the C-terminal region of both the proteins to be required for interaction. Protein modeling studies using known structures of BRCT VII and VIII of TopBP1 and Msh2-Msh3 bound to oligonucleotide could reveal certain residues, which could be important for their interaction.

The crystal structure of BRCT VII and VIII bound to BACH1 identified regions of charged amino acids that bind to the phosphorylated BACH1. The reduced packing between the BRCT domains provides the plasticity to this region for binding various proteins (Leung, Gong et al. 2011). Using the known structure of BRCT VII and VIII bound to BACH1 we could use *in silico* methods to superimpose the MutSα structure to find out the residues which may be mediating the interaction between two proteins. The function of each of the residue in the interaction between TopBP1 and Msh2 could be determined by mutating single as well as in combination and testing the interaction affinities for each mutant using far western analysis.

APPENDIX

Western Blot Buffers

10 X Transfer Buffer (1 l)

29 g Tris

196.5 g Glycine

10 X TBS (1 l)

30 g Tris base

80 g NaCl

2 g KCl

pH 7.6 with HCl

10 X SDS Running Buffer

250 mM Tris

1.92 M glycine

1 % (W/V) SDS

6 X Laemmli buffer (10 mL)

3.5 mL Tris pH6.8

3.6 mL glycerol

1.1 g SDS

0.93 g DTT

1.2 mg Bromophenol Blue

2.9 mL H_2O

Primer sequences for cloning Msh2 into pT7FLAG

Msh2 full-length forward

AACGAGGGCGGCCGCAATGGCGGTGCAGCCGAAGGAGA

Msh2 reverse

AAGGGTACCTCACGTAGTAACTTTTATTCGTGAA

Msh2 Δ1 forward

AACGAGGGCGGCCGCAAGCTTCCATTGGTGTTGTGGGT

Msh2 Δ1-2 forward

AACGAGGGCGGCCGCAACTGGCTCTCAGTCTCTGGCT

Msh2Δ1 -3 forward

AACGAGGGCGGCCGCACAGATGTTCCACATCATTACTGG

Msh2 Δ4 rev

AAGGGTACCTCATTTATCTTTTTCAAAGTATACGTCATT

PCR cycle for amplifying Msh2 full-length and its deletion mutants

95 ºC-2 minutes

95 ºC- 1 minutes
61 ºC- 1 minutes
72 ºC- 2 minutes
} 25 cycles

72 ºC- 5 minutes

4 ºC hold

Phosphate buffer Saline (PBS) (10 X, 1 l)

1.3 M NaCl

27 mM KCl

100 mM Na_2HPO_4

18 mM KH_2PO_4

siRNA sequences for TopBP1 and Msh2

Lac Z siRNA

Sense strand- CGUACGCGGAAUACUUCGA

Antisense strand- UCGAAGUAUUCCGCGUACG

TopBP1 siRNA

Sense strand- GUGGUUGUAACAGCGCAUCUU

Antisense strand - -GAUGCGCUGUUACAACCACUU

Msh2siRNA

Sense strand - ACAGAAUAGAGGAGAGAUUUU

Antisense strand-UUUGUCUUAUCUCCUCUCUAA

Antibodies	Dilution	Purpose
Phospho-Chk1 Ser 345	1:2500	Immunoblotting
Phospho-Chk2 Thr 68	1:1000	Immunoblotting
Chk1	1:1000	Immunoblotting
Chk2	1:1000	Immunoblotting
RPA 32	1:1000	Immunoblotting
Phospho-RPA 32 Thr 21	1:1000	Immunoblotting
GTBP	1:1000	Immunoblotting
Msh2	1:1000	Immunoblotting
TopBP1 (Bethyl)	1:1000	Immunoblotting
α-Tubulin	1:20,000	Immunoblotting
Phospho-Chk1 Ser 317	1:1000	Immunoblotting
TopBP1 (BD Biosciences)	1:100	Immunofluorescence
Msh2 N-20	1:100	Immunofluorescence
BRCA1	1:100	Immunofluorescence

53BP1	1:100	Immunofluorescence
Alexa Fluor 488/568	1:1000	Immunofluorescence
Anti rabbit / mouse HRP	1:10, 000	Immunoblotting

Far western Buffers

AC buffer containing varying concentrations of guanidine –HCl for denaturation and renaturation

Concentration of guanidine HCl (M)	6	3	1	0.1	0
Glycerol (mL)	2.5	2.5	2.5	2.5	2.5
5M NaCl (mL)	0.5	0.5	0.5	0.5	0.5
1M Tris-Cl pH-7.5 (mL)	0.5	0.5	0.5	0.5	0.5
0.5 M EDTA (mL)	0.05	0.05	0.05	0.05	0.05
10 % Tween 20 (mL)	0.25	0.25	0.25	0.25	0.25
Guanidine – HCl (8 M) (mL)	18.75	9.3	3.13	0.31	0
Milk powder (g)	0.5	0.5	0.5	0.5	0.5
1M DTT (µl)	25	25	25	25	25
ddH_2O (mL)	2.45	12.82	18.07	20.89	21.20
Total volume (mL)	25	25	25	25	25
Time/ temperature	60 minutes/ room temperature	60 minutes/ room temperature	60 minutes/ room temperature	60 minutes / 4 ºC	60 minutes/ 4 ºC

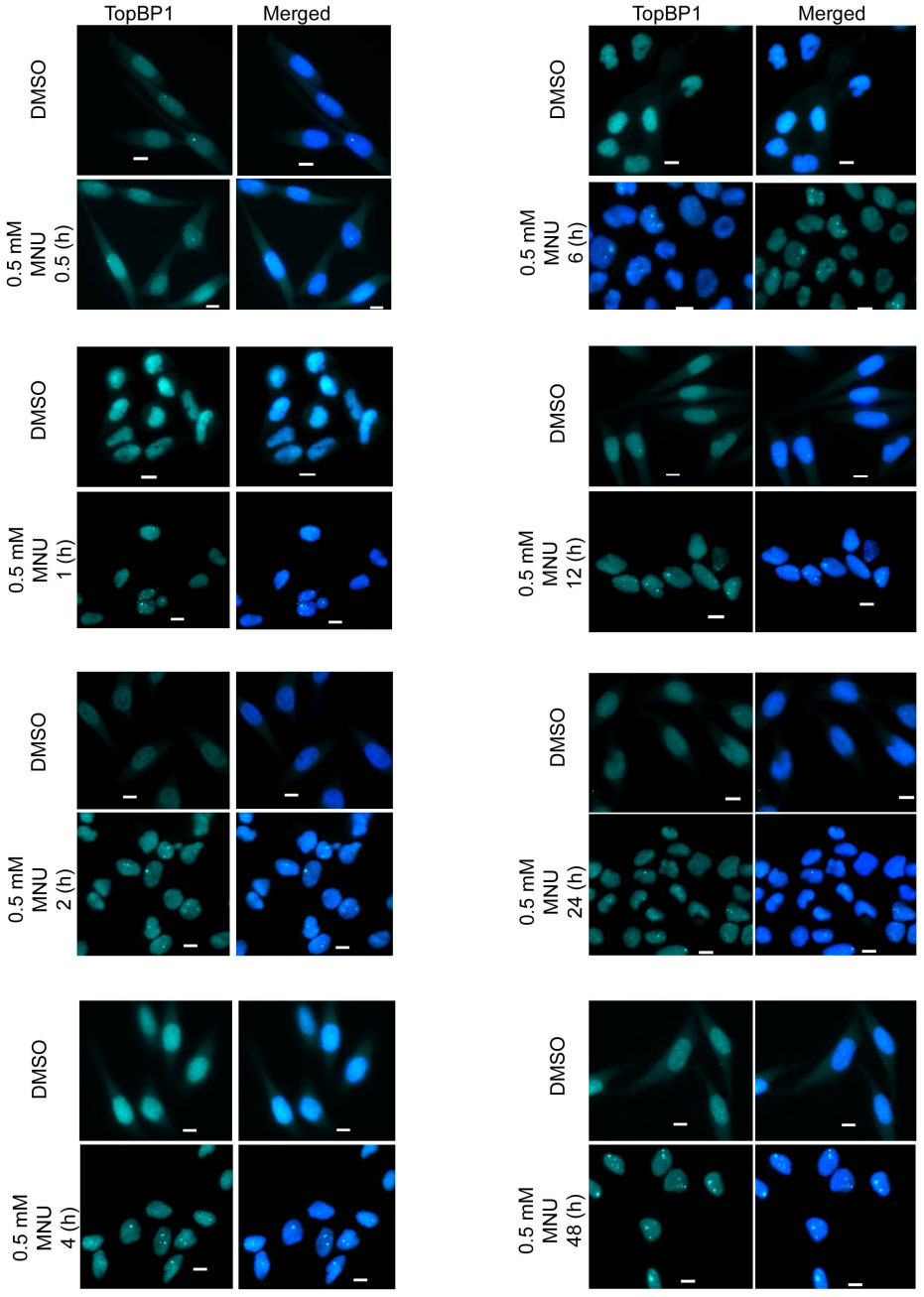

Fig 5.5: TopBP1 foci increase over time upon MNU damage. Number of cells showing TopBP1 foci increase dramatically at 48 hours post MNU exposure. HeLa cells treated with 0.5 mM MNU for different time (0.5, 1,2,4,6,12,24,48 hours) was fixed and immunostained with anti TopBP1 antibody. The cells were scored for presence or absence of TopBP1 foci. The images were taken at a magnification of 63X under fluorescent microscope (Axio Imager Z.1 ApoTome microscope). The bar represents 10 μm.

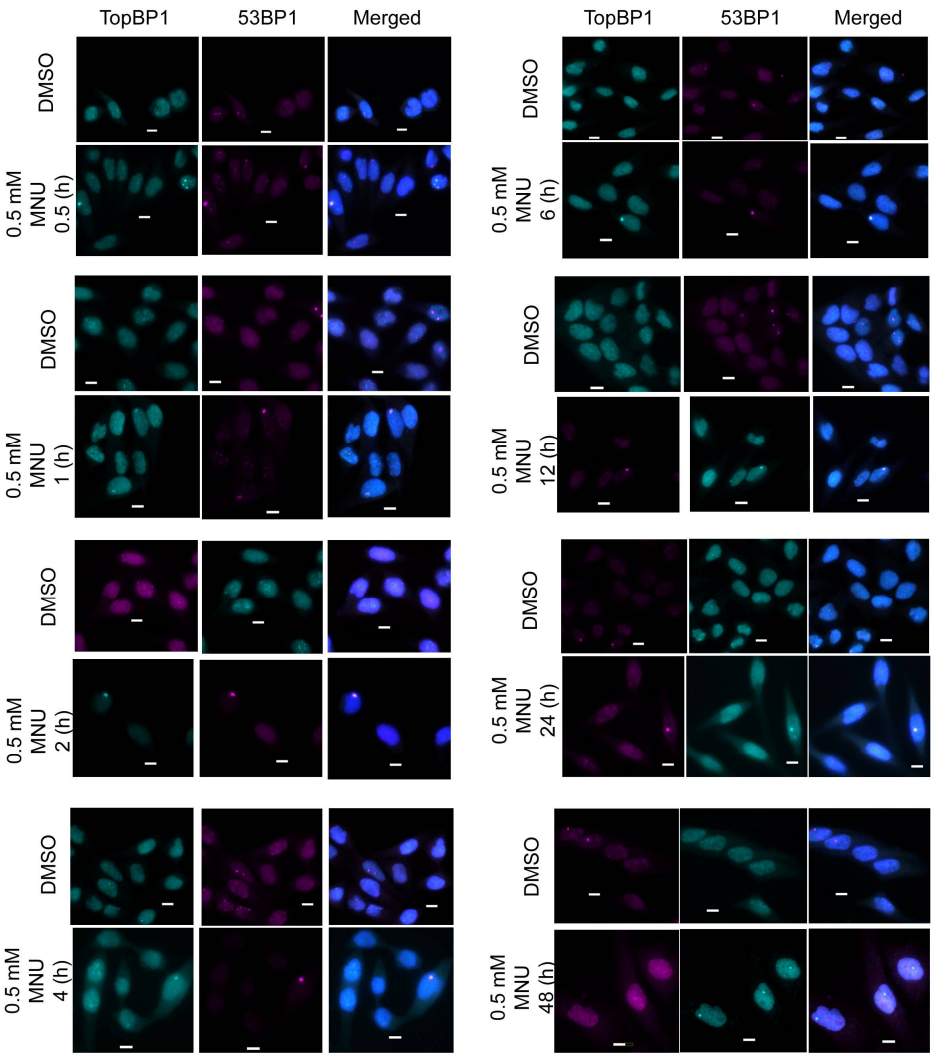

Fig 5.6: TopBP1 and 53BP1 foci co-localized following MNU damage. Around 30 % of the cells show co-localization between TopBP1 and 53BP1 foci following MNU damage. The images were taken under Axio Imager Z.1 ApoTome microscope at 63 X magnification. The bar represents 10 µm.

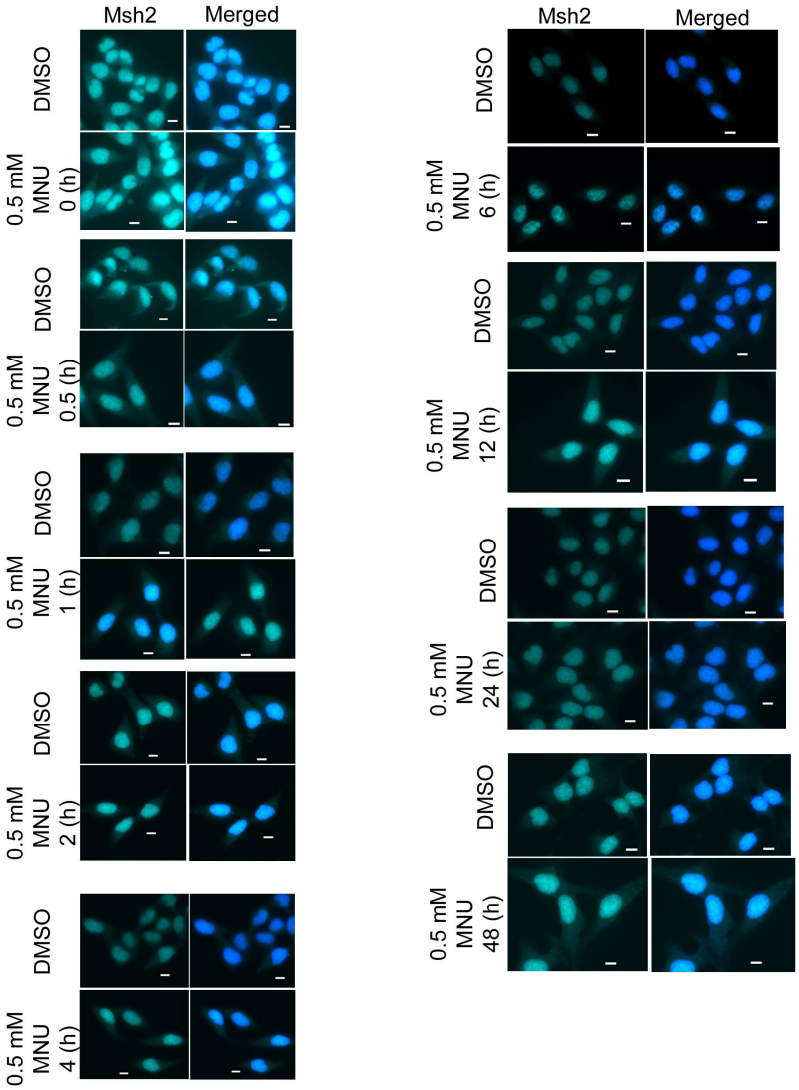

Fig 5.7: Msh2 translocates into nucleus upon MNU damage. HeLa cells treated with 0.5 mM MNU for different time (0.5, 1, 2, 4, 6, 12, 24 and 48 hour) were fixed and immunostained with anti Msh2 antibody. The cells were imaged at 63 X under a fluorescent microscope (Axio Imager Z.1 ApoTome microscope).The bar represents 10 μm.

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