GENOME MAINTENANCE THROUGH TOPBP1

IN YEAST AND MAMMALS

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GENOME MAINTENANCE THROUGH TOPBP1 IN YEAST AND MAMMALS

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Genome integrity is crucial for cell proliferation and organismal survival. In eukaryotes, proper genome maintenance relies on a multifaceted cellular response, often referred as the DNA damage response (DDR), which requires the coordination of DNA replication and cell cycle regulation with DNA repair. Over the last 20 years, studies in yeast, frog, and mammals have revealed conserved roles for the protein scaffold TOPBP1 (Dpb11 in yeast) in initiation of DNA replication and activation of cell cycle checkpoint signaling. In this dissertation, I have investigated the roles of TOPBP1^{Dpb11} in DNA repair, which uncovered an evolutionarily conserved mechanism for the control of recombination-mediated repair and DNA repair pathway choice. Overall, my work reveals how the coordination of cell cycle regulation, DNA replication and DNA repair can be achieved through the action of TOPBP1^{Dpb11}, and provides new insights into the molecular basis of cancer development in patients carrying mutations that impair homologous recombination (HR)-mediated DNA repair.

First, in budding yeast, I showed that Dpb11 plays antagonistic roles in the control of DNA damage checkpoint signaling and HR-mediated repair. Mechanistically, Dpb11 mediates two mutually exclusive interactions with the checkpoint adaptor Rad9 and the repair scaffolds Slx4-Rtt107. Together with graduate student Patrice Ohouo, I found that the binding of Dpb11 to Slx4-Rtt107 prevents aberrant checkpoint hyper-

activation by counteracting Dpb11-mediated stabilization of Rad9. Interestingly, I found that this Dpb11-mediated competition mechanism also controls the role of Rad9 as an anti-resection factor at DNA lesions and thereby regulates HR-mediated repair. Overall these results point to a key role for Dpb11 in the coordination of DNA damage signaling and repair, and establish Dpb11 as a key regulator of DNA end resection.

In humans, I found that TOPBP1 engages in interactions with both the anti-HR factor 53BP1 and the pro-HR factor BRCA1, suggesting that TOPBP1 also mediates opposing functions in HR control. I showed that the hyper-stabilization of 53BP1-TOPBP1 interaction enhances the recruitment of 53BP1 and other anti-HR factors to nuclear foci in S-phase, and induces chromosomal aberrations. These results suggest that TOPBP1 is a key regulator of the repair pathway choice. Collectively, the work in this dissertation supports a model whereby TOPBP1^{Dpb11} functions as a master coordinator of genome replication and maintenance and plays a crucial role in the control of DNA repair.

BIOGRAPHICAL SKETCH

Yi Liu was born in Wuhan, China on September 30, 1986 to Xiaoping Liu and Baoqin Wang. She attended Wuhan Foreign Languages School between 1999-2005 where she gained a solid background in math and science. After high school, Yi enrolled at Wuhan University in 2005 to pursue her undergraduate degree in biological sciences. In the course of her undergraduate studies, she embraced the opportunities to absorb and retain knowledge in a broad range of subjects, such as zoology, botany, biochemistry, cell biology, molecular biology, developmental biology, genetics and virology. In her junior year of college, Yi joined the lab of Dr. Yan Wu and studied abscisic acid signal transduction mechanisms in plant stress responses. This initial exposure to the science research environment enabled her to obtain some basic hands-on experience in molecular and cell biology. Yi graduated from Wuhan University in 2009 with a Bachelor of Science degree and decided to further her graduate studies in the United States. She was fortunate to be admitted to the graduate program of Biochemistry, Molecular and Cell Biology (BMCB) in Cornell University. At Cornell, Yi discovered her interest in understanding the basic molecular mechanisms of human diseases. Hence she joined Smolka Lab in the summer of 2010, where she interrogated the role of a scaffold protein TOPBP1 as a master coordinator of DNA damage responses using two different model systems including budding yeast and cultured mammalian cells. By combining quantitative proteomics approach with classic tools in biochemistry, cell biology and genetics, her PhD work revealed a novel role of TOPBP1 in the control of DNA repair besides its well-established function in DNA replication and damage checkpoint signaling.

This thesis is dedicated to my dear mum,

for her unconditional love, support and inspiration

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LIST OF ABBREVIATIONS

CTR: Constitutive TOPBP1 recognition

DAMP: Dampen adaptor-mediated phospho-signaling

DDC: DNA damage checkpoint signaling

DDR: DNA damage response

DSB: Double-strand break

HR: Homologous recombination

NHEJ: Non-homologous end joining

ssDNA: single-strand DNA

CHAPTER 1

INTRODUCTION

The health and survival of living organisms are highly dependent on the accurate maintenance and transmission of their genetic material. The maintenance of genomic integrity is an essential, yet challenging task, considering each of our cells contains more than 3 billion base pairs of DNA, which needs to be unwound and precisely replicated during every cell division. To achieve this, a repertoire of sophisticated mechanisms that sense DNA lesions and coordinate an elaborate network of cellular responses that safeguard genomic integrity has evolved (Ciccia and Elledge, 2010; Huen and Chen, 2008; Jackson and Bartek, 2009). In eukaryotes, the DNA damage checkpoint (DDC) signaling network plays a key role in the detection of aberrant DNA structures and regulation of numerous cellular processes, including cell cycle arrest and DNA repair (Marechal and Zou, 2013; Zhou and Elledge, 2000). Mammalian TOPBP1 is an evolutionarily conserved scaffold protein that plays key roles in DNA replication and DDC signaling, but whose roles in DNA repair remain poorly understood (Wardlaw et al., 2014). In my research project, I investigated the roles of human TOPBP1, and of its yeast orthologue Dpb11, in DNA repair and explored how $TOPBP1^{Dpb11}$ coordinates DDC signaling and DNA repair for the proper control of genome maintenance. By combining quantitative proteomic approach with classical tools in biochemistry, cell biology and genetics, I found that $TOPBP1^{Dpb11}$ plays a key role in the modulation of DDC signaling and the control of DNA repair by balancing recruitment of both pro- and anti-homologous recombination factors at double-strand breaks and replication-

associated lesions. Overall, the work in this dissertation supports a model whereby TOPBP1^{Dpb11} functions as a master coordinator of genome replication and maintenance and plays crucial roles in DNA replication, DNA damage signaling as well as DNA repair.

1.1 Cellular responses to DNA damage

Faithful duplication and passage of the genetic material from one generation to the next relies heavily on high-fidelity DNA replication and on genome maintenance mechanisms that suppress genomic instabilities such as point mutations, small-scale deletions/insertions, or gross genomic rearrangements including translocations, deletions, duplications and inversions. In order to sustain genomic integrity in the face of both endogenous and exogenous DNA insults, eukaryotic cells have developed evolutionarily conserved surveillance mechanisms collectively known as the DNA Damage Response (DDR) (Fig1.1) (Ciccia and Elledge, 2010; Huen and Chen, 2008; Jackson and Bartek, 2009). DDR consists of multiple highly coordinated signaling pathways that enable the cells to detect damaged DNA structures or nucleotide alterations, to halt cell cycle progression and to assemble DNA repair machineries to restore the original genetic information before the resumption of DNA replication and cell cycle.

1.1.1 Sources of DNA damage

Cellular DNA is constantly attacked by endogenous and exogenous sources of DNA damage such as ionizing radiation, reactive oxygen species, replication stress, etc.

Figure 1.1

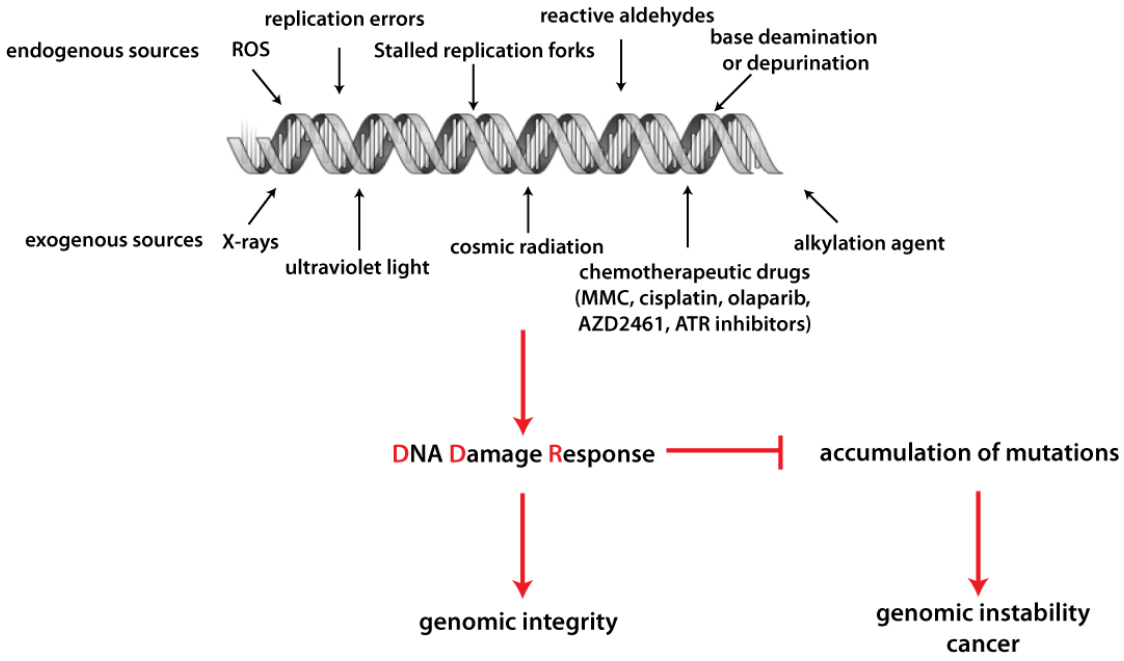


Figure 1.1 DNA damage response and cancer. Genomic integrity is constantly challenged by both endogenous and exogenous sources of DNA damaging factors. To cope with DNA damage, an elaborate set of surveillance mechanisms, collectively termed DNA damage response (DDR), has evolved to prevent the accumulation of genomic instability, which is a major driving force for cancer.

DNA is chemically reactive and is subject to numerous kinds of structural modification and alterations. Upon DNA modification, the resulting aberrant DNA structures, modified nucleotides, damaged bases and mis-incorporated nucleotides need to be repaired prior to DNA replication to prevent erroneous transmission to the daughter cells.

Endogenous sources of DNA damage

A frequent endogenous source of genomic instability arises from ribonucleotide mis-incorporation during DNA replication. While high-fidelity DNA replicative polymerases harbor intrinsic proofreading exonuclease activities and can fulfill their task at extremely low error rate in the range of 10^{-6} to 10^{-8}, a significant number of errors can still accumulate, particularly for large genomes (i.e. human genome contains approximately 3 billion base pairs). In most of the cases, DNA repair mechanisms are capable of successfully restoring the correct genomic DNA information.

Reactive oxygen species (ROS) represent another major endogenous source of DNA damage. Normal cellular metabolism in mitochondria and peroxisomes results in the production of ROS, such as the highly reactive hydroxyl radical (•OH), oxygen (O_2), superoxide($\bullet O_2$) and hydrogen peroxide (H_2O_2). DNA is susceptible to chemical modifications by these compounds. It has been shown that ROS accounts for over one hundred different forms of base lesions (Dexheimer, 2013), of which unsuccessful repair could result in increased genomic instability and cell death. Besides modifications on the nucleotide bases, other examples of oxidatively generated DNA lesions include intra/inter-strand crosslinks and DNA-protein crosslinks (Berquist and Wilson, 2012).

Pathological consequences associated with ROS-related DNA damage have been widely studied in the context of diabetes, cardiovascular diseases, aging and cancer (Davalli et al., 2016; Waris and Ahsan, 2006).

Finally, replication stress is another important source of endogenous DNA damage. Replication stress arises when replication forks stall or collapse by encountering physical barriers such as DNA lesions or stable DNA-protein complexes (Mazouzi et al., 2014; Zeman and Cimprich, 2014). Stalled or collapsed fork structures, if not properly protected or repaired before replication barriers are removed, can lead to intensified DNA damage and genomic instability, which is a major driving force of tumorigenesis (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Consistent with this reasoning, budding yeast mutants with a defective DNA replication machinery display significantly increased rates of gross chromosomal rearrangements (GCRs) (Chen and Kolodner, 1999). In humans, mutations in genes encoding for DNA replication proteins have also been correlated with general growth retardation, developmental defects and susceptibility to cancer (Jackson and Bartek, 2009; O'Driscoll, 2012).

Exogenous sources of DNA damage

In addition to endogenous sources of DNA damage, cells are often also subjected to various kinds of exogenous genotoxic agents. One common example is ultraviolet (UV) radiation from the sun, which can be divided into three groups based on their wavelength and energies (UV-A: 315–400 nm; UV-B: 280–315 nm; UV-C: <280 nm). Fortunately, the most energetic and deleterious UV-C rays are mostly absorbed by the

ozone layer. However, UV-B and UV-A light still penetrate through the atmosphere and are sufficient to harm our DNA. The action of UV light on DNA predominantly results in two types of DNA lesions, cyclobutane pyrimidine dimers, and 6–4 pyrimidine-pyrimidone photoproduct, which both arise from the formation of covalent bonds between two adjacent pyrimidine bases (cytosine-C or thymine-T). These structures can consequently impede replication fork progression and RNA transcriptional machinery, resulting in fork stalling, collapse, or, if not promptly repaired, permanent mutations in the genome.

Another example of an environmental source of DNA damage is ionizing radiation (IR), which is commonly found in cosmic radiation, x-rays and radioactive material. IR generates a wide variety of DNA lesions, among which double-strand break (DSB) is one of the most harmful forms of DNA damage. The inability to accurately and timely repair DSBs results in chromosomal aberrations and eventually cell death.

1.1.2 DNA damage signaling and checkpoint

DNA damage response (DDR) has evolved in order to preserve genomic integrity and maintain cellular homeostasis in the face of DNA damage (Ciccia and Elledge, 2010; Huen and Chen, 2008; Jackson and Bartek, 2009). DDR is composed of an intricate network of signaling pathways, which sense the abnormal DNA structures, promptly signal its presence to other cellular compartments, and assemble DNA repair machineries at the site of lesions to repair the damage (Dexheimer, 2013). Overall, DDR coordinates a set of tightly regulated cellular responses that allows proper repair of the existent damage before resuming the cell cycle.

Central to the DDR is the DNA damage checkpoint (DDC). DDC is a kinase-mediated signaling cascade, highly conserved from unicellular eukaryotic organisms to multicellular vertebrate animals (Marechal and Zou, 2013; Zhou and Elledge, 2000). The term 'checkpoint' was originally coined by Hartwell early in 1989 to describe molecular mechanisms that monitor the completion of earlier cellular events before the initiation of later events (Hartwell and Weinert, 1989). This concept is manifested by the transient cell cycle arrest induced by DDC signaling. However, in the last two decades, our understanding of DDC has expanded significantly. Besides cell cycle regulation, DDC signaling mediates other cellular responses including replication fork stabilization, transcription reprogramming, DNA end resection control, DNA repair and in the case of irreversible damages, triggering senescence or apoptosis (Fig1.2) (d'Adda di Fagagna, 2008; Marechal and Zou, 2013).

In *Saccharomyces cerevisiae*, DDC is mainly orchestrated by the action of the apical checkpoint kinase Mec1, whose functional orthologue is ATR in mammals

Figure 1.2

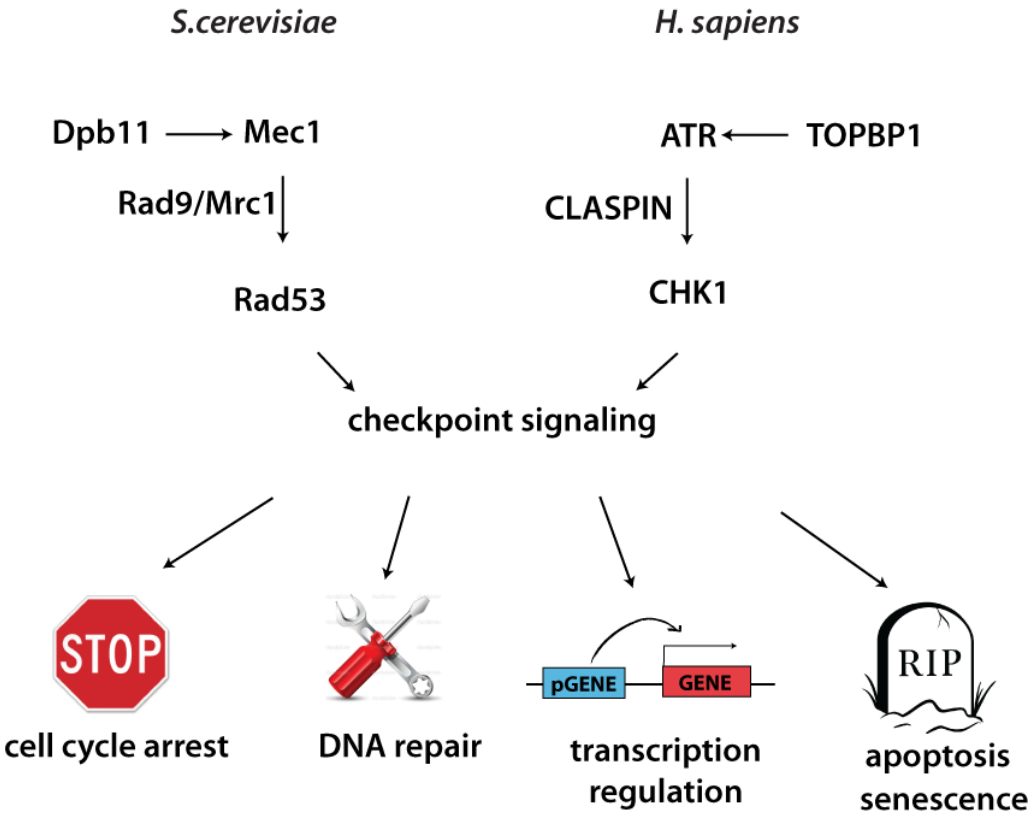


Figure 1.2 Checkpoint kinase ATR^{Mec1}-mediated DNA damage checkpoint signaling. Upon accumulation of ssDNA, ATR^{Mec1} is activated by TOPBP1^{Dpb11} and transduces the signal to the effector kinase CHK1^{Rad53} through the checkpoint adaptors, Rad9/Mrc1 in budding yeast and CLASPIN in humans. The activation of CHK1^{Rad53} eventually elicits a set of highly coordinated cellular events with outcomes including cell cycle arrest, assembly of DNA repair machinery, reprogramming of transcriptional regulation or, in the case of irreparable damages, triggering senescence or apoptosis.

(Fig1.2). Once Mec1 is recruited to the site of lesions, its kinase activity is stimulated by several partially redundant mechanisms involving the Dpb11 scaffold and DNA damage sensor 9-1-1 complex (Bonilla et al., 2008; Majka et al., 2006b; Masumoto et al., 2000; Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008; Navadgi-Patil and Burgers, 2009b). Activated Mec1 can then phosphorylate checkpoint adaptors Rad9 or Mrc1 so as to allow subsequent recruitment and activation of the key effector kinase Rad53 (Alcasabas et al., 2001; Emili, 1998; Osborn and Elledge, 2003; Schwartz et al., 2002; Schwartz et al., 2003; Sun et al., 1998; Sweeney et al., 2005; Tanaka and Russell, 2001; Usui et al., 2009). Activated Rad53, as a mobile kinase, can diffuse away from the site of damage and trigger a nucleus-wide checkpoint response by further phosphorylating other downstream effectors and therefore mediating their functions in cell cycle arrest, DNA damage repair, replication fork stabilization, inhibition of late origin firing, and nuclease regulation to control resection (Branzei and Foiani, 2006; Davidson et al., 2012; Morin et al., 2008; Santocanale and Diffley, 1998; Weinert and Hartwell, 1988; Zegerman and Diffley, 2010; Zhao et al., 2000).

In mammals, DDC is mediated by two major signaling pathways initiated with two apical kinases, Ataxia telangiectasia mutated (ATM) and Ataxia Telangiectasia and Rad3-related (ATR) (Cimprich and Cortez, 2008; Marechal and Zou, 2013; Shiloh and Ziv, 2013). ATR and ATM are master kinases that phosphorylate and thereby regulate the activities of many downstream effectors. Two of the most extensively studied targets of ATR and ATM are the protein kinases CHK1 and CHK2, respectively (Liu et al., 2000; Marechal and Zou, 2013; Matsuoka et al., 1998). The ATR-CHK1 and ATM-CHK2 pathways respond to different types of DNA lesions. While the ATM-CHK2

branch is mainly activated upon the detection of double-strand breaks (DSB), the ATR-CHK1 branch responds to a relatively diverse range of DNA lesions that lead to exposure of single-strand DNA (ssDNA) (Cimprich and Cortez, 2008; Flynn and Zou, 2011). Though it was initially believed that the ATM-CHK2 and ATR-CHK1 pathways act in parallel with some redundant functions, it is now well established that crosstalk between the two signaling pathways is common and both pathways work closely together to coordinate DNA damage responses and ensure genomic integrity. ATR-CHK1 signaling is launched upon the detection of ssDNA. The ssDNA structure can arise from many different types of DNA damage, such as DSB, replication stress, DNA crosslinks or base adducts. Exposed ssDNA is rapidly recognized and coated by a protein named replication protein A (RPA) (Majka et al., 2006a; Wold, 1997; Zou and Elledge, 2003), which further recruits ATR and its cofactor ATRIP (Cortez et al., 2001; Zou and Elledge, 2003). While RPA-ssDNA allows localization of ATR at the site of lesions, full activation of ATR kinase requires the engagement of TOPBP1 (Delacroix et al., 2007; Kumagai et al., 2006), which can be recruited through multiple mechanisms. One way to recruit TOPBP1 is via its physical interaction with the PCNA-like checkpoint clamp 9-1-1 complex (RAD9-HUS1-RAD1) and this interaction is regulated in a phosphorylation-dependent manner (Delacroix et al., 2007; Greer et al., 2003; Rappas et al., 2011; Ueda et al., 2012). In the case of DSBs, ATM is recruited to and activated at the site of lesions through its physical interaction with the DSB sensor MRN complex (MRE11-RAD51-NBS1) (Lee and Paull, 2004; Lee and Paull, 2005; Lee and Paull, 2007; Paull, 2015; Uziel et al., 2003). Once activated, ATM can then phosphorylate its substrate proteins including CHK2 to coordinate DNA damage

responses (Shiloh and Ziv, 2013).

1.1.3 DNA repair mechanisms

Despite the capacity to halt cell cycle progression and DNA replication shortly after the detection of DNA damage, proper lesion repair needs to take place within a relatively limited period of time since prolonged cell cycle arrest is harmful and can result in proliferation suppression or apoptosis (Chen et al., 1998; Ciccia and Elledge, 2010; Clerici et al., 2001; d'Adda di Fagagna, 2008; Orth et al., 2012). Alternatively, if the cell resumes the cell cycle before the lesions are accurately and timely removed, the damage will be transmitted to and amplified in the progeny cells through subsequent cell divisions. Specialized DNA repair mechanisms have evolved to cope with different types of lesions (Friedberg, 2008; Hoeijmakers, 2001). Briefly, base excision repair (BER) is mainly used for the repair of damaged bases, mis-incorporated bases or single-strand breaks (Krokan and Bjoras, 2013). Nucleotide excision repair (NER) is utilized to repair bulky and helix-distorting DNA lesions (Scharer, 2013). Mismatch repair (MMR) is devoted to the repair of non-Watson-Crick mismatches due to nucleotide mis-incorporation or short insertion/deletion (indel) (Li, 2008). Translesion synthesis (TLS) is utilized to bypass replication fork blocking lesions that cannot be timely repaired (Prakash et al., 2005). Lastly, DSB, which is one of the most deleterious forms of DNA lesions, can be repaired by multiple repair mechanisms, among which homologous recombination (HR) and non-homologous end joining (NHEJ) are the two predominant pathways.

Homologous recombination

HR repair is frequently referred to as a relatively error-free mechanism as it utilizes a homologous sequence in the genome (sister chromatids, homologous chromosomes, or repetitive sequences) as template to direct repair synthesis and restore the original DNA sequence (Heyer, 2015; San Filippo et al., 2008). Consequently, HR is mostly restricted to late S and G2 phase of the cell cycle, when the identical sister chromatids are available at close proximity as repair templates. HR-mediated repair proceeds through a highly ordered set of events (Fig1.3b):

(a) Recognition

MRN complex has been shown to play a crucial role in the early stage of HR. DSB ends are first recognized by the MRN complex. The two MRN complexes on the neighboring DNA ends associate with each other through Rad50 dimerization and this keeps two broken ends in close proximity (Hopfner et al., 2002; Moreno-Herrero et al., 2005; Williams et al., 2008). Yeast genetic studies have also implicated cohesin complex in HR repair, presumably due to its function in maintaining close proximity of the two sister chromatids (Losada and Hirano, 2005) (Nasmyth and Haering, 2005). Besides its function in tethering two broken DNA ends, MRN also plays a role in the recruitment and activation of the checkpoint kinase ATM via a physical interaction between NBS1 and ATM (Lee and Paull, 2004; Lee and Paull, 2005).

(b) DNA end resection

Once MRN complex is loaded, DSB ends undergo 5’ to 3’ nucleolytic degradation, which is defined as “resection”, resulting in the generation of 5’ recessed ends and long stretch of 3’ single-strand DNA overhangs (Cejka, 2015). This DNA end

Figure 1.3

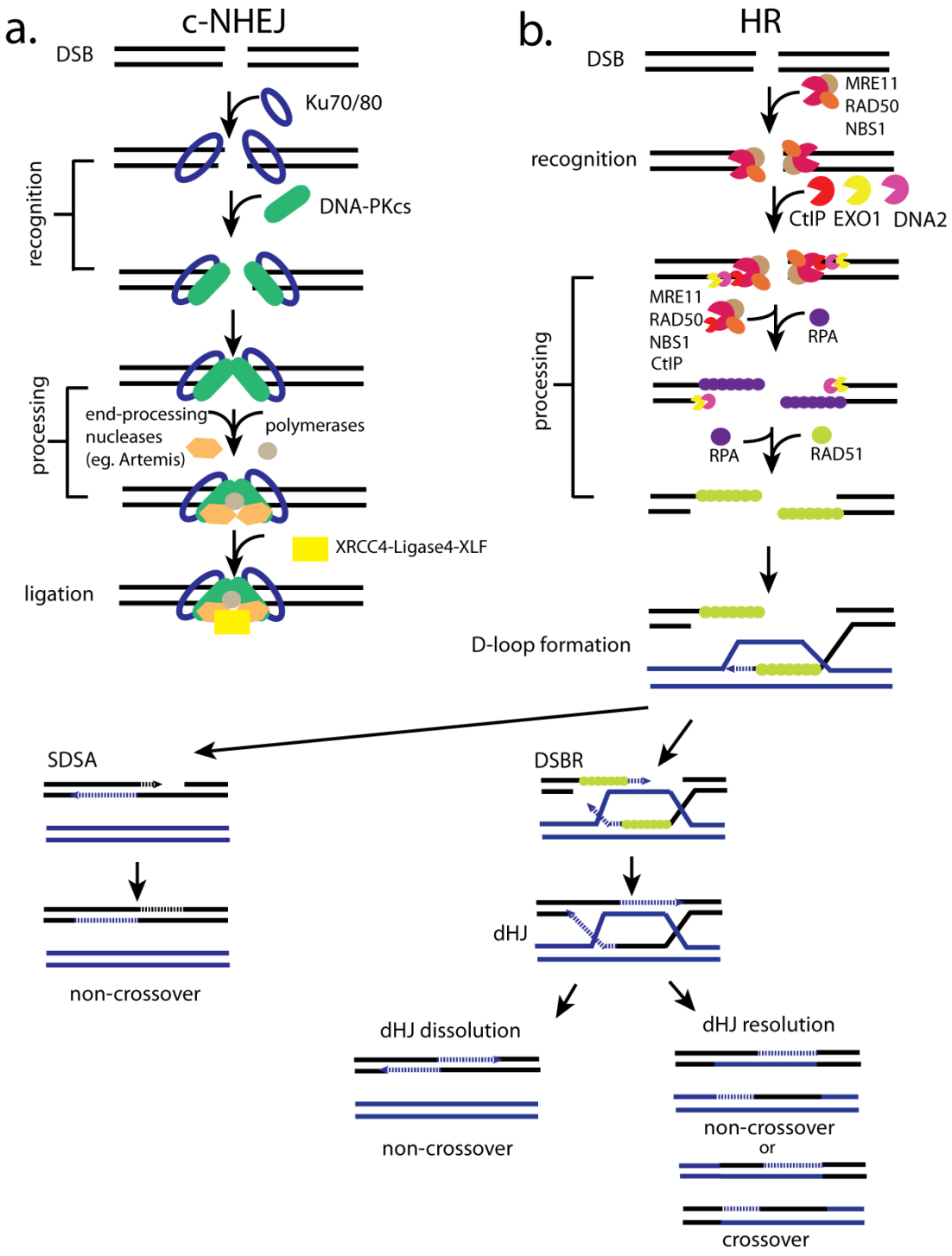

a.
c-NHEJ
DSB
Ku70/80
recognition
DNA-PKcs
processing
end-processing
nucleases
(eg. Artemis)
polymerases
XRCC4-Ligase4-XLF
ligation
b.
HR
DSB
MRE11
RAD50
NBS1
recognition
CtIP EXO1 DNA2
MRE11
RAD50
NBS1
CtIP
RPA
processing
RPA
RAD51
D-loop formation
SDSA
non-crossover
DSBR
dHJ
dHJ dissolution
non-crossover
dHJ resolution
non-crossover
or
crossover

Figure 1.3 Schematic illustration of the major steps in classical non-homologous end joining and homologous recombination DNA repair. (a) Non-homologous end joining is initiated with the recognition of DSB by Ku complex, which subsequently recruits and activates DNA-PKcs kinase. Then DSB ends are processed and ligated by XRCC4-DNA ligase 4-XLF complex to complete the repair. (b) Homologous recombination is initiated by DNA end resection, which is accomplished by joint efforts of several nucleases including MRN-CtIP, EXO1 and DNA2. The exposed ssDNA is then rapidly coated by RPA, which is later displaced by Rad51. The formation of Rad51-ssDNA nucleofilament is required for the subsequent homology search and strand invasion, resulting in the formation of D-loop structure. From this point on, HR repair can be completed by two pathways SDSA or DSBR as depicted in the figure.

resection process is a crucial step that commits the break repair to HR rather than NHEJ. Therefore, resection is carried out under strict regulation. Currently it is widely accepted that DNA end resection is first initiated with an endonucleolytic cleavage by MRN (MRX in yeast) complex and CtIP (Sae2 in yeast), which removes about 30 nucleotides from the 5' break end and results in limited end processing (Cejka, 2015; Cejka et al., 2010; Huertas, 2010; Mimitou and Symington, 2008; Nicolette et al., 2010; Sartori et al., 2007). This short 3' ssDNA tail is then subject to extensive resection by either 5'-3' exonuclease EXO1 or by combined efforts of the Sgs1/Blm-Top3-Rmi1 complex and DNA2 (Limbo et al., 2007; Longhese et al., 2010; Zhu et al., 2008). The range of resection can vary between a few hundred base pairs to tens of kilobases depending on the availability of the homologous template. The stretch of ssDNA resulting from resection is rapidly bound by RPA to stabilize the ssDNA structure. The RAD51 recombinase, which is the key factor required for the later homology search and strand invasion steps, then displaces RPA to assemble a RAD51-ssDNA nucleofilament structure that can engage in strand invasion and homology search (Jasin and Rothstein, 2013; Krejci et al., 2012; San Filippo et al., 2008).

(c) Homology search and resolution

The Rad51-ssDNA nucleofilament is then used for homology search and strand invasion. During strand invasion, a displacement loop (D-loop) structure is formed between 3' ssDNA tail and the homologous duplex DNA (San Filippo et al., 2008). Following D-loop formation, DNA synthesis begins on the 3' end of the invading strand to restore the lost genetic information using the homologous chromosome as template. After end resection, strand invasion and DNA synthesis, HR repair branches out into

two directions, namely synthesis-dependent strand annealing (SDSA) and double-strand break repair (DSBR) (San Filippo et al., 2008). In the case of SDSA, the invading strand is released from the homologous DNA template. The newly synthesized DNA on the 3' of the invading strand now anneals back to the other resected 3' ssDNA tail. Eventually, the SDSA pathway is completed by a second round of DNA synthesis and re-ligation. In the alternative scenario, DSBR is unique in that the other 3' overhang that is not involved in the first around of strand invasion is also captured by the D-loop, resulting in the formation of double Holliday Junctions (Jasin and Rothstein, 2013; Szostak et al., 1983). This joint molecule intermediate structure can be either dissolved by combined actions of Sgs1/BLM-RecQ helicase and a type IA topoisomerase (Top3/TopoIIIα) via branch migration (Wu and Hickson, 2003), or resolved by structure-specific endonucleases such as MUS81/EME1, GEN1 and SLX1/SLX4 leading to crossover or non-crossover products depending on the types of cleavage that occurred (De Muyt et al., 2012; Ho et al., 2010; Wechsler et al., 2011; Zakharyevich et al., 2012).

Classical non-homologous end joining (c-NHEJ)

Although HR is the predominant DSB repair pathway in S/G2, NHEJ is the preferred DSB repair pathway in G1, when the sister chromatids are not available as repair templates. However, NHEJ is constitutively active throughout the cell cycle and can be engaged in S-phase, especially under pathological situations. Compared with HR, NHEJ is a relatively error-prone DSB repair mechanism given that the NHEJ repair machinery directly ligates the two blunt DNA break ends together regardless of the original template sequences (Kass and Jasin, 2010; Lieber, 2010). Of note, the

occurrence of NHEJ may also involve limited DNA end processing, which may give rise to small insertions, deletions, or even translocations when the wrong pieces of DNA are ligated (Deriano and Roth, 2013; McVey and Lee, 2008; Weterings and Chen, 2008). Major steps in NHEJ are shown in Fig1.3a and described as below:

(a) Recognition

NHEJ pathway is initiated by the rapid recognition and binding of the heterodimeric complex Ku70-Ku80 at both DSB ends. The ability of Ku complex to interact with many proteins in vitro allows the Ku-associated DNA end to serve as a docking platform for the assembly of the NHEJ synapse, which bridges two DNA ends together. Specifically, the formation of Ku-DNA complex allows the recruitment of the DNA-PKcs (DNA-dependent protein kinase catalytic subunit) kinase via a physical interaction between Ku and DNA-PKcs (Gottlieb and Jackson, 1993). Next, the Ku complex moves inward away from the DNA terminus and allows DNA-PKcs to contact DNA (Yoo and Dynan, 1999), which is required for the stimulation of DNA-PKcs kinase activity (Yaneva et al., 1997). The inward movement of Ku complex also enables two DNA-PKcs molecules to interact with each other and form a synaptic complex (DeFazio et al., 2002), which results in the tethering of two DNA ends and prevention of excessive end resection. Activated DNA-PKcs can phosphorylate a number of substrates including Ku70, Ku80, XRCC4, XLF, Artemis, DNA ligase IV and DNA-PKcs itself (Douglas et al., 2005; Douglas et al., 2002; Goodarzi et al., 2006; Wang et al., 2004; Yu et al., 2008; Yu et al., 2003b). However the functional relevance of these phosphorylation events in NHEJ is not all clear. It has been suggested that the phosphorylation of DNA-PKcs can induce a conformational change, relieving the

blockage effect imposed by DNA-PKcs and thereby allowing DNA end processing enzymes to access the DNA and prepare the ends for the final step of ligation (Weterings et al., 2003).

(b) Processing

As previously described, for ligation of DNA ends to take place in NHEJ, DNA ends need to be properly processed and converted into 5' -phosphorylated ligatable ends. DNA-PKcs plays a crucial role in the recruitment of end-processing enzymes such as Artemis, polynucleotide kinase/phosphatase (PNKP), AP endonuclease 1 (APE1) and APLF (Davis and Chen, 2013; Deriano and Roth, 2013). The DNA end processing step can either be accomplished through gap filling by polymerases or through resection by nucleases (Weterings and Chen, 2008).

(c) Ligation

Once the broken ends are properly prepared, NHEJ can be completed by the final re-ligation of the two DNA ends. This step requires X4-L4-XLF complex (XRCC4, DNA ligase 4 and XLF), which is recruited by Ku complex, but independent of DNA-PKcs. DNA ligase 4, whose ligase activity is stimulated by XRCC4 and XLF, can ligate blunt ends as well as ends with compatible overhangs (Grawunder et al., 1997). This ligase also has the ability to ligate across gaps or ligate incompatible DNA ends (Gu et al., 2007; Tsai et al., 2007).

Alternative non-homologous end joining (a-NHEJ)

In the absence of the classical NHEJ factors Ku, XRCC4 or DNA ligase 4, NHEJ can still occur through an alternative non-homologous end joining (a-NHEJ) pathway

(Deriano and Roth, 2013; Hartlerode and Scully, 2009; McVey and Lee, 2008). While the classical NHEJ often relies on minimal sequence homology and involves less than 5 base pair insertions or deletions, the microhomology-mediated end joining (MMEJ) pathway, which is a predominant pathway of a-NHEJ, distinguishes itself from c-NHEJ by the use of 5-25 base pair microhomologous sequences during the alignment of two broken ends before ligation (McVey and Lee, 2008). In the case of MMEJ, DNA end resection is often needed in order to reveal the proper microhomolgies that are required for DNA end annealing and ligation. Therefore, MMEJ usually results in deletion of variable size flanking the break site, which accounts for a certain fraction of abnormal chromosomal rearrangements (Bunting and Nussenzweig, 2013).

Repair pathway choice between HR and NHEJ

DSB is one of the most deleterious forms of DNA lesions, which can result directly from radiation or indirectly as a consequence of unrepaired SSBs and collapsed replication forks (Chapman et al., 2012b; Pfeiffer et al., 2000). HR and NHEJ are the two major repair mechanisms involved in DSB repair. In the S/G2 phase of the cell cycle, HR is the predominant repair pathway due to the availability of sister chromatids as repair templates, and NHEJ is mostly suppressed. Defects in HR repair result in the hyper-utilization of NHEJ pathway and increased genomic instability (Bunting et al., 2010; Chapman et al., 2012b; Prakash et al., 2015). The regulation of these two repair pathways is tightly controlled in S phase. Recent findings suggest that HR and NHEJ are coordinated via a competition-based mechanism (Aly and Ganesan, 2011; Bunting et al., 2010; Cao et al., 2009; Daley and Sung, 2014; Kass and Jasin, 2010). In this

competition model, the pro-HR factor BRCA1 and the pro-NHEJ factor 53BP1 compete for the free DSB ends to regulate DNA end processing. How BRCA1 is recruited and stabilized at the DSBs is not yet clear and likely involves multiple co-factors including BARD1 and RAP80 and post-translational modifications such as par-ylation and ubiquitination (Feng et al., 2009; Kim et al., 2007; Li and Yu, 2013; Sobhian et al., 2007; Wang et al., 2007) . Recent studies suggest that the association of BRCA1 at the DNA lesions in conjunction with the nuclease CtIP promotes productive DNA end resection (Cao et al., 2009; Chen et al., 2008; Escribano-Diaz et al., 2013; Yu and Chen, 2004), which is a crucial regulatory step that commits the repair to HR. Meanwhile, the anti-HR factor 53BP1 is also capable of being recruited to and accumulating at the DNA lesions through the recognition of H4K20 methylation by its Tudor domain and H2A-K15 ubiquitination by the UDR domain (Botuyan et al., 2006; Fradet-Turcotte et al., 2013; Panier and Boulton, 2014; Sanders et al., 2004). The stabilization of 53BP1 and its effectors, PTIP and RIF1 at the lesions results in DNA end protection, ultimately channeling DNA repair to NHEJ (Callen et al., 2013; Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Kumar and Cheok, 2014; Prakash et al., 2015; Zimmermann et al., 2013). The competition between 53BP1 and BRCA1 is regulated in a cell-cycle dependent manner (Escribano-Diaz et al., 2013). In S/G2 phase, BRCA1 is able to outcompete 53BP1 at the lesion, facilitating HR repair and therefore suppressing 53BP1-mediated NHEJ repair (Escribano-Diaz et al., 2013). In the absence of a functional BRCA1, 53BP1 can stabilize at DSBs, inhibit resection and promote NHEJ (Chapman et al., 2012b; Escribano-Diaz et al., 2013). This competition model is supported by both mouse genetics and microscopy evidence. First, several research

groups have shown that the embryonic lethality of *brca1*$^{-/-}$ mice due to HR deficiency is rescued by the loss of 53BP1 (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009). Second, super-resolution microscopy shows that BRCA1 antagonizes the localization of 53BP1 at nuclear foci induced by IR (Chapman et al., 2012a). Despite the mounting evidence that supports this competition model, the molecular mechanism that mediates the BRCA1-53BP1 competition remains to be elucidated.

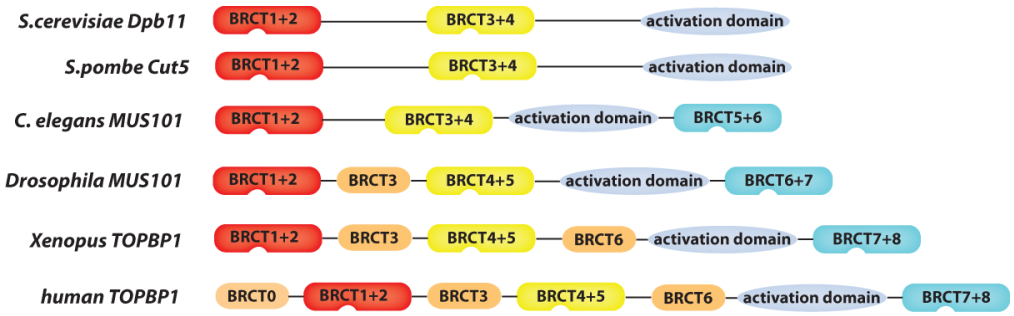
1.2 Overview of Dpb11/TOPBP1

Proper maintenance of genomic integrity involves highly coordinated cellular events in multiple aspects of DNA metabolism, including DNA replication, DNA damage checkpoint signaling, DNA repair and cell cycle control (Jackson and Bartek, 2009). In budding yeast, the Dpb11 scaffold consisting of multiple BRCT domains, plays crucial roles in DNA replication and DNA damage responses by mediating the assembly of multi-subunit complexes (Wardlaw et al., 2014) (Fig1.4a). It is well-established that Dpb11 is essential for the initiation of DNA replication and important for DDC activation. So far the studies have also hinted at a direct role of Dpb11 in DNA repair, yet the precise function of Dpb11 in DNA repair remains elusive (Fig1.4b). Of importance, Dpb11 is a highly conserved protein and the orthologues of Dpb11 in higher eukaryotes have been identified and extensively studied. TOPBP1 (topoisomerase II-binding protein 1), the human orthologue of Dpb11, has also been shown to be essential for DNA replication initiation and activation of the checkpoint kinase ATR (Sokka et al., 2010). Physical interactions of TOPBP1 with several repair factors have also been documented (Greenberg et al., 2006; Gritenaite et al., 2014; Morishima et al., 2007;

Yamane et al., 2002), suggesting the importance of TOPBP1 in DNA repair. However, studying the function of TOPBP1^{Dpb11} in DNA repair has been difficult due to its central and essential roles in DNA replication initiation and DDC signaling. Therefore, identification of separation of function mutants of TOPBP1^{Dpb11} is crucial to tease apart its roles in different aspects of DNA metabolism, which will provide useful insights into the fundamental mechanisms of how TOPBP1^{Dpb11} acts to coordinate DNA replication, DNA damage checkpoint signaling and DNA repair processes (Fig1.4b).

Figure 1.4

a.



b.

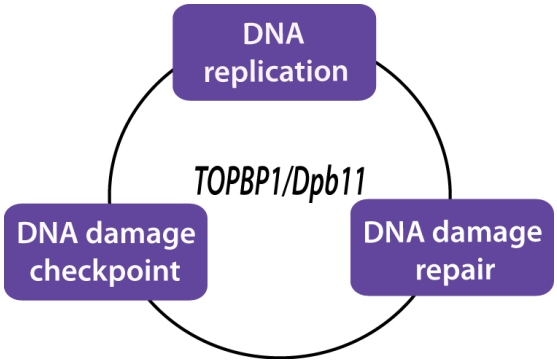


Figure 1.4 Overview of TOPBP1/Dpb11. (a) Schematic illustration of TOPBP1/Dpb11 domain structures. TOPBP1/Dpb11 is an evolutionarily conserved protein that contains multiple BRCT domains and an activation domain that stimulates the activity of apical checkpoint kinase ATR^{Mec1} upon DNA damage. (b) A working hypothesis for the central role of TOPBP1/Dpb11 in the coordination of DNA replication, DNA damage checkpoint and DNA repair processes.

1.2.1 Yeast Dpb11

1.2.1.1 Identification

In budding yeast Dpb11 was initially identified as an interactor of DNA polymerase II (epsilon) in a multicopy suppressor screen (Araki et al., 1995). Overexpression of Dpb11 suppresses the growth defect of a temperature sensitive mutant of Dpb2 and some mutants of pol2, both of which are subunits of polymerase ε. Initial studies revealed that Dpb11 is an essential protein for cell proliferation and required for S-phase DNA damage checkpoint. Consistently, in fission yeast, the orthologue of Dpb11 was identified as cut5 ('cell untimely torn'), which has also been implicated in DNA replication and checkpoint control (Garcia et al., 2005; Hirano et al., 1986)

1.2.1.2 Domain architecture

Dpb11 is a 764 amino acid protein with a molecular weight of 87kDa and contains multiple BRCT *(BRCA1 C-terminus)* domains that are arranged in two pairs, BRCT1/2 and BRCT3/4 (Fig1.4a). Additionally, Dpb11 holds an unstructured C-terminal region that is responsible for the activation of the checkpoint kinase Mec1 upon DNA damage (Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008). The tandem BRCT domains are phospho-binding modules that allow recognition of specific phosphorylation motifs (Rodriguez et al., 2003; Yu et al., 2003a). Through its multiple BRCT domains, Dpb11 recognizes phosphoproteins to dynamically assemble protein complexes with distinct roles in genome replication and maintenance. For example, BRCT-1/2 specifically recognize CDK-mediated phosphorylation in Sld3 during DNA

replication (Zegerman and Diffley, 2007), CDK-mediated phosphorylation in checkpoint adaptor Rad9 upon checkpoint activation (Granata et al., 2010; Pfander and Diffley, 2011) and Mec1/CDK-mediated phosphorylation in repair scaffold Slx4 during DNA repair (Gritenaite et al., 2014; Ohouo et al., 2010; Ohouo et al., 2013). BRCT3/4 has also been shown to recognize Mec1-dependent phosphorylation in Ddc1 upon DNA damage (Puddu et al., 2008; Wang and Elledge, 2002) and CDK-dependent phosphorylation in Sld2 during DNA replication (Tak et al., 2006). The functional relevance of each of these interactions will be described in detail in the following sections.

Orthologues of Dpb11 in higher eukaryotes have also been shown to contain multiple BRCT domains and a checkpoint kinase activation domain with a similar configuration of Dpb11 (Fig1.4a). As organisms expand in complexity, so does the number of BRCT domains. There are a total of 6 BRCT domains identified in the *C. elegans* TOPBP1, and 7 BRCT domains identified in both Drosophila and Xenopus TOPBP1, which is over twice the size of yeast Dpb11. The acquisition of additional BRCT domains at the C-terminus suggests that Dpb11 orthologues in higher organisms acquired extra functions throughout evolution.

1.2.1.3 Functions of Dpb11 in DNA replication, checkpoint signaling and DNA repair

Dpb11 plays a crucial role in the coordination of DNA replication, DNA damage checkpoint signaling and DNA repair. Over the last twenty years, the mechanisms by which Dpb11 controls DNA replication initiation and DNA damage checkpoint

activation have been extensively studied and elucidated, yet the role of Dpb11 in DNA repair is relatively unclear.

Replication initiation

Dpb11 plays an essential role in the DNA replication initiation. The replication machinery is composed of numerous replication proteins whose assembly and movement along the unwounded DNA need to be highly coordinated. DNA replication initiation takes place at specific DNA sequences called replication origins after the sequential loading of specific replication factors (DePamphilis, 2006; Masai et al., 2010) (Fig1.5a). In budding yeast, the 6-subunit origin recognition complex (ORC) binds to replication origins throughout the cell cycle (Bell, 2002). From late M until G1, inactive Mcm2-7 helicase is loaded with the aid of Cdt1 and Cdc6 onto ORC-bound origins to form a pre-replicative complex (pre-RC) (Diffley et al., 1994; Masai et al., 2010). Next, Sld3-Sld7 forms a complex with Cdc45 which is then loaded onto pre-RC in a process that requires activity of DDK (Dbf4-depedent kinase) (Heller et al., 2011; Yabuuchi et al., 2006). Then, upon S-CDK activation at G1/S boundary, pre-loading complex (pre-LC), consisting of GINS complex, Dpb11, Sld2 and Pol ε, is assembled and loaded, resulting in the formation of pre-initiation complex (pre-IC). The assembly of pre-IC allows the formation of CMG (Cdc45-MCM-GINS) complex and stimulation of MCM helicase activity (Gambus et al., 2006; Moyer et al., 2006). Activated CMG complex mediates the unwinding of the origin DNA and leads to the initiation of DNA synthesis. Once DNA synthesis starts, Dpb11 dissociates from the origin.

In the DNA replication initiation process described above, Dpb11 plays a crucial

role in the assembly of the pre-LC complex (Dpb11-Sld2-Polε). More importantly, Dpb11 couples the pre-LC complex with the Sld3-Cdc45-MCM complex at the origin to allow MCM helicase activation. Mechanistically, Dpb11 bridges the Sld3-Polε polymerase complex to the Sld2-MCM helicase complex by recognizing and physically interacting with phosphorylated Sld2 and Sld3 (Araki, 2011; Mueller et al., 2011). Of importance, both interactions of Dpb11 with Sld2 and Sld3 are regulated by CDK-dependent phosphorylation (Masumoto et al., 2002; Zegerman and Diffley, 2007). For the Dpb11-Sld2 interaction, it has been shown that phosphorylation of Sld2 at a total of 11 canonical CDK target sites induced by S-CDK serves as a priming event, rendering another single non-canonical CDK phosphorylation motif T84 accessible to CDK (Tak et al., 2006). This CDK phosphorylated T84 is then directly recognized by Dpb11 BRCT-3/4 to promote the association of Dpb11-Sld2 and the assembly of pre-LC. This sequential phosphorylation mechanism guarantees that Dpb11 only allows replication to start until a certain threshold of CDK activity is reached. For the regulation of the Sld3-Dpb11 interaction, it has been shown that Sld3, once loaded at the origin, is phosphorylated by CDK at T600 and S622 (Zegerman and Diffley, 2007). Phosphorylated Sld3 can then be recognized by the BRCT-1/2 in Dpb11. Taken together, Dpb11 plays an essential role in the ordered assembly of the replication machinery, allowing replication to start.

DNA damage signaling

The role of Dpb11 in DNA damage signaling can be classified into two aspects. First, Dpb11 directly activates the apical kinase Mec1, the yeast homologue of

mammalian ATR (Cimprich and Cortez, 2008), via its C-terminal tail to initiate checkpoint signaling cascade (Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008). Second, the scaffolding function of Dpb11 enables the recruitment and stabilization of checkpoint adaptor proteins via its tandem BRCT domains to the site of lesions to promote checkpoint signaling (Pfander and Diffley, 2011; Puddu et al., 2008).

The mechanism of Mec1 activation has been extensively studied. Upon replication stress, Mec1 can be activated through at least three different mechanisms, one of which involves Dpb11 (Fig1.5b). When replication forks encounter barriers, the helicase continues to unwind the DNA while the progression of the polymerase and other components of the replisome are blocked by the DNA lesion. This helicase-polymerase uncoupling results in the exposure of long stretches of ssDNA, which is rapidly coated by replication protein A (RPA) (Majka et al., 2006a; Wold, 1997; Zou and Elledge, 2003). RPA then recruits Mec1 and its cofactor Ddc2 via a physical interaction between RPA and Ddc2 (Rouse and Jackson, 2000). Independent of Mec1 recruitment, the PCNA-like trimeric clamp 9-1-1 complex (Ddc1-Mec3-Rad17) is also loaded at the 5' ssDNA/dsDNA junctions. Ddc1, the subunit of 9-1-1 complex, can directly activate Mec1 (Bonilla et al., 2008; Majka et al., 2006b; Navadgi-Patil and Burgers, 2009b), which in turn phosphorylates Ddc1 at its C-terminal residue T602 (Puddu et al., 2008). This Mec1-dependent phosphorylation motif in Ddc1 serves as a docking site for the recruitment of Dpb11 via BRCT-3/4 (Navadgi-Patil and Burgers, 2009b; Puddu et al., 2008; Wang and Elledge, 2002). This brings Dpb11 to the vicinity of Mec1, allowing further activation of Mec1 kinase through the C-terminal tail of Dpb11 (Navadgi-Patil and Burgers, 2009a; Navadgi-Patil and Burgers, 2009b).

Besides the role of Dpb11 in directly activating Mec1, Dpb11 also facilitates DDC signaling by physically interacting with at least two checkpoint proteins, Ddc1 and Rad9 (Fig1.5b). Upon DNA damage, Dpb11 interacts with Ddc1 and this phosphorylation-dependent interaction is crucial for the recruitment of Dpb11 and therefore Dpb11-dependent activation of Mec1 (Puddu et al., 2008; Wang and Elledge, 2002). Also, Dpb11 physically interacts with the checkpoint adaptor Rad9 to facilitate Rad9 recruitment at lesions and therefore promote DDC signaling (Granata et al., 2010; Pfander and Diffley, 2011). The stabilization of Rad9 at lesions recruits the downstream checkpoint effector kinase Rad53 to the vicinity of Mec1 to facilitate Mec1-dependent phosphorylation and activation of Rad53 (Schwartz et al., 2002; Schwartz et al., 2003; Sun et al., 1998; Usui et al., 2009). Rad9 is recruited to the sites of lesions via at least three distinct yet somewhat redundant mechanisms, two of which are dependent on the recognition of chromatin marks. First, the tandem BRCT domains at the C-terminus of Rad9 recognize DNA damage induced phosphorylation of histone H2A (Hammet et al., 2007). Second, the Tudor domain of Rad9 binds to constitutive methylated histone H3 lysine 79 (H3K79me), which becomes accessible after chromatin remodeling upon DNA damage (Wysocki et al., 2005). Lastly, a direct physical interaction with Dpb11 allows further stabilization of Rad9 at DNA lesions (Granata et al., 2010; Pfander and Diffley, 2011). This interaction, like many other Dpb11-mediated interactions, is also regulated in a phosphorylation-dependent manner, since Dpb11 BRCT-1/2 recognize CDK-dependent phosphorylation of S462, T474 of Rad9 during S-phase. In this case, by simultaneously interacting with both the Mec1-Ddc2 complex and Rad9, Dpb11 bridges the checkpoint kinase Mec1-Ddc2 and checkpoint adaptor Rad9 together,

forming a ternary complex Mec1-Ddc2-Dpb11-Rad9, which is required for efficient Rad53-mediated DDC activation (Pfander and Diffley, 2011).

DNA damage repair

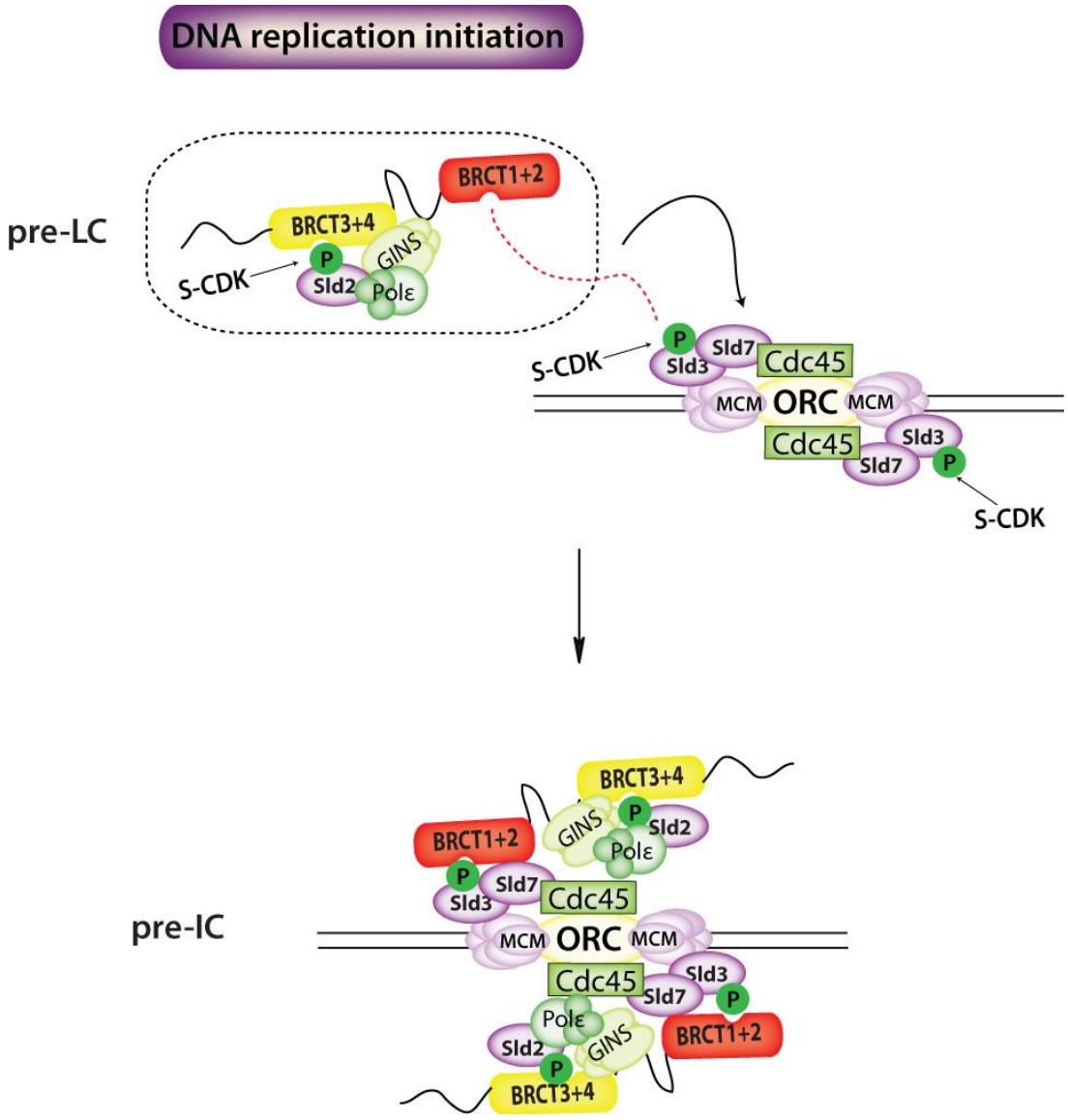
A direct repair role for Dpb11 was first recognized in an epistasis analysis of *dpb11-1* combined with deletion mutants of different repair factors (Ogiwara et al., 2006). While *dpb11-1* is proficient in checkpoint activation at the permissive temperature, it displays severe repair defects upon MMS-induced DNA damage. Yeast genetic analysis showed that *dpb11-1* does not become more sensitive to MMS when combined with deletions in homologous recombination repair factors such as Rad51 and Rad52. These epistatic genetic interactions between *DPB11* and *RAD51/52* suggest that Dpb11 functions in the same pathway as Rad51/52 in recombinational repair.

Another piece of evidence that hints at a role for Dpb11 in DNA repair is the identification of the Dpb11-Slx4 interaction (Ohouo et al., 2010) (Fig1.5c). Slx4 is a well-established repair scaffold that does not possess DNA processing activity on its own but instead facilitates the recruitment and stimulates the activity of other structure-specific endonucleases, such as Slx1, Rad1 and Rad10, to resolve DNA repair intermediate structures (Coulon et al., 2004; Flott et al., 2007; Fricke and Brill, 2003; Li et al., 2008; Rouse, 2009). Interestingly, our lab recently uncovered the interaction between Dpb11 and Slx4, which is enhanced upon MMS-induced replication stress (Ohouo et al., 2010). Furthermore, this interaction appears to be mediated by a cluster of phosphorylation sites on Slx4 targeted by the apical kinase Mec1. Mutations of the seven canonical Mec1 phosphorylation sites in Slx4 result in diminished binding to

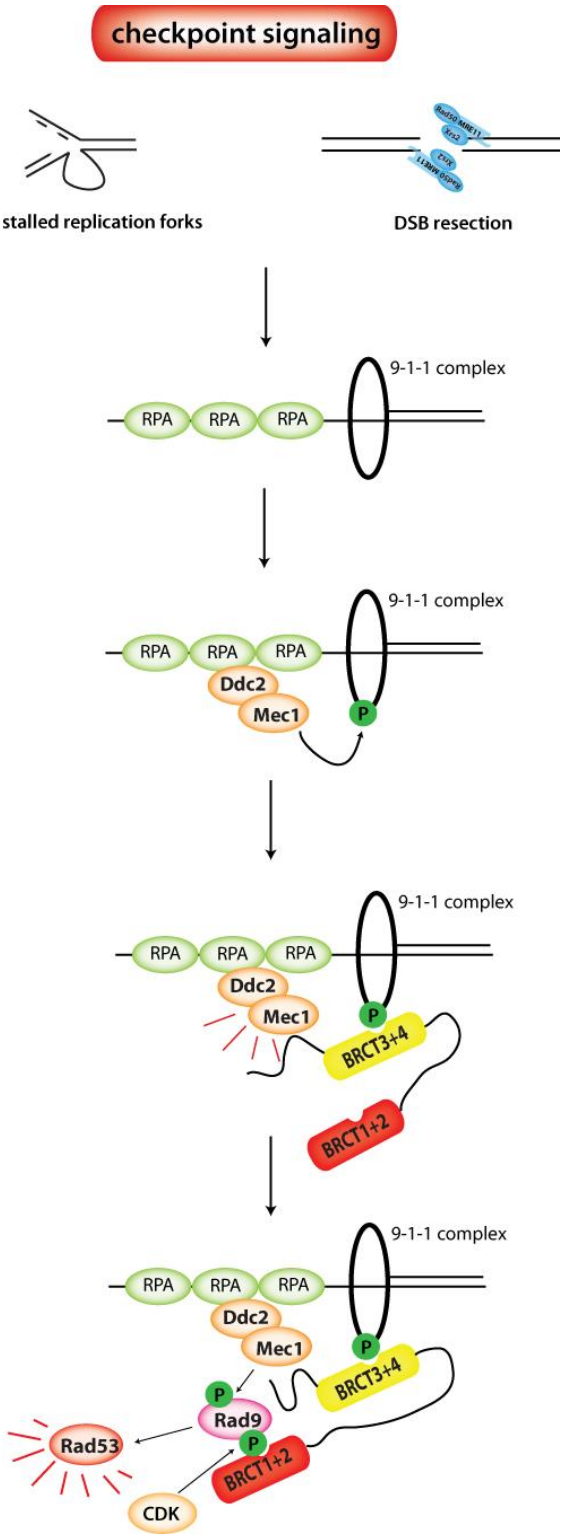
Dpb11, accompanied by severe sensitivity to MMS, pointing to the importance of Dpb11 in DNA repair. While functional relevance of the Dpb11-Slx4 interaction was not clear, this earlier report clearly placed Dpb11 at the interface of DNA checkpoint signaling and damage repair, suggesting a potential direct role of Dpb11 in the regulation of DNA repair. The work in this dissertation addressed the roles of the Dpb11-Slx4 interaction and defined how it is involved in the control of DNA repair and coordination of DDC signaling.

Figure 1.5

a.


DNA replication initiation
pre-LC
BRCT1+2
BRCT3+4
GINS
P
Sld2
Polε
S-CDK
Sld7
Sld3
Cdc45
MCM
ORC
MCM
Cdc45
Sld3
Sld7
P
S-CDK
pre-IC
BRCT3+4
GINS
P
Sld2
Polε
BRCT1+2
P
Sld7
Sld3
Cdc45
MCM
ORC
MCM
Cdc45
Sld3
Sld7
P
Polε
Sld2
GINS
BRCT1+2
P
BRCT3+4

b.


checkpoint signaling
stalled replication forks
DSB resection
9-1-1 complex
RPA
RPA
RPA
9-1-1 complex
RPA
RPA
RPA
Ddc2
Mec1
P
9-1-1 complex
RPA
RPA
RPA
Ddc2
Mec1
P
BRCT3+4
BRCT1+2
9-1-1 complex
RPA
RPA
RPA
Ddc2
Mec1
P
BRCT3+4
P
Rad9
Rad53
P
BRCT1+2
CDK

c.

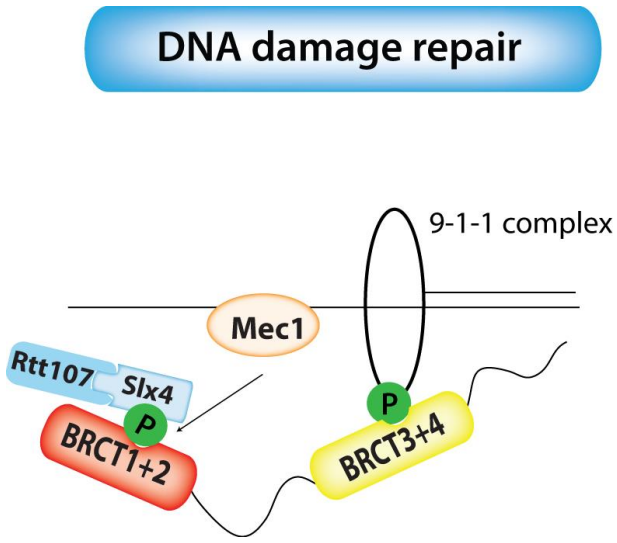

DNA damage repair
9-1-1 complex
Mec1
Rtt107
Slx4
P
BRCT1+2
P
BRCT3+4

Figure 1.5 Schematic illustration for the roles of Dpb11 in DNA replication initiation, checkpoint signaling and DNA repair. (a) Schematic illustration of the role of Dpb11 in DNA replication. Dpb11 is required for the initiation of DNA replication by bridging Sld2-GINS-polymerase ε complex to the Sld3-Cdc45-MCM helicase via the recognition of S-CDK targeted phosphorylation sites on Sld2 and Sld3. (b) Schematic illustration for the role of Dpb11 in checkpoint signaling. Upon accumulation of ssDNA, Dpb11 promotes checkpoint signaling by stimulating Mec1 kinase activity and mediates the recruitment of Rad9 which transduces the signal from Mec1 to downstream kinase Rad53. (c) Schematic illustration for the role of Dpb11 in DNA repair. Dpb11 facilitates the recruitment of Slx4-Rtt107 repair scaffolds to DNA lesion sites through the recognition of Mec1-dependent phosphorylation of Slx4, suggesting a role of Dpb11 in the coordination of DNA damage checkpoint signaling and DNA repair.

1.2.2 Mammalian TOPBP1

1.2.2.1 Identification

The mammalian orthologue of Dpb11, topoisomerase-II-binding protein, TOPBP1, was initially isolated in a two-hybrid screen as factor that interacts with the C-terminal region of Topoisomerase II beta (Yamane et al., 1997). It was predicted to play a role in the repair of double-strand breaks caused by the defective catalytic activity of topoisomerase II (Yamane et al., 1997). Though the functional relevance of the TopBP1-topoisomerase II interaction is still not clear today, the work in the last two decades has established TOPBP1 as a key scaffolding protein in DNA replication and DNA damage checkpoint signaling. This dissertation explores roles for TOPBP1 in DNA repair.

1.2.2.2 Domain architecture

Like Dpb11, TOPBP1 is also a multi-BRCT domain protein. It is composed of 1522 amino acids, with a molecular weight of 180kDa. Bioinformatic analysis has identified 8 BRCT domains (Yamane et al., 1997), acting in three pairs: BRCT-1/2, BRCT-4/5, BRCT-7/8 and 2 singular BRCT domains, BRCT-3 and BRCT-6. The three pairs of BRCT domains in TOPBP1, like in Dpb11, function as phospho-binding modules to recognize specific phosphorylated motifs and mediate phosphorylation-dependent interactions. It is generally acknowledged that BRCT 1/2 and 4/5 are structurally and functionally conserved counterparts of the tandem repeats BRCT-1/2 and BRCT-3/4 in Dpb11 (Wardlaw et al., 2014). These two pairs of canonical tandem BRCT repeats in TOPBP1 can specifically recognize cyclin-dependent kinases or

checkpoint kinase-targeted phosphorylation motifs to mediate DNA damage checkpoint-regulated or cell cycle-controlled protein interactions. However, mammalian TOPBP1 has evolved an extended C terminus harboring extra tandem BRCT repeats 7/8, which allows simultaneous regulation of more phosphorylation-mediated interactions. Interestingly, TOPBP1 possesses two singlet BRCT domains (BRCT 3 and 6), which, unlike canonical phospho-binding BRCT tandem repeats, do not show binding specificity toward phosphorylated motifs, but instead dimerize with other proteins such as E2F1 and PARP1 (Liu et al., 2003; Wollmann et al., 2007). To add even more complexity to the domain architecture of TOPBP1, in 2011, Rappas *et al.* successfully determined the crystal structure of the N-terminal TOPBP1 which surprisingly revealed an additional BRCT "0" at the very N-terminus resulting in a unique triple-BRCT domain configuration (Rappas et al., 2011). However the function of the BRCT-0 is yet to be understood. Since there is no phospho-binding pocket present in BRCT-0, one possible function of this N-terminal BRCT-0 domain is to support and stabilize interactions mediated by BRCT-1/2.

In addition to BRCT domains, TOPBP1 also contains an ATR activation domain (AAD) located between BRCT-6 and BRCT-7. The AAD is required and sufficient for *in vivo* and *in vitro* activation of ATR kinase activity upon DNA damage, which is a crucial step to initiate DNA damage checkpoint signaling (Choi et al., 2010; Kumagai et al., 2006; Xu and Leffak, 2010). It has been shown that the point mutation W1147R in mouse TOPBP1 inactivates AAD activity and results in embryonic lethality (Zhou et al., 2013). Ablation of TOPBP1 AAD activity in MEFs leads to premature senescence and defective checkpoint signaling response upon DNA damage (Zhou et al., 2013).

This suggests that TOPBP1 AAD is essential for embryonic development and cell proliferation.

1.2.2.3 Functions of TOPBP1 in DNA replication, checkpoint signaling and DNA repair

Studies over the last two decades have revealed the crucial roles of TOPBP1 in genome replication, DNA damage checkpoint signaling and transcriptional regulation, establishing TOPBP1 as a central player in the control of DNA metabolism (Wardlaw et al., 2014). In addition, some work has implicated TOPBP1 directly in DNA repair pathways, though further investigation is needed to clearly delineate its role and mechanisms of action.

Replication initiation

In higher eukaryotes, lack of TOPBP1 leads to embryonic lethality and cell death due to either deficient DNA damage response or defective DNA replication, ultimately resulting in apoptosis or cellular senescence (Jeon et al., 2011). While this is partly explained by the required role of TOPBP1 AAD in the activation of ATR kinase activity, like in budding yeast, the scaffolding function of TOPBP1 is also vital for replication initiation (Boos et al., 2011; Makiniemi et al., 2001) . To draw parallels between the function of scDpb11 and hTOPBP1, the counterparts of Sld3 and Sld2 in humans have been proposed to be Treslin/Ticcr and RecQ4 respectively (Mueller et al., 2011) (Fig1.6a). It has been reported in several model organisms that Treslin is required for the loading of Cdc45 onto the chromatin thereby promoting DNA replication

(Kumagai et al., 2010; Sansam et al., 2010). Treslin is known to bind to TOPBP1 in a phosphorylation-dependent manner and this interaction is required for proficient DNA replication initiation (Boos et al., 2011; Kumagai et al., 2010; Kumagai et al., 2011). Specifically, Treslin is phosphorylated by CDK2 at a canonical CDK target residue S1001, which then is recognized by and bound to BRCT1/2 of TOPBP1 (Boos et al., 2011; Kumagai et al., 2010; Kumagai et al., 2011). The substitution of Treslin S1001 by alanine or the lack of TOPBP1 N terminal BRCT domains results in diminished Treslin-TOPBP1 binding and consequently DNA replication defect (Boos et al., 2011; Kumagai et al., 2011). While relevance of the TOPBP1-Treslin interaction has been relatively well understood, it is not clear if RecQ4 functions similarly to Sld2. RecQ4 was originally proposed to be a candidate for the human Sld2 orthologue based on its N-terminus sequence homology to Sld2 by bioinformatics analysis (Matsuno et al., 2006). In Xenopus, it has been shown that the N-terminus segment of RecQ4 is essential for the initiation of DNA replication. The same domain in RecQ4 also mediates its interaction with Xenopus TOPBP1 (Matsuno et al., 2006; Ohlenschlager et al., 2012). Interestingly, the ability of RecQ4 to interact with TOPBP1 is correlated with its ability to support replication initiation (Matsuno et al., 2006). This suggests that RecQ4 together with TOPBP1 plays a crucial role in the assembly of the replication machinery, mirroring the function of the Sld2-Dpb11 complex in budding yeast. However, unlike Sld2-Dpb11, which is regulated by CDK-dependent phosphorylation, the RecQ4-TOPBP1 interaction is independent of phosphorylation as the binding is not impaired upon phosphatase treatment (Matsuno et al., 2006). However, it has been reported recently, in *C. elegans*, that RecQ4 appears to be a substrate of CDK both *in vitro* and

in vivo. CDK-dependent phosphorylation of RecQ4 is required for its interaction with the orthologue of TOPBP1 in *C. elegans* through the extended C terminal tandem BRCT5/6. Mutations at the CDK target sites in RecQ4 results in diminished interaction with TOPBP1 as well as defective replication initiation (Gaggioli et al., 2014). Further work is needed to fully delineate the regulatory mechanism of this interaction in mammals.

DNA damage checkpoint signaling

TOPBP1, similarly to Dpb11, plays important roles in DNA damage signaling. The function of TOPBP1 in DDC signaling is mainly executed by either direct activation of ATR kinase activity via its AAD domain or the recruitment of checkpoint factors via the scaffolding function of its tandem BRCT domains (Wardlaw et al., 2014). Often, these two aspects of the TOPBP1 function are intertwined and dependent on each other. While in *Saccharomyces cerevisiae*, multiple activators of Mec1 kinase have been identified (Kumar and Burgers, 2013; Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008; Navadgi-Patil and Burgers, 2009b; Puddu et al., 2008), in mammals TOPBP1 is the only well-established activator of ATR (Kumagai et al., 2006). To depict the process of ATR activation, I will use the scenario of replication stress or DSB damage as examples (Fig1.6b). Like in yeast, long stretches of ssDNA can accumulate upon fork stalling due to functional uncoupling of MCM helicase and DNA polymerase activities or upon DSB resection mediated by nucleases. Exposed ssDNA is rapidly coated by replication protein A (RPA) to protect and prevent faulty processing of this ssDNA structure. Then, ATR is recruited together with its co-factor ATR-interacting protein

(ATRIP), orthologue of yeast Ddc2, to the site of lesions via a direct physical interaction between RPA and ATRIP (Cortez et al., 2001; Zou and Elledge, 2003). Independently of ATR recruitment, the PCNA-like trimeric clamp 9-1-1 complex, is loaded onto the 5' ssDNA/dsDNA junctions. Concurrently, the loading of 9-1-1 allows the recruitment of TOPBP1 via a BRCT 1/2 –mediated phosphorylation-dependent interaction with the C-terminal tail of Rad9, a subunit of the 9-1-1 complex (Delacroix et al., 2007; Greer et al., 2003; Rappas et al., 2011; Ueda et al., 2012). This brings TOPBP1 and ATR in close proximity and ultimately allows the stimulation of ATR kinase activity by TOPBP1 through its ATR-activation domain (AAD) (Delacroix et al., 2007; Kumagai et al., 2006). However, the detailed mechanism of how TOPBP1 activates ATR remains yet to be elucidated. In a speculative model, upon independent recruitment of TOPBP1 and ATR-ATRIP complex, TOPBP1 AAD touches ATR and drives the conformational change of ATR-ATRIP complex to stimulate its kinase activity (Mordes et al., 2008a).

Besides direct activation of ATR, TOPBP1 also promotes checkpoint signaling by facilitating the recruitment of checkpoint proteins mediated by its BRCT domains. Like in budding yeast, one of the most extensively studied TOPBP1 interacting proteins is RAD9, the orthologue of yeast Ddc1, whose interaction with TOPBP1 facilitates TOPBP1 recruitment to the lesion sites and ATR activation (Delacroix et al., 2007; Lee et al., 2007; Ohashi et al., 2014; St Onge et al., 2003). This evolutionarily conserved interaction is accomplished through the recognition of phosphorylation at serine341 and serine387 at the C-terminal tail of RAD9 by the N-terminal BRCT 1/2 in TOPBP1 (Ueda et al., 2012). While the yeast Dpb11-Ddc1 interaction is only stimulated by Mec1 activity, RAD9 S341 and S387 are constitutively phosphorylated by casein kinase II

(CKII), suggesting that TOPBP1-RAD9 interaction is also constitutive even in the absence of DNA damage (St Onge et al., 2003; Takeishi et al., 2010; Ueda et al., 2012).

BRCT-4/5 in TOPBP1 has also been shown to physically interact with two checkpoint signaling factors: MDC1 during the replication checkpoint and 53BP1 during the G1 checkpoint (Cescutti et al., 2010; Wang et al., 2011). Upon replication stress, MDC1 binds to TOPBP1 through the recognition of phospho-SDT repeats in MDC1 by TOPBP1 BRCT-4/5 (Leung et al., 2013; Wang et al., 2011). This interaction facilitates TOPBP1 stabilization and accumulation at the stalled forks, thereby promoting replication checkpoint activation. In addition, it has also been reported that TOPBP1 can bind to 53BP1 via BRCT-4/5 in response to DSB to facilitate TOPBP1 localization at the lesions and ensure proper G1 checkpoint (Cescutti et al., 2010).

The extreme C terminal BRCT-7/8, which exclusively exists in the TOPBP1 of higher eukaryotes, is also capable of mediating phosphorylation-dependent interactions. As an example, the FANCJ/BACH1 helicase has been shown to interact with TOPBP1 via BRCT7/8 in response to replication stress (Gong et al., 2010; Leung et al., 2011). This interaction is mediated by S-phase specific phosphorylation at T1133 in FANCJ/BACH1 and has been shown to be important for the efficient RPA loading on ssDNA and therefore productive ATR-CHK1 signaling upon replications stress.

DNA repair

Despite the extensive studies on TOPBP1 in the last two decades, its precise role in repair remains elusive. This could be possibly because the repair function of TOPBP1 is tightly associated with its other functions in checkpoint signaling and replication

initiation. In other words, it is likely that the same functional modules in TOPBP1 are required for the regulation of both DNA damage repair and checkpoint signaling, rendering it difficult to distinguish a direct repair defect from indirect consequence of defective checkpoint signaling. However, recently there is increasing evidence suggesting a bona fide role of TOPBP1 in DNA repair. One of the earliest findings that implicates TOPBP1 in repair processes is that TOPBP1 depletion results in a reduced HR repair rate in response to DSB and hyper-sensitizes these cells to MMC and IR (Morishima et al., 2007). This is consistent with the very recent observation that TOPBP1 knockdown results in hyper-sensitivity to PARP inhibitor as a result of impaired Rad51 foci formation and defective HR repair (Moudry et al., 2016). Mechanistically, it was suggested that TOPBP1 physical interacts with Polo-like kinase 1 (PLK1) and promotes PLK1-mediated phosphorylation of Rad51 at serine 14, a prerequisite for Rad51 foci formation.

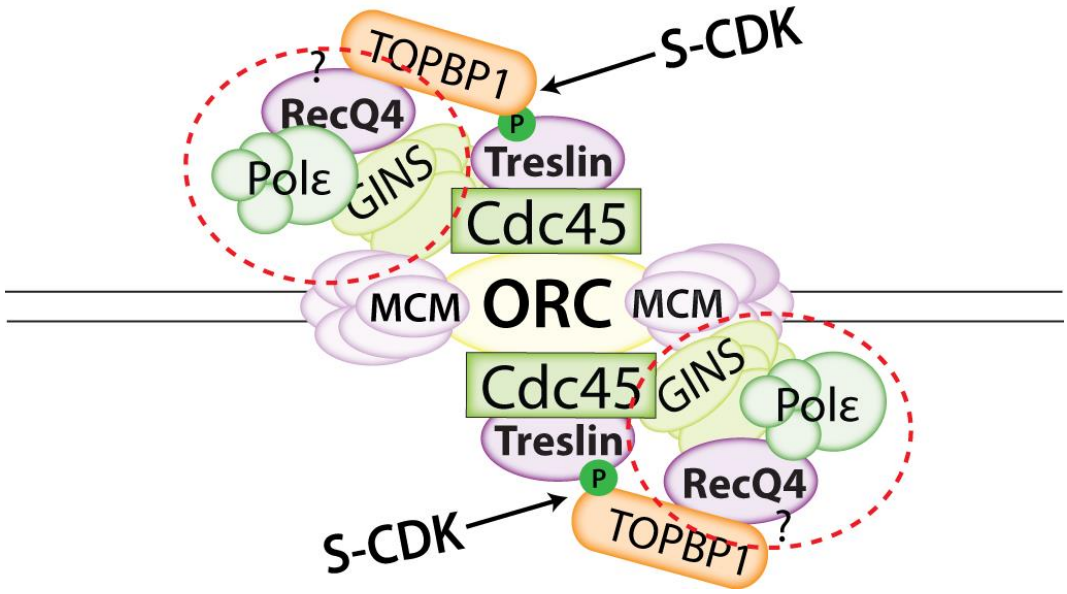
Besides a potential role in HR regulation, TOPBP1 has also been shown to interact with an extensive network of repair factors including BRCA1, 53BP1, SLX4 and BLM, which are involved in the processing of different types of DNA lesions (Fig1.6c). It has long been shown that TOPBP1 forms a stable complex with BRCA1-BARD1 heterodimer in response to IR (Greenberg et al., 2006). Though this TOPBP1-BRCA1 interaction requires PIKK-dependent phosphorylation in BRCA1, it is still not clear if this interaction is direct and, if so, which pair(s) of BRCT domains in TOPBP1 mediate the binding. The functional relevance of this interaction also remains to be elucidated. Besides BRCA1, TOPBP1's interaction with another DSB repair protein 53BP1 has been documented (Yamane et al., 2002). While this interaction is required

in the G1 checkpoint as previously described, it is not clear if or how the repair function of 53BP1 is modulated by TOPBP1. In a third example, TOPBP1 binds to the repair scaffold SLX4 (Gritenaite et al., 2014). Human SLX4 is an evolutionarily conserved repair scaffold protein that serves as a docking platform for the assembly of multiple structure-specific endonucleases including XPF-ERCC1, MUS81-EME1, and SLX1. However, unlike the yeast Dpb11-Slx4 interaction, human TOPBP1-SLX4 binding is not enhanced upon genotoxic stress (unpublished result). Instead the human TOPBP1-SLX4 interaction is cell cycle regulated, as this interaction is attenuated by the addition of the CDK inhibitor roscovitine. Consistently, mutation at the canonical CDK phosphorylation site T1260 in SLX4 significantly impairs its association with TOPBP1. Yet it is not clear if this interaction is mediated by TOPBP1 BRCT domains and its functional relevance also remains to be elucidated. Further TOPBP1 has been shown to interact with BLM (Bloom syndrome helicase) also in a phosphorylation-dependent manner, yet the functional relevance and the molecular basis of this interaction remain controversial (Blackford et al., 2015; Wang et al., 2013).

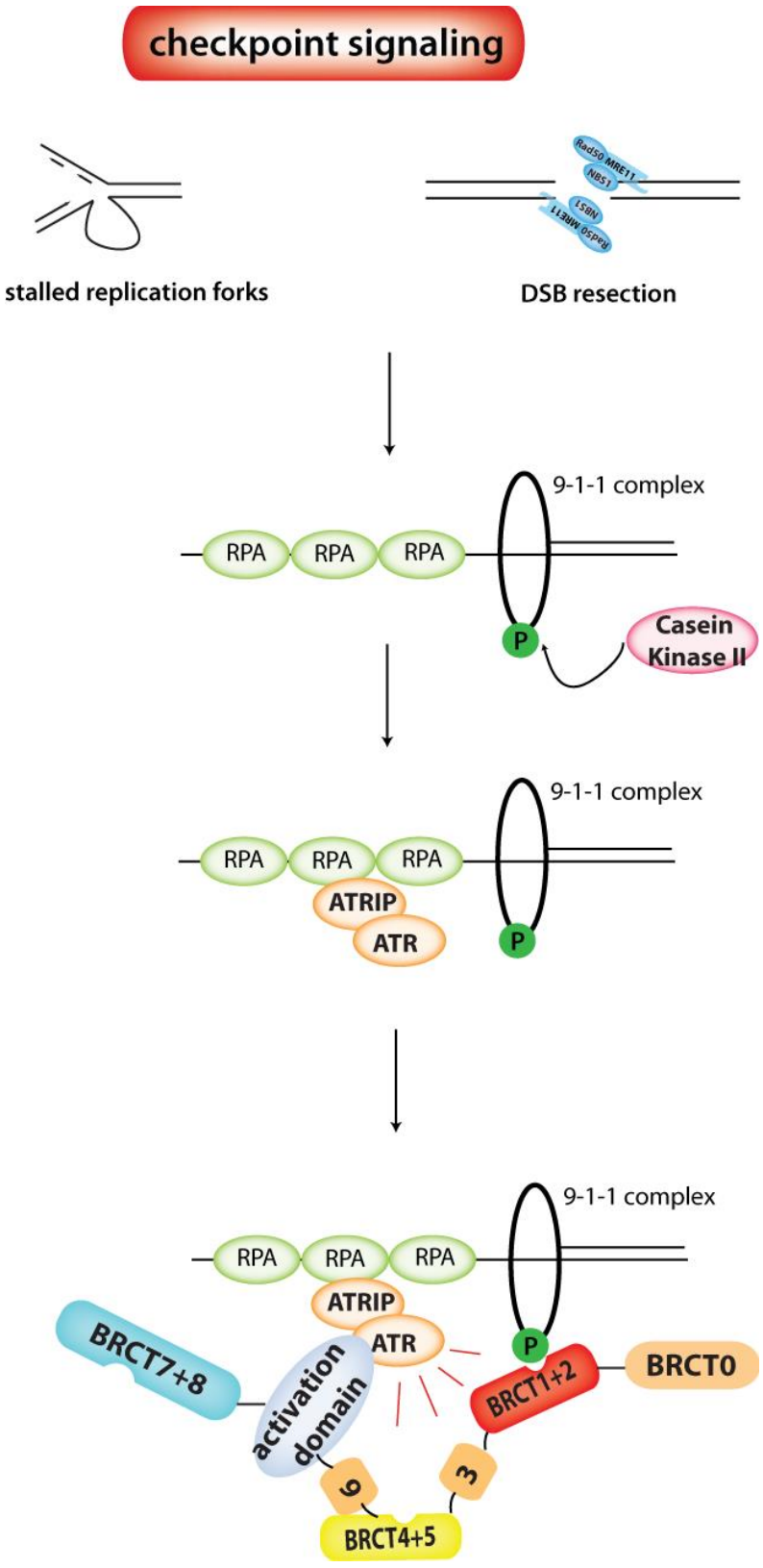
Taken together, the physical interactions of TOPBP1 with a wide range of repair factors point to a potential role for TOPBP1 in the coordination of multiple DNA repair pathways thereby allowing the handling of different types of DNA lesions.

Figure 1.6

a.


TOPBP1
S-CDK
?
RecQ4
Polε
GINS
P
Treslin
Cdc45
MCM
ORC
MCM
Cdc45
GINS
Polε
Treslin
P
RecQ4
?
S-CDK
TOPBP1

b.


checkpoint signaling
stalled replication forks
DSB resection
Rad50 MRE11
NBS1
9-1-1 complex
RPA
RPA
RPA
P
Casein Kinase II
ATRIP
ATR
BRCT7+8
activation domain
BRCT1+2
BRCT0
9
3
BRCT4+5

c.

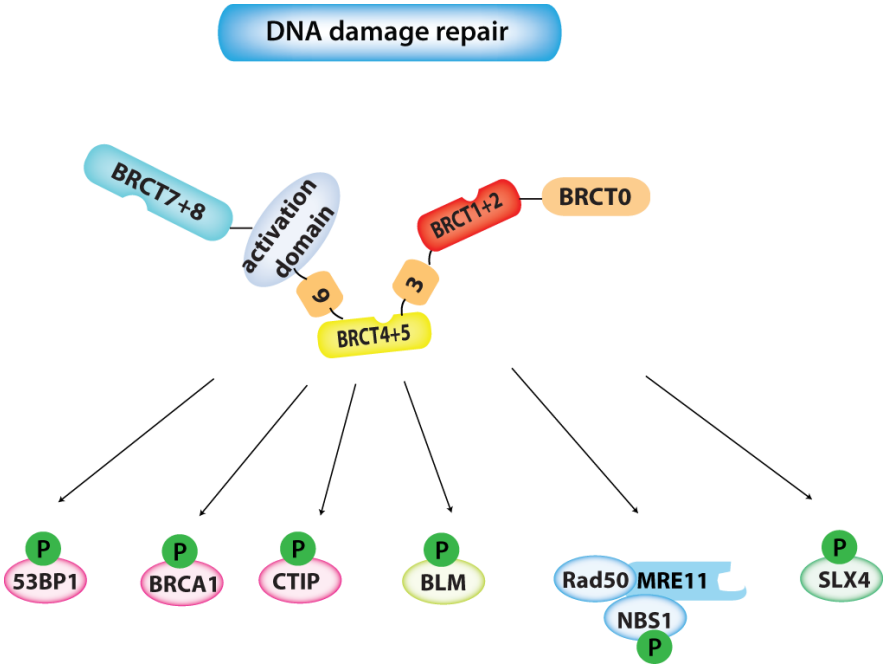


Figure 1.6 Schematic illustration for the roles of TOPBP1 in DNA replication, checkpoint signaling and DNA repair. (a) Schematic illustration for the role of TOPBP1 in DNA replication. Mammalian TOPBP1-Trelsin and TOPBP1-RecQ4 interactions are believed to mirror Dpb11-Sld3 and Dpb11-Sld2 interactions in *S. cerevisiae* respectively and assist the assembly of Pre-IC to allow replication initiation. (b) Schematic illustration for the role of TOPBP1 in checkpoint signaling. TOPBP1, recruited via a phosphorylation-mediated interaction with Rad9, a subunit of 9-1-1 complex, activates ATR kinase activity upon damage. (c) Schematic illustration for the role of TOPBP1 in DNA repair. While TOPBP1 is known to interact with numerous DNA repair factors, the exact function of TOPBP1 in DNA repair remains to be elucidated.

Transcriptional control

Besides the roles of TOPBP1 in DNA replication and DDR, TOPBP1 has also been shown to perform important functions in transcriptional regulation in both transcription activation and transcription repression (Wright et al., 2006). Of importance, BRCT-7/8 in TOPBP1 specifically recognizes AKT-dependent phosphorylation at serine 1159 in TOPBP1, leading to TOPBP1 oligomerization (Liu et al., 2006), which has been proposed to be a key event that switches TOPBP1 function from DDC signaling to transcriptional regulation (Liu et al., 2013).

1.3 TOPBP1 and cancer

Proper maintenance of the genome relies on a sophisticated network of signaling pathways (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). In humans, defects in DNA damage signaling and repair result in increased genomic instability, which is a hallmark of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Hoeijmakers, 2009; Jackson and Bartek, 2009). Harmful mutations in the DDR components are frequently associated with genetic disorders, characterized by growth retardation, developmental defects, neurological problems, infertility, immunological disorders, premature aging, hyper-sensitivity to DNA damage factors and, most importantly, predisposition to cancer (Jackson and Bartek, 2009; O'Driscoll, 2012). Despite the important roles of TOPBP1 in multiple DNA metabolic events, not much is understood regarding the role of TOPBP1 mutations in cancer. In previous studies, it has been reported that TOPBP1 protein expression level is significantly increased in breast cancer tissue, especially in poorly differentiated tumor cells, which tend to grow

quicker and are generally more aggressive (Forma et al., 2012). In addition to the altered expression level, significant changes of TOPBP1 protein localization were also observed in cancer. While TOPBP1 staining is mostly nuclear in normal breast tissue, there is a substantial accumulation of or even exclusive TOPBP1 localization in the cytoplasm in breast cancer tissue (Forma et al., 2012; Going et al., 2007). This aberrant expression pattern of TOPBP1 is likely to impair its function in genome protection and eventually result in the accumulation of genomic instability and progression to cancer. Supporting the role of TOPBP1 in tumorigenesis, TOPBP1 polymorphisms have been associated with cancer susceptibility. In a screen for TOPBP1 mutations in familial breast cancer and ovarian cancer, the Arg309Cys TOPBP1 variant was identified to be associated with a two-fold higher risk of breast and/or ovarian cancer, based on 125 Finnish cancer families (Karppinen et al., 2006). However, a contrary result was obtained in an independent study based on a screen of a German population, where no difference of Arg309Cys frequency was found between cancer patient families and the corresponding control population (Blaut et al., 2010). This latter study is also consistent with another previous report showing that there was no association of TOPBP1 with ovarian cancer risk in a total of more than 2000 BRCA1/2 mutation carriers examined (Rebbeck et al., 2009). Besides, another TOPBP1 single nucleotide polymorphism (SNP) c.*229C>T in the 3'UTR region that causes increased expression level has been identified to be associated with increased risk of breast cancer, ovarian cancer and endometrial cancer (Forma et al., 2013; Forma et al., 2014; Karppinen et al., 2006; Liu et al., 2009; Liu et al., 2011). This corroborates the finding that TOPBP1 overexpression is frequently found in breast cancer and is associated with poor prognosis (Liu et al.,

2009). Taken together, further studies are necessary to reach a definitive conclusion regarding the exact role TOPBP1 in carcinogenesis.

Despite many unanswered questions, prior work has placed TOPBP1 as a potential therapeutic target for cancer treatment by perturbing its function in the regulation of p53 and E2F1. It has been shown the Calcein AM compound can sensitize cultured cancer cells by impairing the function of BRCT7-8 to block TOPBP1 oligomerization and therefore suppress the deregulated p53 and E2F1 activity (Chowdhury et al., 2014; Liu et al., 2009).

1.4 Summary

Overall, current studies establish TOPBP1^{Dpb11} as a key player in DNA replication initiation and DNA damage checkpoint signaling, while its function in DNA repair remains unclear. In my dissertation, I investigated the roles of TOPBP1^{Dpb11} in DNA repair and explored the molecular mechanism of how it coordinates DNA replication, checkpoint signaling with DNA repair during genome maintenance.

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CHAPTER 2

DNA Repair Scaffolds Slx4-Rtt107 Dampen Checkpoint Signaling by Counteracting the Adaptor Rad9

Patrice Y. Ohouo, Francisco M. Bastos de Oliveira, **Yi Liu**, Chu Jian Ma, Marcus B. Smolka

The work presented in this chapter was published in *Ohouo et.al, 2013**. My contribution (Figs 2.1b-d and Fig2.2) to this work estabished the role of Dpb11 in coordinating the competition between the repair scaffolds Slx4-Rtt107 and the checkpoint adaptor Rad9, and hinted the importance of the phosphorylation at serine 486 in Slx4 for its interaction with Dpb11 (Fig 2.3 by Patrice Y. Ohouo and Francisco M. Bastos de Oliveira).

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2.1 Introduction

Replication stress is one of the major sources of genomic instability. During DNA replication, forks are prone to stall or collapse when they encounter obstacles that impede their progression, such as DNA lesions, highly transcribed regions or stable DNA-protein complexes (Barbour and Xiao, 2003). Stalled or collapsed forks can lead to intensified DNA damage and genomic instability, which is a hallmark of cancer (Hanahan and Weinberg, 2011). In order to maintain genomic integrity, a set of highly conserved surveillance mechanisms have evolved, collectively termed DNA damage response (DDR). The activation of DDR halts cell cycle progression, protects

replication fork stability and promotes fork repair (Flynn and Zou). In budding yeast, proper coordination of DDR events highly depends on the action of apical DNA damage checkpoint kinase Mec1, orthologue of human ATR. Cells lacking functional Mec1 exhibit increased gross chromosomal rearrangement and hyper-sensitivity to genotoxins. Mec1 kinase exerts its function in DDR mostly by targeted phosphorylation of many checkpoint proteins and DNA repair factors in a spatio- and temporal- regulated manner. Among numerous Mec1 targets, DNA repair scaffolds Slx4-Rtt107 have been shown to be crucial for proper replication stress response and cellular resistance to DNA alkylating agents such as methyl methanesulphonate (MMS), which blocks replication and induces the DNA damage checkpoint signaling (Fricke and Brill, 2003; Roberts et al., 2006; Rouse, 2004). Slx4 is an evolutionarily conserved DNA repair scaffolding protein (Fekairi et al., 2009; Fricke and Brill, 2003; Munoz et al., 2009; Svendsen et al., 2009), and mutations in human SLX4 were recently linked to Fanconi anaemia (Kim et al., 2011; Stoepker et al., 2011). The previous work from our lab has revealed that Mec1 signaling promotes the assembly of Dpb11-Slx4-Rtt107 complex upon replication stress to mediate MMS-induced replication stress response (Ohouo et al., 2010). Ohouo et al showed that Mec1-dependent phosphorylation of Slx4 induced by MMS stabilizes an interaction between Dpb11 and Slx4-Rtt107 repair scaffolds. This interaction is required for cellular resistance to MMS. Mutations of the canonical Mec1 phosphorylation sites in Slx4 (*slx4-7mut*) specifically disrupt Slx4's interaction with Dpb11 and results in hyper-sensitivity to MMS. This work points to a key function for the repair scaffolds Slx4-Rtt107 at the interface of DNA damage signaling and DNA repair in replication stress response.

While the importance of the Dpb11-Slx4-Rtt107 complex formation has been appreciated, the exact role of the Slx4-Rtt107 scaffolds, which confer MMS resistance, remain elusive. It has been previously reported that Slx4 or Rtt107 deletion leads to prolonged Rad53 activation during the recovery from MMS-induced DNA damage. In wildtype cells, controlled Rad53 activation is beneficial by enforcing a transient cell cycle arrest to allow DNA repair to take place. However, hyper-activated Rad53-dependent checkpoint signaling could result in persistent cell cycle arrest, which is detrimental to cell survival and therefore Rad53 activation needs to be closely monitored. We hypothesized that Slx4-Rtt107 scaffolds serve as the modulator of DNA damage checkpoint (DDC) signaling to prevent aberrant Rad53 hyper-activation and ensure proper checkpoint activation. Indeed, we found that Slx4-Rtt107 scaffolds, independent of their repair function, serve to antagonize Rad53 activation by counteracting checkpoint adaptor protein Rad9 via a competition-based mechanism at replication-induced lesions. We show that Slx4 counteracts Dpb11-mediated recruitment of Rad9 by physically interacting with Dpb11 through the same BRCT domains that mediate Dpb11-Rad9 interaction. Collectively, these results support a competition-based model whereby Dpb11 physically interacts with Slx4-Rtt107 scaffolds to balance the engagement of Rad9 at replication stress-induced lesions so as to dampen Rad9-mediated Rad53 signaling. This work provides mechanistic understanding to the crosstalk between DDC signaling and DNA repair. My work in this project provides evidence that establishes the central role of Dpb11 in coordinating the competition between Slx4 and Rad9.

2.2 Materials and Methods

2.2.1 Yeast strains and plasmids

Strains generated in this study were derived either from MBS164 or MBS191 (both congenic to S288C). Unless indicated, all proteins were tagged at the C terminus and the expression was verified by western blotting. Tagged strains were assayed for sensitivity to MMS to ensure they behaved similarly to the wild-type strain. Sensitivity assays were independently confirmed in strains derived from freshly sporulated diploids. Standard cloning methods were used to generate the plasmids for this study. Plasmids containing domains of Dpb11 tagged at the amino terminus with a PATH tag (2× protein A + TEV cleavage site + 6× His) (Smolka et al., 2006) were based on the pET21a vector (Novagen). Wild-type alleles cloned into the pFA6a vector (Addgene) were linearized before integration into the respective endogenous loci. SLX4 and DPB11 constructs containing an ADH1 or a TDH3 promoter were generated by fusing the respective promoters (800 base pairs upstream of the start codon) to the corresponding open reading frame. The resultant PCR products were subsequently cloned into the pRS416 or pFA6a vector. All point mutations were generated by site-directed mutagenesis using either the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) or the PFU Ultra II kit (Agilent).

2.2.2 Yeast cell culture, cell synchronization and genotoxin treatment

Yeast cells were grown in yeast peptone dextrose (YPD) or drop-out medium at 30 °C. Log phase cultures (optical density at 600 nm $\approx$ 0.3) were subjected to α-factor (0.5 μg/ml) treatment for G1 arrest. Cells were then washed and resuspended in warm

medium containing the indicated genotoxin.

2.2.3 Immunoprecipitation

For immunoprecipitation (IP), approximately 100 mg frozen cell pellet was lysed by bead beating at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, Complete EDTA-free Protease Inhibitor Cocktail, 5 mM sodium fluoride and 10 mM β-glycerophosphate). After adjusting protein concentrations to about 6 mg/ml, inputs were aliquoted, and lysates were incubated with either anti-HA or anti-Flag agarose resin (Sigma) for 2–3 h at 4 °C. After three washes in lysis buffer, bound proteins were eluted with three resin volumes of SDS elution buffer (100 mM Tris-HCl, pH 8.0, and 1% SDS) for HA IP or of Flag peptide (Sigma) solution (0.5 μg/ml in 100 mM Tris-HCl and 0.2% Tergitol) for Flag IP. SDS loading buffer with DTT was added, and samples were analyzed by western blotting with the indicated antibodies.

2.2.4 Immunoblot

For immunoblot analysis, about 50 mg frozen cell pellet was lysed by bead beating at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% tergitol, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), Complete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche)). SDS loading buffer with 60 mM dithiothreitol (DTT) was added. Samples were separated by standard SDS-polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were detected using the following antibodies: anti-Rad53 (yc-19, 1:10,000, Santa Cruz Biotechnology), anti

H2A^{pS129} (07-0745, 1:10,000, Millipore), anti-HA (12CA5, 1:10,000, Roche) and anti-Flag (M2, 1:5,000, Sigma) antibodies.

2.2.5 Pull-down with recombinant BRCT domain

Protein domains (for Dpb11 BRCT1/2, amino acids 1–270; and BRCT3/4, amino acids 271–582) containing an N-terminal PATH tag (see Yeast strains and plasmids) were expressed in Escherichia coli, bound to human IgG–agarose resin (GE Healthcare) and then used as bait for pull-downs from yeast lysates as previously described (Smolka et al., 2006).

2.2.6 SILAC labelling of yeast

For mass spectrometry experiments, cells were grown in (-) Arg (-) Lys drop-out medium ('light' version complemented with normal arginine and lysine; 'heavy' version complemented with lysine $^{13}C_6$, $^{15}N_2$ and arginine $^{13}C_6$, $^{15}N_4$) for at least five generations.

2.2.7 Purification of phosphopeptides by immobilized metal-ion affinity chromatography (IMAC)

For the PATH-pulldown mass spectrometry analysis, eluted 'heavy' and 'light' proteins from IgG agarose beads bound with purified recombinant BRCT1/2- or C-term-PATH were mixed together, reduced, alkylated and precipitated. Proteins were resuspended in a solution of 2 M urea and 12.5 mM Tris-HCl, pH 8.0, and digested with trypsin for 16 h at 37 °C. Phosphopeptides were enriched using an 'in-house' IMAC

column, then eluted with 10% ammonia and 10% acetonitrile and dried in a SpeedVac evaporator.

2.2.8 Mass spectrometry analysis

IMAC elutions or HILIC fractions were dried in a SpeedVac evaporator, reconstituted in 0.1% trifluoroacetic acid and analysed by LC-MS/MS using a 125 μM ID capillary C18 column and an Orbitrap XL mass spectrometer coupled with an Eksigent nanoflow system. Database searching was performed using the SORCERER system (Sage-N Research) running the program SEQUEST. After searching a target-decoy budding yeast database, results were filtered either based on probability score to achieve a 1% false positive rate or manual inspection. Quantification of heavy/light peptide isotope ratios was performed using the Xpress program as previously described (Smolka et al., 2007).

2.3 Results

2.3.1 Slx4 binding to Dpb11 counteracts Rad53 activation and Dpb11-Rad9 interaction.

While investigating the activation status of the *S. cerevisiae* DDC kinase Rad53 in *slx4Δ* cells and *slx4-7MUT* cells in which the mutated Slx4 is specifically defective in binding to Dpb11, we noted that MMS treatment leads to hyperphosphorylation, and thus hyperactivation, of Rad53 compared with wildtype cells (Fig2.1a), which is consistent with a previous report (Roberts et al., 2006). The activation of Rad53 in response to MMS mostly depends on the checkpoint adaptor Rad9 (Fig2.1a lower

panel), suggesting that Slx4 has a crucial role in counteracting aberrant Rad9-dependent Rad53 activation and, in particular, Slx4-Dpb11 interaction is important for preventing Rad53 hyper-activation (Fig2.1a).

Because Dpb11 binds to Rad9 to positively regulate Rad9-dependent Rad53 activation (Granata et al., 2010; Pfander and Diffley, 2011) and physically interact with Slx4 to prevent aberrant Rad53 hyper-activation, we hypothesized that these two molecular events are mutually exclusive. If this hypothesis is valid, we predicted that upon DNA damage Slx4 sequesters Dpb11 and restricts Rad9 from binding to Dpb11. Indeed, we monitored Dpb11-Rad9 interaction in both wildtype and slx4Δ cells and found that the absence of Slx4 results in a significantly enhanced interaction between Dpb11 and Rad9 upon MMS treatment (Fig2.1b-c), suggesting that Slx4 and Rad9 bind to Dpb11 in a mutually exclusive manner. Taken together, these results support a model whereby Slx4 binds to Dpb11 to counteract Rad53 signaling and Dpb11-Rad9 interaction.

Figure 2.1

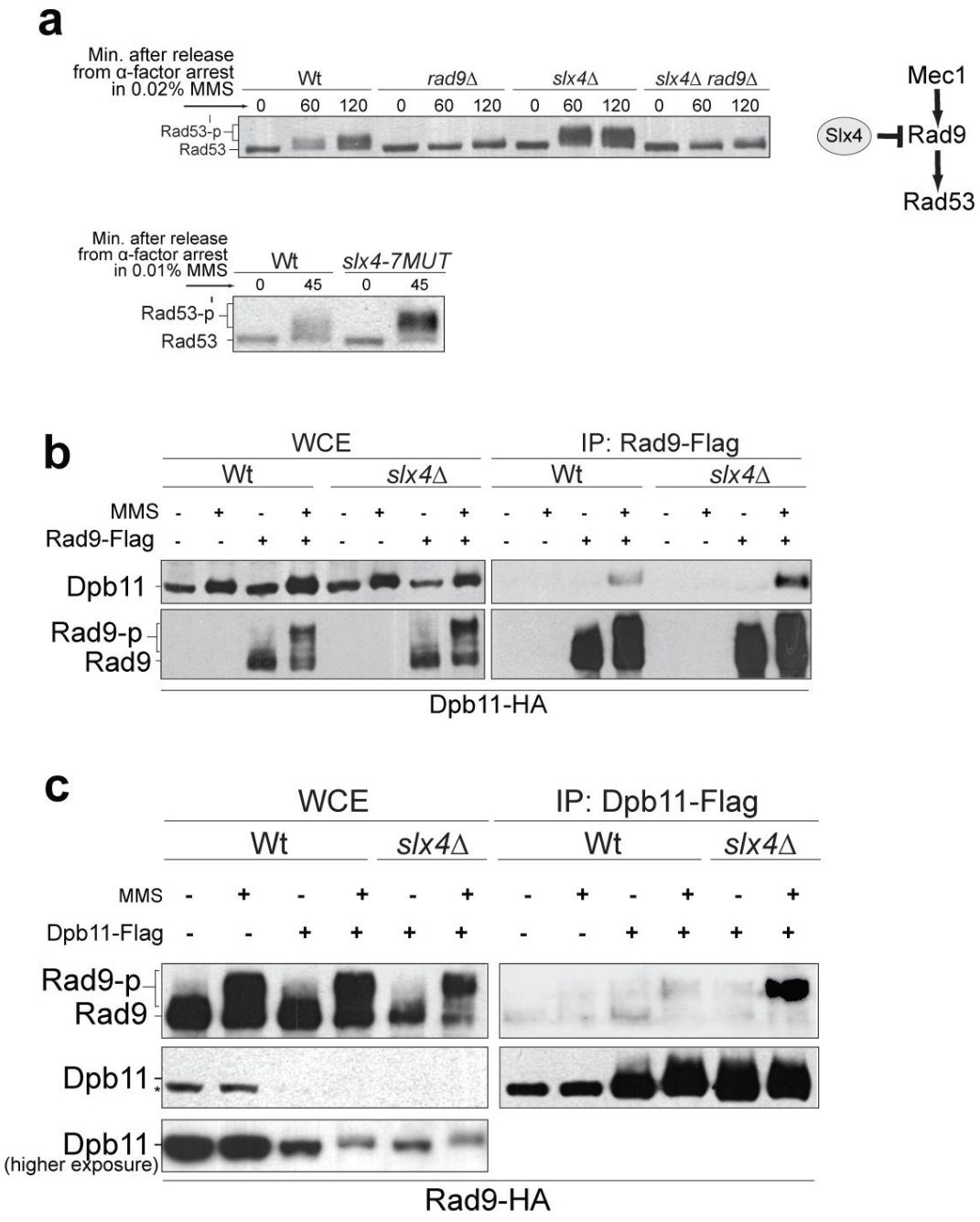
a
Min. after release from α-factor arrest in 0.02% MMS
Wt
rad9Δ
slx4Δ
slx4Δ rad9Δ
0 60 120 0 60 120 0 60 120 0 60 120
Rad53-p
Rad53
Mec1
Slx4
Rad9
Rad53
Min. after release from α-factor arrest in 0.01% MMS
Wt
slx4-7MUT
0 45 0 45
Rad53-p
Rad53
b
WCE
IP: Rad9-Flag
Wt
slx4Δ
Wt
slx4Δ
MMS
Rad9-Flag
Dpb11
Rad9-p
Rad9
Dpb11-HA
c
WCE
IP: Dpb11-Flag
Wt
slx4Δ
Wt
slx4Δ
MMS
Dpb11-Flag
Rad9-p
Rad9
Dpb11
*
Dpb11
(higher exposure)
Rad9-HA

Figure 2.1 Slx4 binding to Dpb11 counteracts Rad53 activation and Dpb11-Rad9 interaction. (a) Western blot showing the phosphorylation status of Rad53–HA in the indicated strains after MMS treatment and a model for the role of Slx4 in antagonizing Rad53 activation by counteracting Rad9. Rad53-p, phosphorylated Rad53; WT, wild type. (b) Co-immunoprecipitation (co-IP) of Dpb11-HA and Rad9-FLAG in wildtype and *slx4Δ* strains. Input levels and phosphorylation status of Rad9 are shown. (c) Reciprocal co-IP between Dpb11-FLAG and HA-Rad9 in wildtype and *slx4Δ* cells. Asterisk indicates a contaminating band that is specifically present in Dpb11 untagged cells.

2.3.2 Slx4 and Rad9 compete for binding to BRCT domains 1/2 in Dpb11.

Dpb11 contains two pairs of BRCT domains and an unstructured C-terminal domain (Fig2.2a). Because BRCT domains are phospho-binding modules, and the interactions of Dpb11 with Slx4 and Rad9 have been shown to be regulated by Mec1 and CDK respectively, we predicted that both of the interactions are mediated by BRCT domains. In order to find out which pairs of BRCT domains in Dpb11 are responsible for its binding to Slx4 and Rad9, we performed mass spectrometry analysis over the PATH-pulldown sample of BRCT1/2 and C-terminal domain of Dpb11 (Fig2.2b). Interestingly, we found that several phospho-peptides originated from DDR or stress response factors such as Xrs2, Mre11, Rck2, Rad9 and Slx4, specifically interact with recombinantly expressed BRCT1/2 (Fig2.2c). Of interest, both phosphorylated Rad9 and Slx4 from MMS-treated yeast cells appear to be recognized by BRCT1/2 in Dpb11 (Fig2.2c). This mass spectrometry result is further confirmed by western blotting analysis (Fig2.2d), suggesting that Slx4 and Rad9 compete for binding to Dpb11 at BRCT domains 1/2, and their interactions with Dpb11 are mutually exclusive.

Figure 2.2

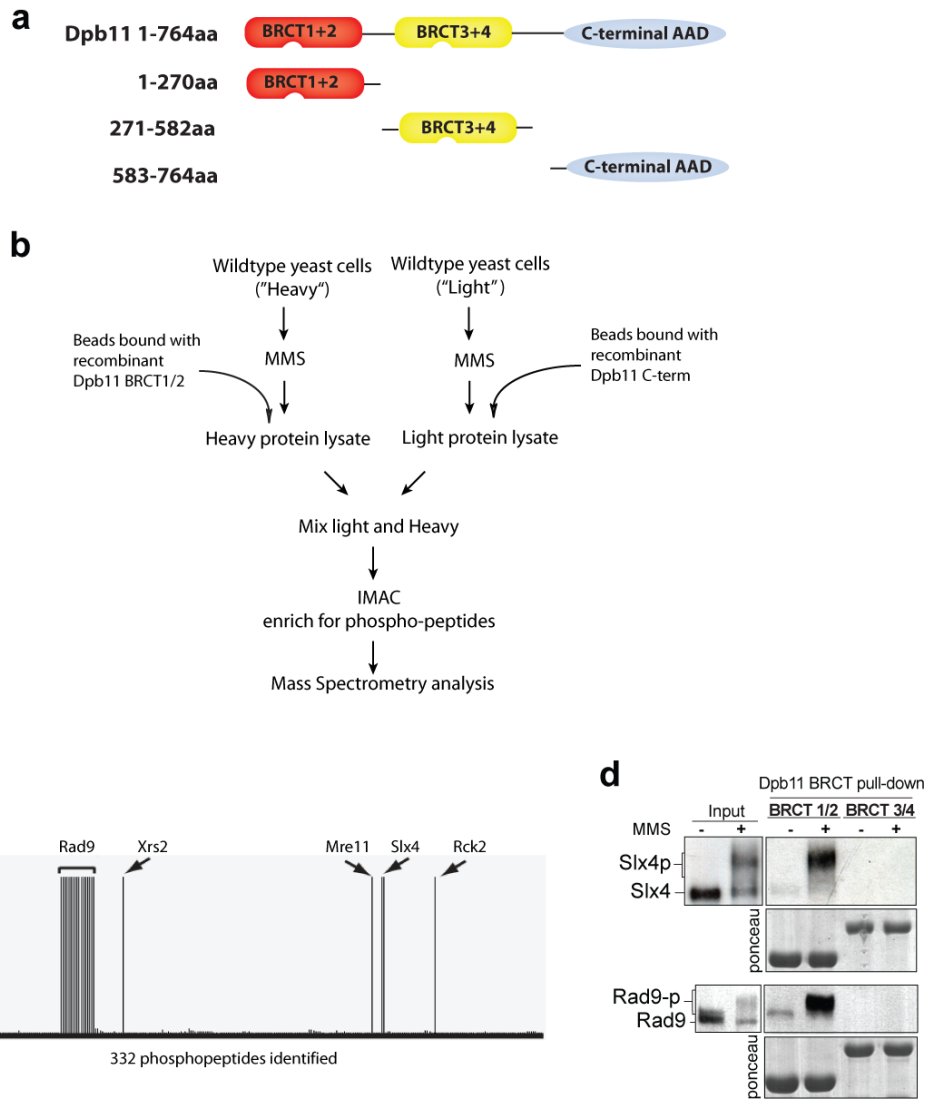
a
Dpb11 1-764aa
BRCT1+2
BRCT3+4
C-terminal AAD
1-270aa
BRCT1+2
271-582aa
BRCT3+4
583-764aa
C-terminal AAD
b
Wildtype yeast cells ("Heavy")
Wildtype yeast cells ("Light")
Beads bound with recombinant Dpb11 BRCT1/2
MMS
MMS
Beads bound with recombinant Dpb11 C-term
Heavy protein lysate
Light protein lysate
Mix light and Heavy
IMAC
enrich for phospho-peptides
Mass Spectrometry analysis
c
Rad9
Xrs2
Mre11
Slx4
Rck2
>100
Ratio B1/2 IP/C-term IP
1
332 phosphopeptides identified
d
Dpb11 BRCT pull-down
Input
BRCT 1/2
BRCT 3/4
MMS
- + - + - +
Slx4p
Slx4
ponceau
Rad9-p
Rad9
ponceau

Figure 2.2 Slx4 and Rad9 compete for binding to BRCT domains 1/2. (a) Schematic illustration of Dpb11 domain architecture. BRCT1/2 and C-terminus truncations are used in the following pull-down experiment. (b) A flow chart of the procedures for PATH pull-down-mass spectrometry experiment. (c) Mass spectrometry analysis result from the pull-down of the recombinant BRCT1/2 and C-terminal of Dpb11. A total of 322 phospho-peptides were identified. Rad9, Slx4, Mre11, Xrs2 and Rck2 show high affinity specifically for recombinant BRCT1/2. (d) Pull-down of Slx4–Flag or Rad9–HA from *S. cerevisiae* lysates using recombinant BRCT1/2 and BRCT3/4 from Dpb11; ponceau staining of the membrane is also shown.

2.3.3 A proline-directed phosphorylation site S486 in Slx4 is crucial for its interaction with Dpb11.

A Slx4 phospho-peptide containing proline-directed phosphorylation site at serine 486 (S486) is consistently identified in our previous PATH-pulldown mass spectrometry analysis, suggesting that S486 could be a potential target recognized by BRCT1/2. Since interactions of Dpb11 with both Sld3 and Rad9 have also been shown to be mediated by cyclin-dependent kinase (CDK)-dependent phosphorylation (S/TP) to either initiate DNA replication (Tanaka et al., 2007; Zegerman and Diffley, 2007) or promote DDC signaling (Granata et al., 2010; Pfander and Diffley, 2011), we predicted that phosphorylation at S486 in Slx4 is crucial for its binding to Dpb11. Further supporting our prediction, we found that the region in Slx4 flanking S486 site highly resembles the Dpb11-binding region in Sld3 and Rad9 through manual alignment (Fig2.3a). In particular, two canonical CDK-targeted phosphorylation sites within the region of 465-488aa in Slx4 align well with S600 and S622 in Sld3, which are known to be important for the Dpb11–Sld3 interaction (Tanaka et al., 2007; Zegerman and Diffley, 2007). Indeed, we showed that mutation of S486 to alanine in Slx4 results in abrogated interaction with Dpb11 and hyper-activation of Rad53 upon MMS treatment (Fig 2.3b, d). Therefore, these results together suggest that the Slx4–Dpb11 interaction is mediated by the coordinated action of both Mec1 and a proline-directed kinase, likely CDK. Furthermore, consistent with the competition model, we detected hyper-phosphorylated Rad9 in a Dpb11 pull-down of cells expressing Slx4 from its endogenous promoter, but we did not detect Rad9 in a Dpb11 pull-down using cells that overexpressed Slx4 (Fig2.3c). This effect was specific for S486. These findings together

support our model that Slx4 counteracts DDC signaling by binding to Dpb11 and preventing its stable interaction with Rad9 (Fig2.3e).

Figure 2.3

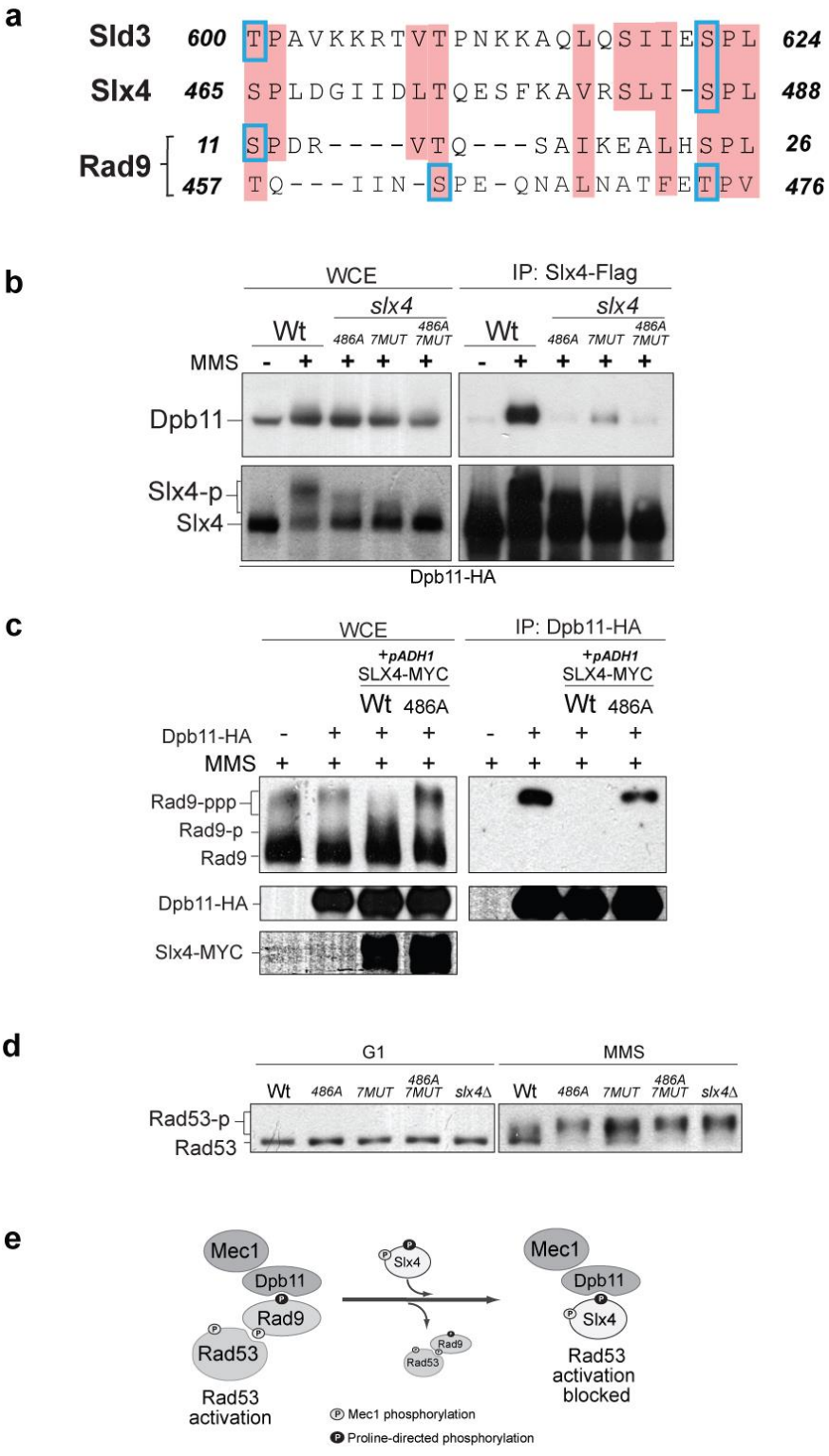
a
Sld3 600 TPAVKKRTVTPNKKAQLQSIIESPL 624
Slx4 465 SPLDGIIDLTQESFKAVRSLI-SPL 488
Rad9 11 SPDR----VTQ---SAIKEALHSPL 26
Rad9 457 TQ---IIN-SPE-QNALNATFETPV 476
b
WCE
IP: Slx4-Flag
slx4
Wt
486A
7MUT
486A 7MUT
MMS
Dpb11
Slx4-p
Slx4
Dpb11-HA
c
WCE
IP: Dpb11-HA
+pADH1 SLX4-MYC
Wt 486A
Dpb11-HA
MMS
Rad9-ppp
Rad9-p
Rad9
Dpb11-HA
Slx4-MYC
d
G1
MMS
Wt 486A 7MUT 486A 7MUT slx4Δ
Rad53-p
Rad53
e
Mec1
Dpb11
Rad9
Rad53
Rad53 activation
Slx4
Rad53 activation blocked
Mec1 phosphorylation
Proline-directed phosphorylation

Figure 2.3 A proline-directed phosphorylation site S486 in Slx4 is crucial for its interaction with Dpb11. (a) Manual alignment of proline-directed sites in Slx4, Rad9 and Sld3 important for binding to Dpb11. Blue boxes indicate the residues that have been shown to be important for Dpb11 interaction with Sld3 (Tanaka et al., 2007; Zegerman and Diffley, 2007). Rad9 (Granata et al., 2010; Pfander and Diffley, 2011) and Slx4 (this study). Red shade indicates sequence similarities. (b) Interaction of the indicated Flag-tagged Slx4 mutants with Dpb11–HA; input levels and phosphorylation status of Slx4 are shown. (c) Co-IP of Dpb11 and Rad9 in cells overexpressing Slx4. (d) Western blot showing the phosphorylation status of Rad53. (e) A Model for the mechanism by which Slx4 counteracts Rad53 activation. G1, G1 cell-cycle phase; Rad9-p, hypophosphorylated Rad9; Rad9-ppp, hyperphosphorylated Rad9; WCE, whole cell extract.

2.3.4 A working model

Taken together, in the integrated model shown in Fig2.4, we propose that Dpb11 plays an antagonistic role in fine-tuning the checkpoint by coordinating the competition between Rad9 and the Slx4-Rtt107 complex. Upon replication fork stalling or DSB resection, Mec1, with its cofactor Ddc2, is recruited to RPA-coated ssDNA and independently DNA damage sensor 9-1-1 complex is loaded onto 5' ss-dsDNA junction to initiate Mec1 activation. Next Ddc1, a subunit of 9-1-1 complex, recruits Dpb11 to the lesion site to fully activate Mec1. Once Dpb11 anchors to the lesion site, Dpb11 mediates Rad9 recruitment to promote DDC signaling via a CDK phosphorylation-dependent interaction. Meanwhile Dpb11 also forms a tight complex with Slx4 scaffold upon Mec1 activation and this Mec1-induced interaction can outcompete Rad9 from the sites of lesion, thereby preventing Rad53 hyper-activation. In addition, Rad9 is also counteracted by Rtt107 as both Rad9 and Rtt107 compete for binding to $H2A^{pS129}$. Overall our work points to a model whereby Dpb11 plays a key role in fine-tuning DDC signaling by controlling the disengagement of Rad9 from the lesion sites and maintaining genomic integrity.

Figure 2.4

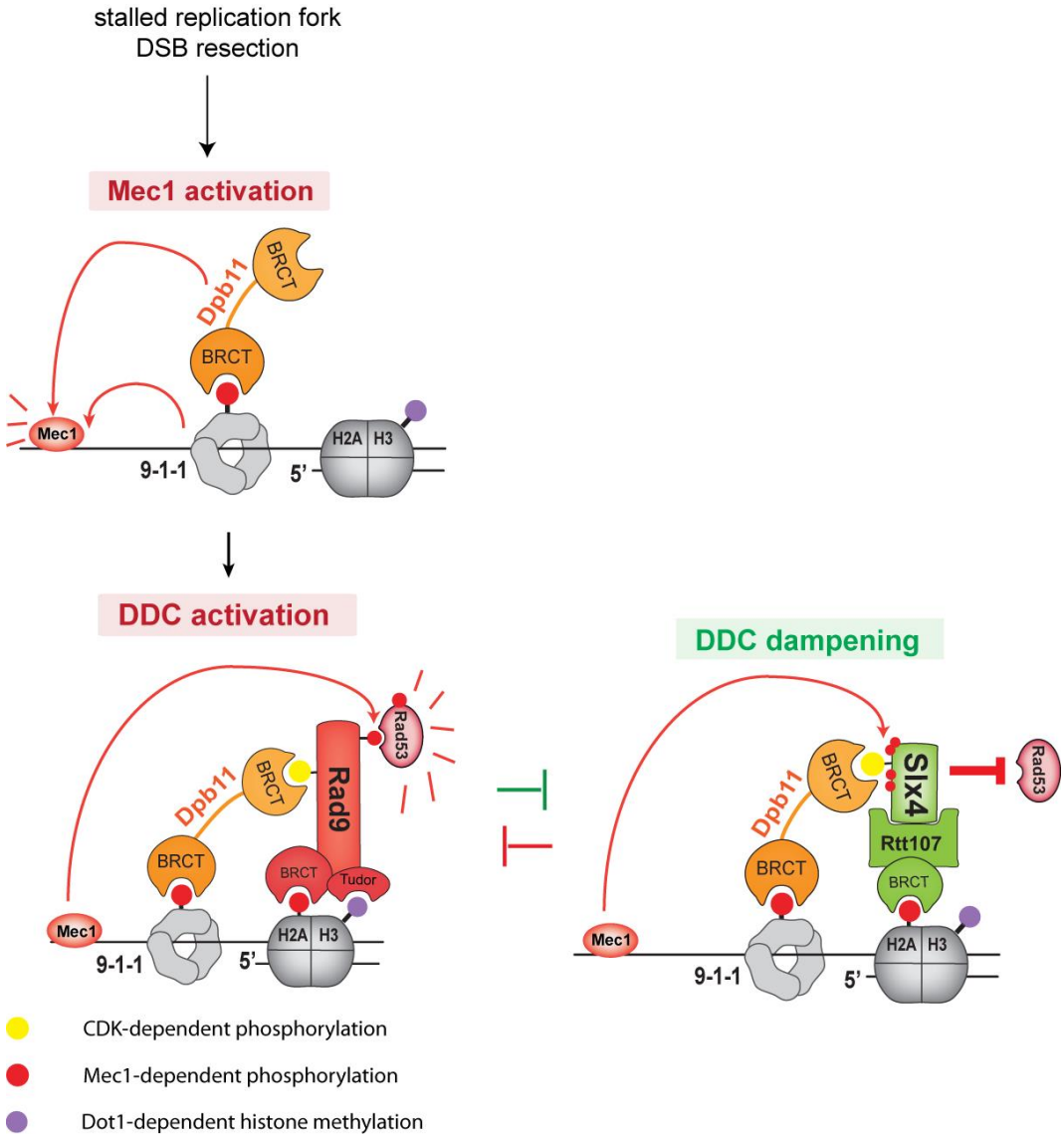

Figure 2.4 A model for the role of Dpb11 in DDC signaling modulation via a competition-based mechanism.

2.4 Discussion

One crucial outcome of DDC signaling is cell cycle arrest, which puts a 'pause' on DNA synthesis and mitotic division before DNA lesions are properly removed. Therefore mechanisms that down-regulate DDC signaling must exist to allow replication forks to restart and the cell cycle to resume after the completion of DNA repair. Failure to timely terminate DDC signaling prevents the cells from re-entering the cell cycle and suppresses cell growth (Clerici et al., 2001; Heideker et al., 2007). Because DDC signaling consists of a conserved kinase cascade and is mainly coordinated by the action of an upstream kinase Mec1, which can then phosphorylate downstream kinase Rad53 and many other factors, a phosphatase-mediated mechanism involving Pph3, Ptc2 and Ptc3 has been relatively well-characterized for fine-tuning the levels of downstream Rad53 activation. Surprisingly, our work here has revealed a distinct mechanism, named DAMP (dampens adaptor-mediated phospho-signaling) that achieves the down-regulation of Rad53 activity independent of phosphatase actions. We show that DNA repair scaffolds Slx4-Rtt107 prevent aberrant Rad53 hyper-activation via a competition-based mechanism by counteracting the checkpoint adaptor protein Rad9. Our results suggest that there are two critical points of regulation that Slx4-Rtt107 scaffolds act to dampen Rad53 signaling in this competition model, with the first one being Dpb11 and the second being phosphorylated histone H2A ($H2A^{pS129}$). On the one hand, both Slx4 and Rad9 interact with Dpb11 in a mutually exclusive manner by competing for binding to the same N-terminal BRCT domains in Dpb11. This competition is further evidenced by the increased Rad9-Dpb11 interaction in the absence of Slx4. On the other hand, Rtt107, anchored to the DNA lesions via its physical

interaction with the phosphorylated histone H2A, outcompetes Rad9 from the lesion sites. Our work provides a mechanistic understanding of how checkpoint signaling is tightly coordinated with and monitored by DNA repair to ensure genomic integrity.

The follow-up work published by Cussiol et al. (Cussiol et al., 2015) further dissected the molecular mechanism of the DAMP function for the Slx4-Rtt107 repair scaffolds. It was found that the multi-BRCT domain protein Dpb11, which establishes the competition between Slx4 and Rad9 in the DAMP model, appears to be a key player in the coordination of DDC signaling and DNA repair via BRCT domain-mediated recognition of distinct phospho-proteins and therefore the assembly of different multi-subunit complexes. While it is well-established that Dpb11 couples Sld3-Cdc45-MCM helicase with Sld2-polymerase ε complex to initiate DNA replication, Cussiol et al shows that Dpb11 fine-tunes DDC signaling by physically bridging between Slx4-Rtt107 and Ddc1, a subunit of the 9-1-1 complex to downregulate Rad53 activation, and bridging between Rad9 and Ddc1 to promote Rad53 activation. It was also proposed that Dpb11 ties Slx4-Rtt107 with nuclease complex Mus81-Mms4 to facilitate the resolution of joint molecules such as DNA repair intermediates, suggesting that DNA repair is tightly controlled by DDC signaling. Particularly, Cussiol et al engineered minimal Multi-BRCT-domain module (MBD) that dampens checkpoint and functionally mimics the action of Dpb11-Slx4-Rtt107 complex. To generalize the mode of action for Dpb11, Dpb11 executes its scaffolding function by anchoring to the lesion sites via a BRCT-mediated phosphorylation-dependent interaction and simultaneously tethering to another phosphorylated effector protein through a distinct pair of BRCT domains, in a way that it stabilizes the recruitment of its binding partners to promote

their engagement at the DNA lesions. Collectively we propose that Dpb11 acts as a scaffold to mediate the assembly of multiple ternary complexes in order to achieve spatio-temporal regulation of DDR events.

Despite their important functions in DDC signaling, both Slx4-Rtt107 scaffolds and Rad9 are also involved in DNA repair. Interestingly, while Rad9 has long been shown to block resection at the lesions (Clerici et al., 2014; Ferrari et al., 2015; Lazzaro et al., 2008), recent work suggests that Slx4-Rtt107 function as a positive regulator for DNA resection (Dibitetto et al., 2016). Because Slx4-Rtt107 scaffolds and Rad9 also play opposing roles in the modulation of DDC signaling, it is tempting to speculate that the pro-resection function of Slx4-Rtt107 and anti-resection role of Rad9 are similarly regulated by the Dpb11-mediated competition mechanism. This led us to investigate the competition model in the context of DNA end resection in Chapter 3.

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CHAPTER 3

TOPBP1/Dpb11 Controls DNA Repair

Through the Coordinated Recruitment of 53BP1/Rad9

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(Jose Cussiol and Diego Dibitetto performed the experiments in Fig3.1 and Fig3.2 and Jennie Sims and Yi Liu performed metaphase spread analysis in Fig3.6)

Abstract

Genome maintenance and cancer suppression require homologous recombination (HR) DNA repair. In yeast and mammals, the protein scaffold TOPBP1^{Dpb11} has been implicated in HR, although its precise function and mechanism of action remain elusive. Here we show that yeast Dpb11 controls DNA repair through mutually antagonistic functions. BRCT domains of Dpb11 mediate opposing roles in the control of DNA end resection by coordinating both stabilization and exclusion of Rad9 from DNA lesions. In human cells, we find that TOPBP1 engages in interactions with the anti-resection factor 53BP1 and the pro-HR factor BRCA1, suggesting that TOPBP1 also mediates opposing functions in HR control. Consistent with a pro-NHEJ function for TOPBP1, hyper-stabilization of the 53BP1-TOPBP1 interaction enhances the recruitment of 53BP1 and NHEJ factors to nuclear foci in S-phase and induces chromosomal aberrations. Our results support a model in which TOPBP1^{Dpb11} is a regulator of repair pathway choice.

3.1 Introduction

The proper repair of double-strand breaks (DSBs) occurring during DNA replication is heavily dependent on error-free homologous recombination (HR) (Heyer, 2015; Schwartz and Heyer, 2011). However, DSBs may also be repaired by the joining and direct ligation of DNA ends through non-homologous end joining (NHEJ). Because of the risk of ligating wrong ends and/or deleting DNA sequences, NHEJ is considered an error-prone repair mechanism. During DNA replication, NHEJ repair is particularly deleterious due to the intrinsic increased incidence of breaks, especially one ended DSBs, whose inappropriate joining leads to dicentric chromosomes that initiate break-fusion cycles and complex chromosome rearrangements (Gaillard et al., 2015; Gelot et al., 2015). In fact, NHEJ-mediated mutagenic repair is a major contributor to genomic instabilities and tumorigenesis that arise when the HR machinery is deficient (Deng and Wang, 2003; Prakash et al., 2015). During DNA replication, the ability of cells to inhibit NHEJ and favor HR is therefore essential for genome integrity.

A critical step in regulating the choice of HR or NHEJ for repair is the control of 5'-to-3' nucleolytic processing of DNA ends (also referred as resection), as formation of long 3' ssDNA tails naturally promotes HR while preventing NHEJ (for review see (Chapman et al., 2012b; Prakash et al., 2015)). 53BP1 is a scaffolding protein that plays a major role in limiting resection (Bothmer et al., 2010; Bunting et al., 2010). While the mechanism by which 53BP1 limits resection remains incompletely understood, it involves 53BP1-dependent recruitment of the additional anti-resection factors such as RIF1 and PTIP (Callen et al., 2013; Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Kumar and Cheok, 2014; Zimmermann et al., 2013). In S-phase, the tumor suppressor BRCA1 is proposed to play a key role in pro-HR by

counteracting the recruitment of 53BP1 to DSBs, therefore enabling resection (Bunting et al., 2010). This model is supported by the genetic data in mice showing that the loss of 53BP1 suppresses embryonic lethality, genomic rearrangements and tumorigenesis seen in mice lacking functional BRCA1 (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009; Prakash et al., 2015). DNA end resection is inhibited during S-phase in cells lacking BRCA1, and the increased recruitment of 53BP1 to replication-induced lesions results in mutagenic NHEJ and increased chromosomal aberrations (Bunting et al., 2010; Escribano-Diaz et al., 2013). Collectively, these observations support a model for repair pathway choice in which BRCA1 and 53BP1 compete for the sites of DNA lesions to promote HR or NHEJ. However, there is a fundamental gap of knowledge concerning how BRCA1 counteracts the engagement of 53BP1 in S-phase at the lesions to avoid genomic instability and cancer.

While many aspects of the mammalian DNA repair are conserved in budding yeast, it remains unknown whether key mechanisms of DNA repair pathway choice are also conserved. Notably, a clear sequence homolog or a functional analog of BRCA1 has not been identified in fungi. On the other hand, the 53BP1 ortholog Rad9 has been shown to play a conserved role in resection block (Clerici et al., 2014; Ferrari et al., 2015; Lazzaro et al., 2008). Cells lacking *RAD9* resect DSBs faster and more extensively (Chen et al., 2012; Clerici et al., 2014; Lazzaro et al., 2008). Of importance, it was recently proposed that a complex formed by the DNA repair scaffolds Slx4 and Rtt107 is able to counteract the engagement of Rad9 at replication-induced lesions to dampen checkpoint signaling (Ohouo et al., 2013). Given the roles of Rad9 in blocking resection, it was predicted that the ability of Slx4-Rtt107 to counteract Rad9 recruitment

to DNA lesions would help avert the block, therefore promoting resection. Indeed, recent work presented experimental evidence that the Slx4-Rtt107 complex favors resection of DSBs (Dibitetto et al., 2016).

TOPBP1 (Dpb11 in yeast) is an essential scaffolding protein that plays evolutionarily conserved roles in the initiation of DNA replication and activation of DNA damage checkpoint signaling (Boos et al., 2011; Navadgi-Patil and Burgers, 2008; Pfander and Diffley, 2011; Puddu et al., 2008; Tanaka et al., 2007; Zegerman and Diffley, 2007). TOPBP1^{Dpb11} is comprised of multiple BRCT (BRCA1 C-terminus) domains (9 in humans and 4 in yeast), which are protein interacting modules that often recognize phosphorylated motifs (Manke et al., 2003; Rodriguez et al., 2003; Yu et al., 2003). TOPBP1^{Dpb11} recognizes phospho-proteins via its BRCT domains and have been shown to assemble them into distinct multi-subunit complexes required for replication initiation or checkpoint activation (Boos et al., 2011; Pfander and Diffley, 2011; Tak et al., 2006; Zegerman and Diffley, 2007). Although TOPBP1 has been implicated in HR DNA repair (Germann et al., 2011; Liu and Smolka, 2016; Morishima et al., 2007; Moudry et al., 2016), its precise role and mode of action remain largely elusive. Here we show that in budding yeast, Dpb11 plays a decisive role in the control of DNA end resection, the first key step in HR, by establishing a competition between the anti-resection protein Rad9 and the pro-resection scaffolds Slx4-Rtt107 for DNA lesions. In humans, we find that TOPBP1 coordinates the recruitment of 53BP1 via a physical interaction that appears to be mutually exclusive with that of the pro-HR factor BRCA1. Our results support a model in which TOPBP1^{Dpb11} controls the mutually exclusive

engagement of antagonistic regulators of HR-mediated DNA repair for the proper control of repair pathway choice and genome maintenance.

3.2 Material and methods

3.2.1 Yeast strains and plasmids

Strains generated in this study were derived from MBS164 or MBS1050 (both congenic to S288C) or JKM179 or YMV80 (where indicated). All proteins were tagged at the C terminus and the expression was verified by western blotting. Tagged strains were assayed for sensitivity to MMS to ensure they behaved similarly to the wild-type strain. Standard cloning methods were used to generate the plasmids for this study. B3/4-Rad9 chimera was generated using a stitch PCR protocol. Briefly, we fused the Rad9 promoter (450 base pairs upstream of the start codon) to the Dpb11 BRCT3/4 (292-600aa). The resulting PCR product was subjected to another round of stitch PCR with the Rad9-3xFlag sequence (see Fig3.1b for the schematic illustration of the chimeric protein). The final PCR product was subsequently cloned into pRS416 (for ectopic expression) or pFa6a (for integration at the *RAD9* genomic locus). All point mutations were generated by site-directed mutagenesis using the Primestar® Max DNA Polymerase (Takara). All yeast strains and plasmids used in this study are described in Supplementary Tables S1 and S2, respectively.

3.2.2 Yeast cell culture and immunoprecipitation procedures

Yeast cells expressing genes with the indicated epitope tags were cultured in YPD or in synthetic complete medium lacking uracil (SC-URA) when carrying an

expression plasmid with *URA3* selection marker. Cells were grown to log phase, subjected to MMS treatment as specified in the figures and collected by centrifugation. For each immunoprecipitation experiment, about 0.1 g of the cell pellets were lysed by bead beating at 4°C in 1ml of the lysis buffer (50 mM Tris-HCl pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche), 5 mM sodium fluoride and 10 mM β-glycerol-phosphate. The lysate was cleared by centrifugation and then incubated with anti-HA or anti-FLAG agarose resin (Sigma-Aldrich) for 2hrs at 4°C. The immunoprecipitated proteins were then washed with the above lysis buffer for three times and then eluted with 3 resin volumes of the elution buffer (0.5 μg/mL of FLAG peptide in 100 mM Tris-HCl for anti-FLAG resin; 0.2% Tergitol and 100 mM Tris-HCl pH 8.0, 1% SDS for anti-HA resin).

3.2.3 Measurement of resection at HO-induced DSB

HO-induced DSB resection was measured in JKM139 background by quantitative PCR analysis as described previously (Ferrari et al., 2015). Cells were arrested in G2/M by nocodazole treatment before HO induction. Genomic DNA was extracted and digested or mock-treated with RsaI restriction enzyme (NEB), which cuts inside the amplicons at 0.15 kb, 1.4 kb and 4.8 kb from the HO-cut site, but not in the *PRE1* control region on chromosome V. PCR values are then normalized by the cut efficiency calculated by southern blot analysis.

3.2.4 Mammalian cell culture and immunoprecipitation procedures

Human U2OS and HEK293T cell lines were grown in DMEM supplemented with 10% BCS, non-essential amino acid and penicillin/streptomycin (Corning). Microscopy and immunoprecipitation experiments were performed 48 hours post-transfection followed by the according drug treatment. HEK293T Cells were subjected to either 24 hrs of HU (1 mM) or 14hrs of nocodazole (100 ng/mL) post-transfection before harvesting. For the ATR inhibition experiment, cells were pretreated with 10μM ATR inhibitor (VE821) for 45min before the additional 2.5 mM HU treatment for another 30min. Cell pellets were lysed for 30min on ice in modified RIPA buffer (50 mM Tris-HCl PH 7.5, 150 mM NaCl, 1% tergitol, 0.25% sodioum deoxycholate, 5mM EDTA) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche), 5 mM sodium fluoride, 10 mM β-glycerol-phosphate, 1 mM PMSF and 0.4 mM sodium orthovanadate. Proteins lysate was cleared by 10 min centrifugation to pellet cell debris and then incubated with anti-TOPBP1 resin, anti-HA or FLAG agarose resin (Sigma-Aldrich) as specified for 4 hrs at 4°C. Immunoprecipitates were then washed three times with the modified RIPA buffer and then eluted using 3 resin volumes of the elution buffer (0.5 μg/mL of FLAG peptide in 100 mM Tris-HCl for anti-FLAG resin; 0.2% tergitol and 100 mM Tris-HCl pH 8.0, 1% SDS for others).

3.2.5 Immunoblotting analysis

For both yeast and mammalian immunoprecipitation experiments, whole cell lysates and eluents were denatured with 3x SDS sample buffer (composed of bromophenol blue, stacking gel buffer, 50% glycerol, 3% SDS and 60 mM DTT) and resolved on SDS-PAGE gels. Proteins were then transferred onto polyvinylidene

fluoride (PVDF) membranes and probed with desired antibodies.

3.2.6 Mass spectrometry analysis

For mammalian SILAC experiments, HEK293T cells were grown in SILAC DMEM media lacking arginine and lysine (ThermoFisher Scientific 88425) supplemented with 10% dialyzed FBS and penicillin/streptomycin. "Light" DMEM media were supplemented with "light" arginine and lysine; ''heavy'' DMEM media were supplemented with "heavy" lysine $^{13}C6$, $^{15}N2$ and "heavy" arginine $^{13}C6$, $^{15}N4$. Cells were treated with 1mM HU for 24hrs or 100ng/ml nocodazole for 14hrs accordingly before harvesting. TOPBP1 was immunoprecipitated using affinity-purified TOPBP1 antibodies (kindly provided by Dr. Raimundo Freire) or antibodies that recognize the according epitope tags. Immunoprecipitated proteins were then reduced, alkylated, precipitated and digested by trypsin. The peptides were then desalted, dried, reconstituted in 80% acetonitrile and 1% formic acid and then fractionated by Hydrophilic Interaction Chromatography (HILIC). Fractions were dried, reconstituted in 0.1% trifluoroacetic acid, and analyzed by LC-MS/MS using an Orbitrap XL mass spectrometer as described (Ohouo et al., 2010). Database search and quantitation of heavy/light peptide isotope ratios were performed as described (Smolka et al., 2007).

3.2.7 Chemicals

PARP inhibitor (AZD2461) and ATR inhibitor (VE821) were purchased from Selleckchem. Nocodazole was purchased from Calbiochem. Hydroxyurea and MMS

was purchased from Acros organics.

3.2.8 Antibodies

Rad53 and yeast epitope tagged proteins were probed using specific antibodies: anti-Rad53 antibody (clone Mab EL7, 1:30 dilution); anti-FLAG (M2 F1804; Sigma, 1:5000 dilution), anti-HA (12CA5; Roche, 1:10000 dilution), ECL HRP-linked secondary antibody (NA931-GE, 1:10000 dilution). The following antibodies were used in the mammalian part: anti-FLAG (M2, F1804; Sigma), anti-HA.11 (MMS-101P; Covance), 53BP1 (NB100-304; Novus Biologicals), RIF1 (sc-55979; Santa Cruz), phospho-KAP-1(S824) (A300-767A-T; Bethyl), phospho-CHK1 (Ser345) (#2341; Cell Signaling), BRCA1 (#OP92, MS110; Calbiochem). TOPBP1 and BRCA1 antibodies were provided by Dr. Raimundo Freire. The antibody against PTIP was provided by Dr. Kai Ge.

3.2.9 Immunofluorescence

U2OS cells grown on glass coverslips, after subjected to the desired drug treatment, were fixed with 3.7% formaldehyde/PBS for 15min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 5min at room temperature, blocked with 5% BSA for 30min at 37°C and incubated with primary antibodies for 1hr at room temperature. This was followed by three times of PBS washes and secondary antibody incubation (alexa fluor 568 donkey anti-mouse, 647 donkey anti-goat). Next cells were washed with PBS three times and mounted using vectashield antifade mounting medium with DAPI (H1200; Vector Laboratories).

3.2.10 Microscopy analysis

The images were acquired using a CSU-X spinning disc confocal microscope (Intelligent Imaging Innovations) on an inverted microscope (DMI600B; Leica), with 63×, 1.4 NA objective lens (mammalian cells), 100×, 1.46 NA objective lens (yeast cells) and a charge-coupled device camera (cool-SNAP HQ2, Photometrics) for mammalian cells or electron-multiplying charge-coupled device camera (QuantEM; Photometrics) for yeast cells. SlideBook software (Intelligent Imaging Innovations) was used to obtain Z-stack images. Maximum intensity projections were created in Slidebook software and exported for analysis in Image J.

For mammalian RIF1 and 53BP1 foci analysis, more than 150 transfected cells for each condition were imaged and analyzed per replicate. Cells with more than 10 distinct RIF1 foci or 53BP1 variant foci were scored as foci-positive cells. The percentage of RIF1 foci or 53BP1 variant foci-positive cells was calculated. The arithmetic mean and standard error of the mean (SEM) derived from 3 replicates were used for the plot. A two-tailed Student's t-test with 95% confidence interval was used to determine if the difference between the means of the two sets of values was significant.

For yeast RPA and Rad52 foci analysis, cells were grown in SC media until log phase (OD = 0.3) and MMS was added to the cells at a concentration of 0.033% for 2 hrs at 30°C. Next, cells were washed in sterile water and resuspended in fresh SC media. Live yeast cultures were mounted on an agarose slide pad (1.2% agarose in SC–TRP media). More than 150 cells were scored for each replicate. The percentage of cells

with Rad52-mRuby2 or RPA-mRuby2 foci was calculated based on the presence of Rad52 foci and the presence of single RPA focus or multiple RPA foci. The graph is plotted using the mean±SEM from three independent experiments.

3.2.11 Metaphase spread preparation

HEK293T cells were co-transfected with 53BP1 or CTR-53BP1 and H2B-GFP, purchased from Addgene (addgene plasmid #11680), as a marker for successful transfection when capturing metaphase images. Cells grown in DMEM media were treated with colcemid (150ng/ml) for 1hr and collected by trypsination followed by centrifugation. Cell pellets were resuspended in hypotonic buffer (0.034 M KCl) for 6min at 37°C and then fixed in fixation buffer (3:1 of methanol and acetic acid) overnight. Fixed cells were then washed, spotted onto microscope slide, and mounted using vectashield antifade mounting medium with DAPI. Cells in metaphases were then imaged with the CSU-X spinning disc confocal microscope with 100×, 1.46 NA objective. Chromosomal aberrations were then scored. Each condition is repeated at least two times independently and more than 45 metaphases were analyzed per replicate. The two-tailed Student's t-test was used for statistical analysis.

3.3 Results

3.3.1 BRCT domains of Dpb11 mediate mutually antagonistic functions in DNA end resection

In budding yeast, Dpb11 has been shown to recruit Rad9 to the 9-1-1 clamp loaded at DNA lesions to promote activation of the DNA damage checkpoint (Fig3.1a)

(Abreu et al., 2013; Granata et al., 2010; Pfander and Diffley, 2011; Wang et al., 2012). Since Rad9 and its human ortholog 53BP1 have both been shown to block DNA end resection, we hypothesized that the role of Dpb11 in mediating the recruitment of Rad9 to DNA breaks plays a decisive role in resection control and HR-mediated DNA repair. To test this, we fused full-length Rad9 with the BRCT domains 3/4 of Dpb11, which mediates an interaction with the checkpoint clamp 9-1-1 complex (Fig3.1b), with the expectation that this chimera will hyper-stabilize Rad9 at DNA lesions. Using a system to induce a persistent DSB at the MAT locus by over-expression of HO endonuclease (Lee et al., 1998; White and Haber, 1990) we found that the Dpb11$^{BRCT3/4}$-Rad9 chimera (hereinafter referred to as 'B3/4-Rad9') was robustly detected at 0.15kb from the break site using ChIP-qPCR (Fig3.1c). Of importance, a point mutation corresponding to K544A in Dpb11, known to disrupt the ability of BRCT-3/4 to recognize the phosphorylated 9-1-1 clamp, prevented the stabilization of B3/4-Rad9 near the site of DSB (Fig3.1c). Taking advantage of this system, we assessed the effect of Dpb11-mediated Rad9 hyper-stabilization on DNA end resection using an assay to monitor the accumulation of ssDNA flanking an irreparable HO-induced DSB site (Dibitetto et al., 2016; Ferrari et al., 2015). Although we did not observe an impact on resection at 0.15 kb from the break, resection was significantly inhibited at 1.4 kb and severely blocked at 4.8 kb from the break site upon expression of the B3/4-Rad9 chimera (Fig3.1d). The K544A mutation that impairs BRCT-3/4 fully restored resection, arguing for a key role of Dpb11 in bridging Rad9 to the 9-1-1 complex for inhibition of resection.

We have recently proposed a model in which Dpb11 also mediates the controlled disengagement of Rad9 from lesions for dampening checkpoint signaling (Fig3.1e)

(Cussiol et al., 2015; Ohouo et al., 2013). In this model, the Slx4-Rtt107 scaffolding complex competes with Rad9 for Dpb11 interaction, ultimately preventing Rad9 from stabilizing at DNA lesions. We hypothesized that this competition mechanism is also crucial to control the roles of Rad9 in DNA repair and could provide the molecular basis to understand how 53BP1 recruitment is regulated in mammals. We predicted that a fusion of the Slx4-Rtt107 complex with BRCT-3/4 should be able to antagonize the B3/4-Rad9 chimera and restore resection. We have previously shown that a fusion of Dpb11 BRCT3/4 with Rtt107 BRCT-5/6 (referred as MBD: minimal multi-BRCT domain module) (Fig3.1f), functionally mimics the role of the Slx4-Rtt107-Dpb11 complex in checkpoint dampening (Cussiol et al., 2015). Here we found that expression of MBD prevents hyper-stabilization of the B3/4-Rad9 chimera at DSBs (Fig3.1g) and, strikingly, fully suppresses the resection block induced by B3/4-Rad9 (Fig3.1h). Collectively, the above results are consistent with a model in which Dpb11 play mutually antagonistic roles in resection by coordinating the stabilization as well as exclusion of Rad9 from DNA lesions (Figs3.1e and 3.1i).

Figure 3.1

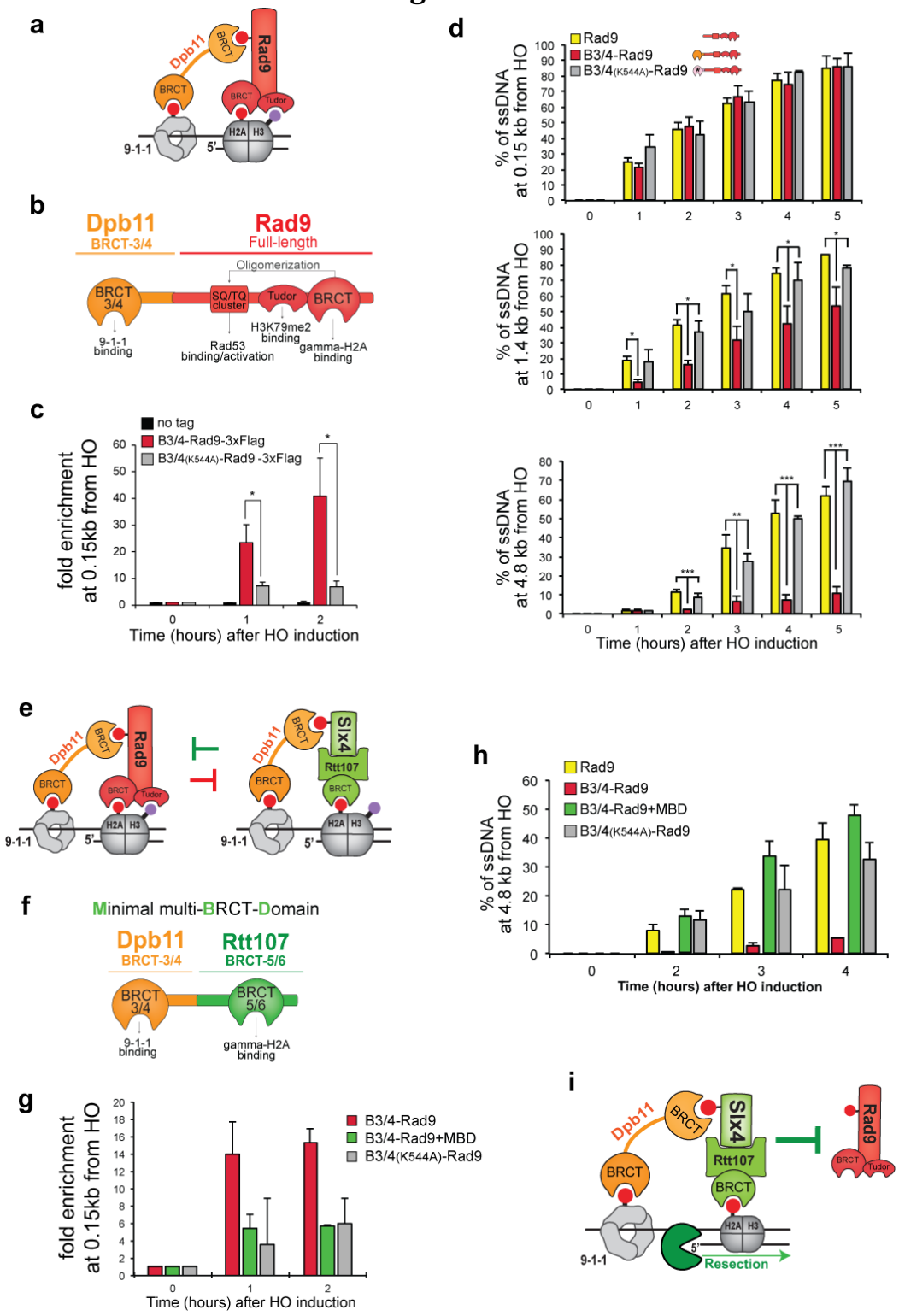

a
Dpb11
BRCT
Rad9
BRCT
BRCT
Tudor
H2A
H3
9-1-1
5'
b
Dpb11
BRCT-3/4
Rad9
Full-length
Oligomerization
BRCT 3/4
SQ/TQ cluster
Tudor
BRCT
9-1-1 binding
Rad53 binding/activation
H3K79me2 binding
gamma-H2A binding
c
no tag
B3/4-Rad9-3xFlag
B3/4(K544A)-Rad9 -3xFlag
fold enrichment at 0.15kb from HO
Time (hours) after HO induction
d
Rad9
B3/4-Rad9
B3/4(K544A)-Rad9
% of ssDNA at 0.15 kb from HO
% of ssDNA at 1.4 kb from HO
% of ssDNA at 4.8 kb from HO
Time (hours) after HO induction
e
Dpb11
BRCT
Rad9
BRCT
BRCT
Tudor
H2A
H3
9-1-1
5'
Slx4
Rtt107
f
Minimal multi-BRCT-Domain
Dpb11
BRCT-3/4
Rtt107
BRCT-5/6
BRCT 3/4
BRCT 5/6
9-1-1 binding
gamma-H2A binding
g
B3/4-Rad9
B3/4-Rad9+MBD
B3/4(K544A)-Rad9
fold enrichment at 0.15kb from HO
Time (hours) after HO induction
h
Rad9
B3/4-Rad9
B3/4-Rad9+MBD
B3/4(K544A)-Rad9
% of ssDNA at 4.8 kb from HO
Time (hours) after HO induction
i
Dpb11
BRCT
Slx4
Rtt107
BRCT
BRCT
Rad9
BRCT
Tudor
H2A
H3
9-1-1
5'
Resection

Figure 3.1 BRCT domains of Dpb11 mediate mutually antagonistic functions in DNA end resection. (a) A model for the recruitment of Rad9 mediated by Dpb11 at the DNA lesions. (b) Schematic illustration of the B3/4-Rad9 (Dpb11$^{BRCT3/4}$-Rad9) chimera. (c) ChIP-qPCR analysis to determine the recruitment of Rad9 chimera variants at 0.15kb from HO-induced DSB site at the indicated time points. Cells expressing the indicated chimera proteins derived from JKM179 strain were arrested by nocodazole and HO endonuclease was then induced to trigger an irreparable DSB. Rad9 chimera variants were then subjected to chromosome immunoprecipitation and qPCR analysis. The graph is plotted using mean±SEM from at least two independent experiments. P value is determined based on single-tailed Student's t test (*for P<0.05, ** for P<0.01 and *** for P<0.001). (d) DSB resection analysis by qPCR to determine the effect of Rad9, B3/4-Rad9 or B3/4(K544A)-Rad9 chimera expression on the resection efficiency flanking the HO-induced cut. The graph is plotted using mean ± SEM from at least two independent experiments and P value is determined based on single-tailed Student's t test. (e) A working model for the role of Dpb11-Slx4-Rtt107 complex in antagonizing Rad9 recruitment at the lesions sites for the modulation of checkpoint signaling. (f) Schematic illustration of the MBD (Minimal multi-BRCT-domain module) chimera. (g) ChIP-qPCR analysis to show the effect of MBD expression on the recruitment of B3/4-Rad9 at 0.15kb from the HO-induced DSB site at indicated time points. For this experiment, B3/4-Rad9 and MBD chimeric proteins were integrated into the Rad9 and Slx4 locus respectively. (h) DSB resection analysis by qPCR to determine the effect of MBD expression on the resection block imposed by B3/4-Rad9 chimera at the indicated time points after HO induction. (i) A model for the antagonistic roles of Dpb11 in

resection control by coordinating the competition between Slx4 and Rad9 at the DSBs.

3.3.2 Dpb11-mediated recruitment of Rad9 impairs HR-mediated repair in response to replication stress

Slx4 and Rtt107 have been shown to be particularly important in the response to MMS-induced replication stress (Ohouo et al., 2010). We therefore asked whether the B3/4-Rad9 chimera also impairs the control of resection and HR-mediated repair in cells treated with MMS, a DNA alkylating agent that blocks replication fork progression. Expression of B3/4-Rad9 chimera results in strong MMS sensitivity, which is suppressed by the corresponding K544A mutation in B3/4 (Fig3.2a). While MMS treatment leads to the formation of multiple RPA foci in cells expressing the mutated BRCT-3/4(K544A)-Rad9 chimera, expression of the chimera B3/4-Rad9 bearing functional BRCT-3/4 prevented most cells from accumulating multiple RPA foci (Fig3.2b), consistent with the inhibition of DNA end resection. This defect in RPA foci formation is accompanied by a severe reduction in the formation of the Rad52 foci (Fig3.2c), pointing to an impairment of HR-mediated repair. These results show that Dpb11-mediated recruitment of Rad9 also plays an important role in coordinating DNA end resection and HR repair in the response to replication blocks. Because the resection block imposed by B3/4-Rad9 at DSB is counteracted by the Slx4-Rtt107-Dpb11-mimicking module MBD as is shown in Fig3.1, we speculated that the impaired HR-mediated repair induced by B3/4-Rad9 at replication-induced lesions could also be rescued by the co-expression of MBD. Indeed, MBD was sufficient to rescue B3/4-Rad9-induced MMS sensitivity (Fig3.2d). Expression of B3/4-Rad9 led to hyper-activation of Rad53 in cells treated with MMS as evaluated by the mobility shift of Rad53, consistent with the Dpb11-mediated function of Rad9 in promoting checkpoint

signaling (Fig3.2e). This aberrant Rad53 hyper-phosphorylation as well as the appearance of a hyper-shifted form of B3/4-Rad9 was suppressed by the co-expression of MBD, which is in agreement with the reduced binding of B3/4-Rad9 nearby an HO-induced DSB upon MBD expression. These data again reinforce the competition-based model in which Dpb11 regulates HR repair by coordinating the mutually exclusive recruitment of Slx4 and Rad9, and reveal that Dpb11 plays these antagonistic roles in DNA repair also in the context of replication stress.

According to our model, the regulation of Dpb11 interactions with Slx4 or Rad9 is expected play a key role in the control of DNA end resection. Therefore, the decision to specifically stabilize the Dpb11-Slx4 should comprise the discerning molecular event that transitions Dpb11's function from blocking resection to favoring resection. Since interactions of Dpb11 with Slx4 and Rad9 are both dependent on CDK (Gritenaite et al., 2014; Ohouo et al., 2013; Pfander and Diffley, 2011; Wang et al., 2012), we reasoned that CDK activity is unlikely to be the discerning molecular event that commands the choice of Slx4 versus Rad9 stabilization at DNA lesions. Previously, we have shown that the Dpb11-Slx4 interaction requires the Mec1 kinase (Ohouo et al., 2010) and here we show that Mec1 seems to be specifically required to enhance the Dpb11-Slx4 interaction but not the Dpb11-Rad9 interaction (Fig3.2f). Therefore, we propose a model in which resection block imposed by the Rad9-Dpb11 complex is counteracted by Mec1 signaling through the formation of a Dpb11-Slx4-Rtt107 complex (Fig3.2g).

Figure 3.2

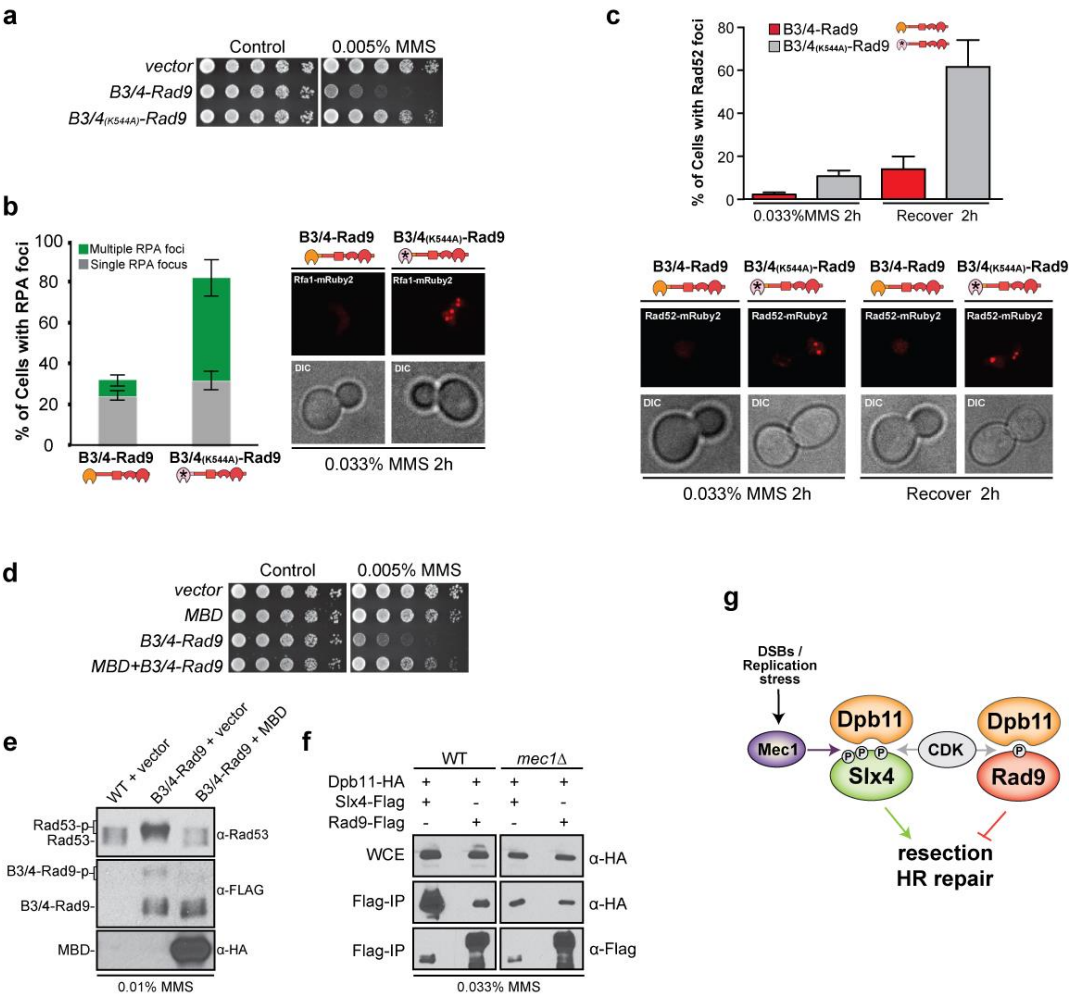

a
Control
0.005% MMS
vector
B3/4-Rad9
B3/4(K544A)-Rad9
b
% of Cells with RPA foci
Multiple RPA foci
Single RPA focus
B3/4-Rad9
B3/4(K544A)-Rad9
Rfa1-mRuby2
DIC
0.033% MMS 2h
c
% of Cells with Rad52 foci
B3/4-Rad9
B3/4(K544A)-Rad9
0.033%MMS 2h
Recover 2h
Rad52-mRuby2
d
Control
0.005% MMS
vector
MBD
B3/4-Rad9
MBD+B3/4-Rad9
e
WT + vector
B3/4-Rad9 + vector
B3/4-Rad9 + MBD
Rad53-p
Rad53-
α-Rad53
B3/4-Rad9-p
B3/4-Rad9-
α-FLAG
MBD-
α-HA
0.01% MMS
f
WT
mec1Δ
Dpb11-HA
Slx4-Flag
Rad9-Flag
WCE
Flag-IP
α-HA
α-Flag
0.033% MMS
g
DSBs / Replication stress
Mec1
Dpb11
Slx4
CDK
Rad9
resection
HR repair

Figure 3.2 Dpb11-mediated hyper-stabilization of Rad9 impairs proper HR repair of replication-induced lesions. (a) MMS sensitivity induced by the expression of B3/4-Rad9 chimera. Four-fold serial dilutions were spotted on SC-URA plates and grown for 2–3 days at 30°C. (b) RPA-mRuby2 foci were quantified in MMS-treated cells (0.033% for 2hrs) expressing either B3/4-Rad9 chimera or the mutated chimera B3/4(K544A)-Rad9. Percentage of cells with one RPA focus or multiple RPA foci were plotted as indicated. More than 150 cells were scored per replicate. (c) Rad52-mRuby2 foci were quantified in cells expressing either B3/4-Rad9 or the mutated chimera B3/4(K544A)-Rad9. Cells were treated with 0.033% MMS for 2hrs or recovered for 2hrs after MMS treatment. Cells were evaluated based on the presence or the absence of Rad52-mRuby2 foci. More than 150 cells were scored per replicate. (d) MBD expression rescues the MMS sensitivity induced by B3/4-Rad9 chimera. (e) Immunoblots showing the phosphorylation status of Rad53 in cells expressing B3/4-Rad9 chimera or co-expressing B3/4-Rad9 chimera with MBD. (f) Co-IP in MMS treated cells (0.033% for 2hrs) determining the effect of *MEC1* deletion in the interactions of Dpb11-HA with Slx4-Flag and Rad9-Flag respectively. (g) A model for the key role of Dpb11 in resection control via the coordination of Slx4 and Rad9 competition that ensures the proper engagement of Rad9 at DSB or replication-induced lesions.

3.3.3 Proteomic analysis of TOPBP1 interactions in human cells reveals regulated interactions with antagonistic regulators of HR-mediated repair

Based on our findings in yeast, we speculated that TOPBP1 also plays a role in coordinating the recruitment of antagonistic factors for the proper control of DNA repair in mammals. Previous reports revealed that TOPBP1 indeed interacts with the pro-NHEJ protein 53BP1 and a range of pro-HR factors, including BRCA1-associated proteins (Greenberg et al., 2006; Gritenaite et al., 2014; Morishima et al., 2007; Yamane et al., 2002; Yoo et al., 2009). We reasoned that TOPBP1 interactions specifically induced by replication stress should reveal pro-HR functions for TOPBP1. We therefore performed an unbiased mass spectrometry analysis to define the network of TOPBP1 interactions in cells treated with hydroxyurea (HU) to induce replication stress, and in cells treated with nocodazole to reveal interactions that are independent of replication stress (Fig3.3a). We also measured the changes of the identified interactions by directly comparing cells treated with HU and nocodazole in order to specifically reveal interactions induced by replication stress (Fig3.3b). While over 35 proteins were found to interact with TOPBP1 in our analyses (Fig3.3a), most interactions did not display major changes in our comparison (Fig3.3b). Similar to what we previously observed for yeast Dpb11 (Ohouo et al., 2010), the interaction of human TOPBP1 with a pro-HR factor, in this case BRCA1, was strongly induced by replication stress (Fig3.3b). Of interest, interaction of TOPBP1 with 53BP1 followed an opposite trend as compared to the TOPBP1-BRCA1 interaction (Figs3.3b,c).

Figure 3.3

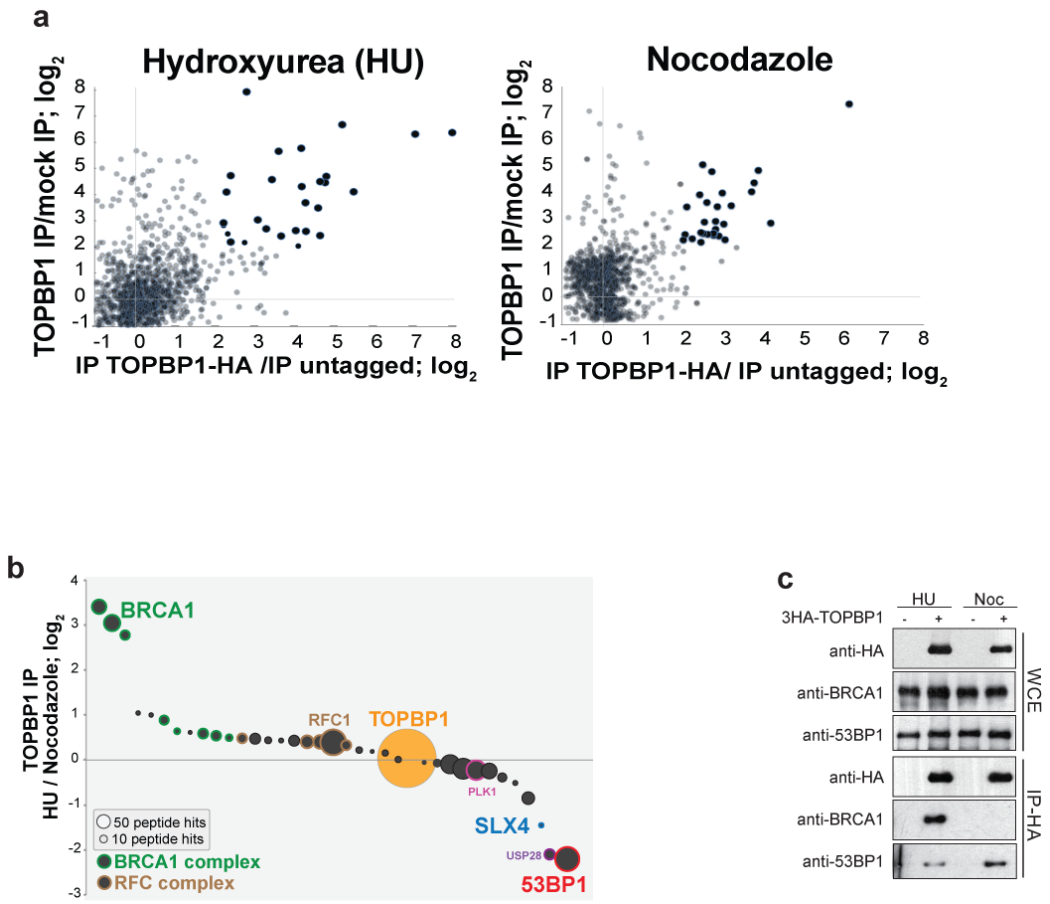

a
Hydroxyurea (HU)
Nocodazole
TOPBP1 IP/mock IP; log2
IP TOPBP1-HA /IP untagged; log2
IP TOPBP1-HA/ IP untagged; log2
b
TOPBP1 IP
HU / Nocodazole; log2
BRCA1
RFC1
TOPBP1
PLK1
SLX4
USP28
53BP1
50 peptide hits
10 peptide hits
BRCA1 complex
RFC complex
c
HU
Noc
3HA-TOPBP1
anti-HA
anti-BRCA1
anti-53BP1
WCE
IP-HA

Figure 3.3 Proteomic analysis of TOPBP1 interactions in human cells reveals regulated interactions with antagonistic regulators of HR-mediated repair. (a) TOPBP1 interactions in cells arrested with HU and nocodazole that are determined by quantitative mass spectrometry analyses. HEK293T cells grown in "light" and "heavy" SILAC DMEM media were both treated with 1mM hydroxyurea (HU) for 24hrs to identify proteins that interact with TOPBP1 in response to replication stress. Either the endogenous TOPBP1 is purified using antibodies against TOPBP1 or over-expressed TOPBP1 tagged with the epitope tag HA is purified using the anti-HA resin. Proteins with a ratio >4 identified in both experiments are considered as TOPBP1 interactors. Each dot in dark color represents an identified TOPBP1 interaction. Similarly procedures are performed for cells treated with 100 ng/ml nocodazole for 14 hrs to define TOPBP1 interactions in G2/M. (b) Comparison of the identified TOPBP1 interactions in cells treated with HU and nocodazole based on quantitative mass spectrometry analyses. (c) The interactions of TOPBP1 with BRCA1 and 53BP1 are reciprocally regulated in cells treated with HU and nocodazole, confirming the mass spectrometry result in Fig3.3b.

3.3.4 The interactions of TOPBP1 with BRCA1 and 53BP1 are regulated a mutually exclusive manner

The opposite regulation of the TOPBP1 interactions with BRCA1 and 53BP1 led us to hypothesize that the formation of these two complexes are mutually exclusive. We created three TOPBP1 mutants with point mutations that disrupt the functionality of the three distinct sets of BRCT domains (BRCT-1/2: K154A and K155A; BRCT-5: K704A and W711R; BRCT-7: R1314Q) and we assessed the ability of these BRCT domain mutants of TOPBP1 to interact with BRCA1 or 53BP1 compared to wildtype TOPBP1. Interestingly we found that both of these interactions required functional BRCT-1/2 and BRCT-5 domains of TOPBP1 (Fig3.4a), further supporting the notion that 53BP1 and BRCA1 interact with TOPBP1 in a mutually exclusive manner. Because 53BP1 and BRCA1 localize to sites of DNA lesions in a mutually exclusive manner (Chapman et al., 2012a), and have been proposed to compete for DNA lesions to dictate repair pathway choice (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009; Chapman et al., 2012a), our findings suggest that TOPBP1 could be the mediator of such competition, similar to the role of Dpb11 in the control of Rad9 and Slx4 in yeast. We therefore hypothesized that the interaction of BRCA1 with TOPBP1 induced by replication stress antagonizes the TOPBP1-53BP1 interaction. This hypothesis is supported by the observation that the TOPBP1-53BP1 interaction was significantly enhanced upon knockdown of *BRCA1* (Figs 3.4b, c). It is noteworthy that the mutually exclusive interactions of TOPBP1 with BRCA1 and 53BP1 is reminiscent of the Dpb11-mediated competition between Rad9 and Slx4 in budding yeast, highlighting the similarity in DNA damage response between the two model systems.

Figure 3.4

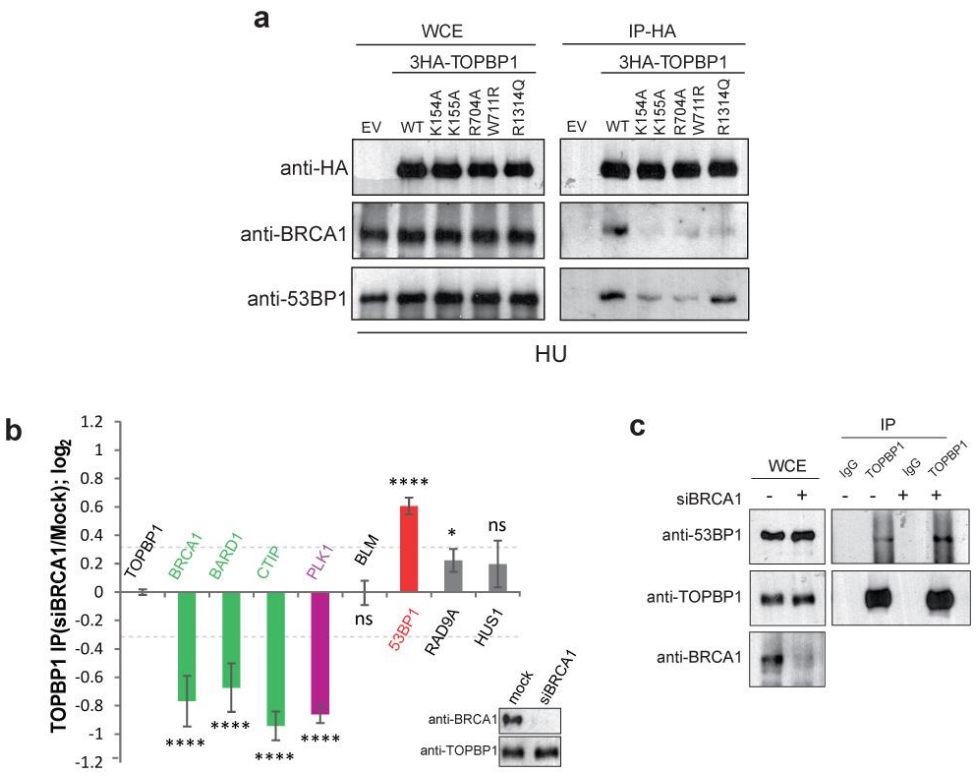


Figure 3.4 The interactions of TOPBP1 with BRCA1 and 53BP1 are regulated a mutually exclusive manner**.** (a) co-IP experiment determining the contribution of each pair of BRCT domains in TOPBP1 for stabilizing TOPBP1 interactions with BRCA1 and 53BP1. (b) Mass spectrometry analysis determining how the absence of BRCA1 impacts the interaction of TOPBP1 with 53BP1. TOPBP1 is immunoprecipitated from HEK293T cells treated with 1mM HU for 24hrs. P value is determined by comparing the light/heavy ratio values of each single identified interactor against that of TOPBP1 using Mann-Whitney test. (c) Co-IP experiment to confirm the effect of BRCA1 knockdown on the TOPBP1-53BP1 interaction using U2OS cells.

3.3.5 ATR signaling stimulates the interaction of TOPBP1 with the pro-HR factor BRCA1

In yeast, the interaction of Dpb11 with the pro-resection factor Slx4 is selectively enhanced upon the checkpoint kinase Mec1 activation. In order to find out if the TOPBP1-BRCA1 interaction is also regulated by the checkpoint kinases, we measured the binding of TOPBP1 to BRCA1 and 53BP1 upon inhibition of each of the checkpoint kinases in mammals, ATR, ATM and DNA-PK. Similar to the scenario in yeast, the orthologue of Mec1, ATR kinase, plays an important role in promoting the interaction of TOPBP1 with BRCA1, while not being required for enhancing the TOPBP1-53BP1 interaction (Fig3.5a). Further, ATM and DNA-PK do not play major roles in the regulation of the TOPBP1-BRCA1 interaction in early response to replication stress. This again mirrors our previous observation in yeast that Mec1 is required for Dpb11-Slx4 interaction but not as important for Dpb11-Rad9 complex formation. These results are suggestive of a key role for ATR^{Mec1} in promoting HR-mediated repair by enhancing the BRCA1-$TOPBP1^{Slx4\text{-}Dpb11}$ interaction upon replication stress. Overall, these findings support a model whereby yeast Dpb11 and mammalian TOPBP1 play roles in coordinating the action of antagonistic repair factors via a competition-based mechanism (Fig3.5b). We note that human SLX4 was also identified as a TOPBP1 interactor (Fig3.3b), consistent with a previous report (Gritenaite et al., 2014). The interaction of TOPBP1 with SLX4 was not enhanced by replication stress, suggesting a fundamentally distinct mode of interaction compared to the Dpb11-Slx4 interaction in yeast. However, we do not exclude the possibility that the SLX4-TOPBP1 interaction plays an important role in controlling HR-mediated repair.

Figure 3.5

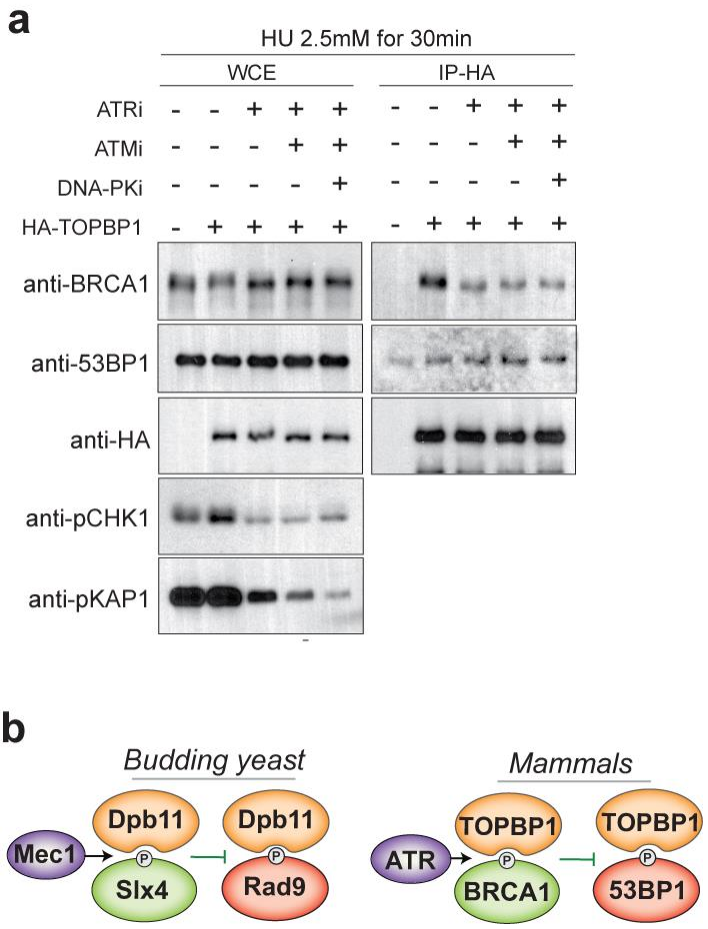


Figure 3.5 ATR signaling stimulates the interaction of TOPBP1 with the pro-HR factor BRCA1. (a) Co-IP experiment determining the importance of ATR, ATM and DNA-PK kinase activity on the interactions of TOPBP1 with BRCA1 or 53BP1. HEK293T cells transfected with HA-TOPBP1 are pretreated with ATR, ATM or DNA-PK inhibitors as indicated for 45min followed by 30min acute HU treatment. (b) A model for a conserved role of TOPBP1^{Dpb11} in coordinating the competition between the pro-HR factor BRCA1^{Slx4} and the anti-HR factor 53BP1^{Rad9}.

3.3.6 Hyper-stabilization of the TOPBP1-53BP1 interaction promotes 53BP1 recruitment to nuclear foci in S-phase

Based on our findings in yeast, we hypothesized that human TOPBP1 controls the recruitment of 53BP1 to DNA lesions and is important to promote 53BP1-mediated DNA repair. To test this hypothesis, we engineered a system for stabilizing the 53BP1-TOPBP1 interaction. We were unable to generate a similar chimeric protein as we did in yeast. A fusion of 53BP1 with BRCT domains of TOPBP1 expresses poorly in human cells lines. To circumvent this issue, we fused 53BP1 to a 120 amino acid region from the N-terminal domain of RFC1 (replication factor C subunit 1), which we found to constitutively interact with TOPBP1 (Fig3.3b). Thus, by fusing the N-terminus of RFC1 (hereinafter referred to as Constitutive TOPBP1-interacting Region, CTR) to 53BP1 (Fig3.6a), we reasoned that the interaction of this chimera with TOPBP1 would be stabilized and enhanced during replication stress. Indeed, the CTR-53BP1 chimeric protein displayed enhanced interaction with TOPBP1 in hydroxyurea (Fig3.6b). This system provided us the opportunity to assess the role of the 53BP1-TOPBP1 interaction in the recruitment of 53BP1. Strikingly, the CTR-53BP1 chimera formed significantly more nuclear foci compared to 53BP1 alone in cells progressing through S-phase following release from an HU-induced arrest (Figs 3.6c, d), suggesting enhanced recruitment of CTR-53BP1 to replication-induced lesions.

Once recruited to the lesion site, 53BP1 plays established roles in promoting the recruitment of PTIP and RIF1, two proteins believed to function as effectors of NHEJ (Callen et al., 2013; Chapman et al., 2013; Munoz et al., 2007; Zimmermann et al., 2013). To investigate whether the increased recruitment of CTR-53BP1 functionally

impacts 53BP1-mediated DNA repair, we first monitored PTIP and RIF1 status. As shown in Fig3.6b, CTR-53BP1 pulled-down more PTIP compared to 53BP1 alone, despite the relatively lower expression level of CTR-53BP1. In addition, while we were unable to monitor PTIP foci using available antibodies, we found that CTR-53BP1 induced a significant increase in the number of RIF1 foci in S-phase cells released from a HU arrest (Figs 3.6 c-d). Since RIF1 and PTIP recruitment to DNA lesions is believed to require DNA damage-induced phosphorylation of 53BP1 (Callen et al., 2013; Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Kumar and Cheok, 2014; Munoz et al., 2007; Zimmermann et al., 2013), our results strongly suggest that the enhanced interaction with TOPBP1 increases the engagement of CTR-53BP1 at sites of lesions, culminating on its phosphorylation and increased recruitment of RIF1, and likely PTIP. Of note, the foci formed by CTR-53BP1 co-localized with H2AX and RIF1 but not with RPA or RAD51 (Fig3.6e), suggesting that the chimera is engaging in NHEJ-mediated DNA repair in a mutually exclusive manner with the HR-machinery.

Figure 3.6

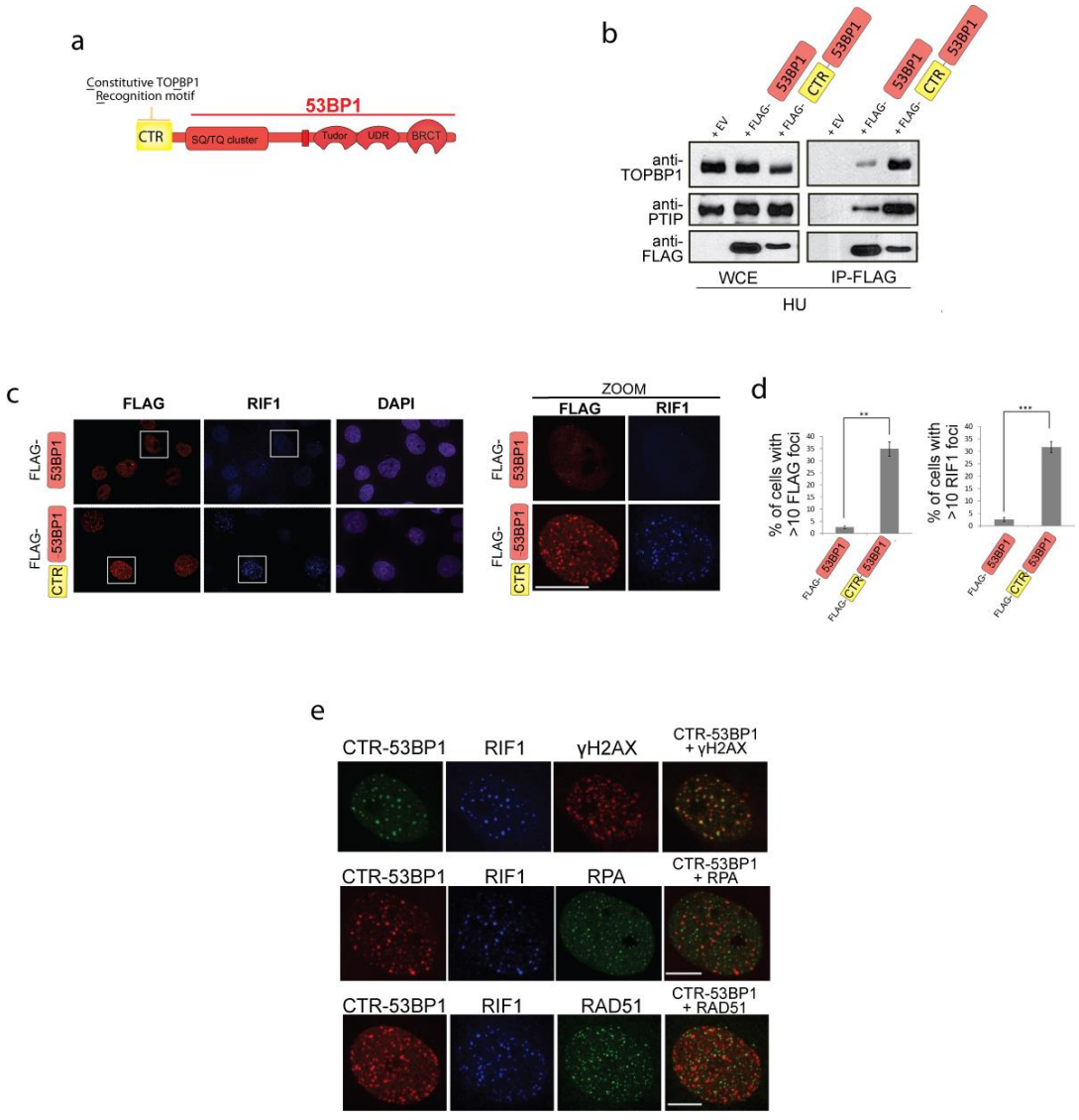

a
Constitutive TOPBP1 Recognition motif
53BP1
CTR
SQ/TQ cluster
Tudor
UDR
BRCT
b
53BP1
CTR
+ EV
+ FLAG-
anti-TOPBP1
anti-PTIP
anti-FLAG
WCE
IP-FLAG
HU
c
FLAG
RIF1
DAPI
ZOOM
FLAG-
53BP1
CTR
d
% of cells with >10 FLAG foci
% of cells with >10 RIF1 foci
e
CTR-53BP1
RIF1
γH2AX
CTR-53BP1 + γH2AX
RPA
CTR-53BP1 + RPA
RAD51
CTR-53BP1 + RAD51

Figure 3.6 Hyper-stabilization of the TOPBP1-53BP1 interaction promotes 53BP1 recruitment to nuclear foci in S-phase. (a) Schematic diagram showing the configuration of the engineered CTR-53BP1 chimera constituted of a 120 amino acid fragment in N-terminal domain of RFC1 (CTR) and full length 53BP1. (b) Co-IP experiment determining the effect of CTR motif fusion on the interactions of 53BP1 with TOPBP1 and PTIP individually. HEK293T cells were treated with 1mM HU for 24hours. (c) Immunofluorescence of the U2OS cells transfected with FLAG-53BP1 or FLAG-CTR-53BP1. Cells were arrested with 1mM HU for 24hrs and released for 3hrs to allow cells to progress through S phase. Scale bar, 10μm. (d) Quantification of the FLAG-stained and RIF1 foci number in the transfection-positive cells in (c). More than 150 transfected cells were scored per replicate. (e) Immunofluorescence of the U2OS cells transfected with FLAG-CTR-53BP1 showing that FLAG-CTR-53BP1 colocalizes with γH2A.x, but not with RPA or Rad51. Cells were arrested with 1mM HU for 24hrs and released for 3hrs before fixation. Scale bar, 10μm.

3.3.7 Hyper-stabilization of the TOPBP1-53BP1 interaction leads to increased chromosomal aberrations.

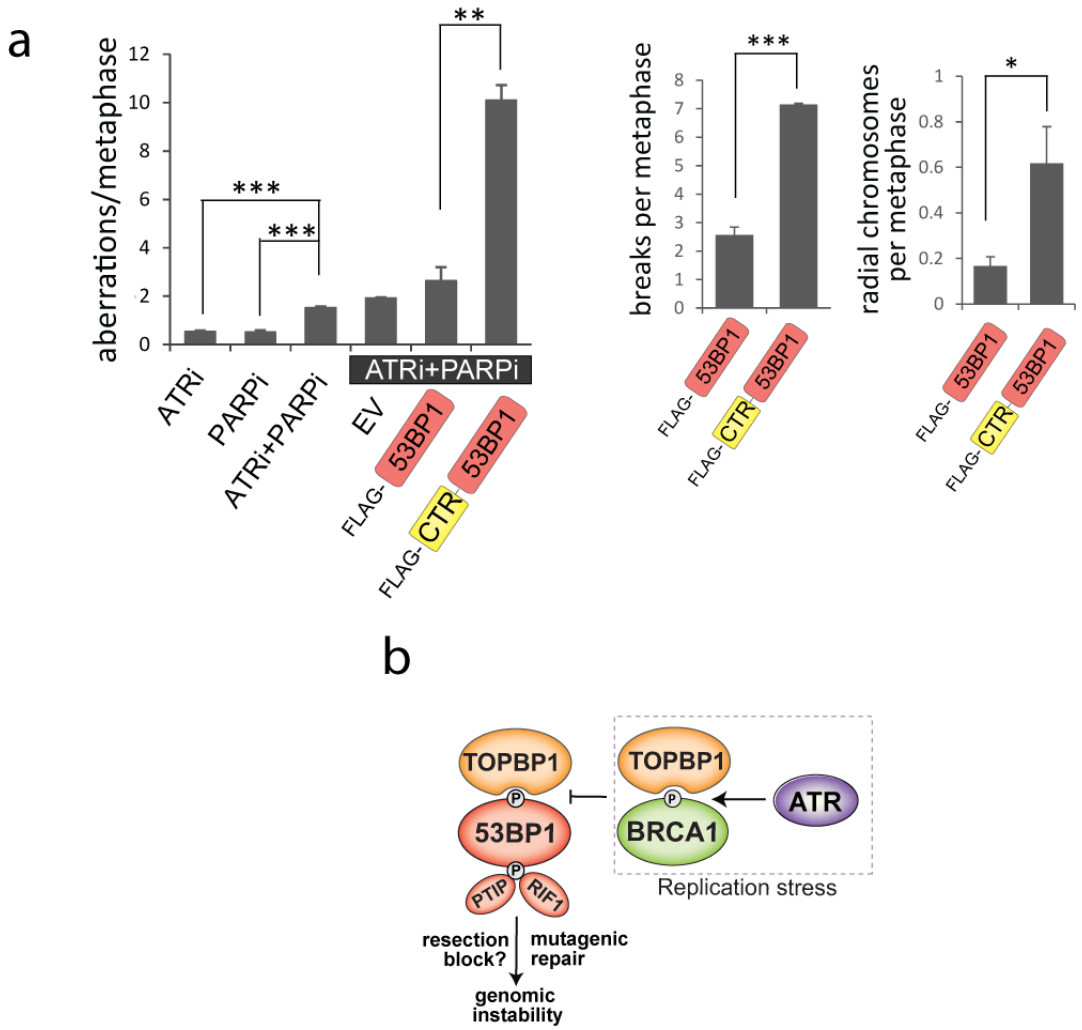
Give that hyper-stabilization of the TOPBP1-53BP1 interaction promotes the engagement of 53BP1 and other pro-NHEJ factors at the lesions, we reasoned that expression of CTR-53BP1 would induce genomic instability by promoting mutagenic NHEJ repair and/or deregulating HR-mediated repair. Indeed, we observed a significant increase in the number of chromosomal aberrations induced by CTR-53BP1, but not by ectopic 53BP1, in response to replication stress induced by a combination of PARP inhibitor (AZD2461) and ATR inhibitor (VE821) (Fig3.7a). The increase in chromosomal aberrations was highly dependent on this particular drug combination, as neither PARP inhibitor nor ATR inhibitor alone does not lead to a specifically increased chromosomal aberrations in cells over-expressing CTR-53BP1. We attribute the dependency on this drug combination to the formation of collapsed replication forks and concomitant inhibition of the TOPBP1-BRCA1 interaction, among other crucial ATR functions. This result again supports our hypothesis that TOPBP1 plays a key role in mediating the recruitment of 53BP1 to DNA lesions to promote 53BP1-dependent mutagenic DNA repair and genomic instability (Fig3.7b).

3.3.8 A working model

Our results in budding yeast and mammal together point to a conserved function of TOPBP1^{Dpb11} in DNA repair control by balancing the engagement of the anti-HR factor 53BP1^{Rad9}. We propose a model whereby TOPBP1^{Dpb11} plays antagonistic roles in HR repair control by coordinating the competition between 53BP1^{Rad9} and a pro-HR

factor, BRCA1 in humans and Slx4 in budding yeast (Fig. 3.7b). Upon DSB or replication-induced lesions, ATR^{Mec1} signaling selectively enhances the interaction between $TOPBP1^{Dpb11}$ and BRCA1/Slx4, resulting in the sequestration of TOPBP1 and therefore counteracting the action of 53BP1 by preventing $TOPBP1^{Dpb11}$-mediated $53BP1^{Rad9}$ stabilization at the lesions and its engagement in mutagenic repair.

Figure 3.7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PTIP
RIF1
resection block?
mutagenic repair
genomic instability
TOPBP1
P
BRCA1
ATR
Replication stress

Figure 3.7 Hyper-stabilization of the TOPBP1-53BP1 interaction induces chromosomal aberrations. (a) Analysis of chromosomal abnormalities in metaphases of HEK293T cells that were treated with 1 μM ATR inhibitor and/or 3 μM PARP inhibitor AZD2461. n>45 metaphases were analyzed in each replicate. (b) A model for the crucial role of TOPBP1 in DNA repair by balancing the engagement of 53BP1 at the DNA lesions to ensure proper regulation of DNA end resection and genomic integrity.

3.4 Discussion

In the last two decades, it has been well-established that TOPBP1 plays key roles in both the initiation of DNA replication and activation of checkpoint signaling. While the importance of TOPBP1 in DNA repair has been appreciated, the precise function of TOPBP1 in the control of DNA repair pathways remains unclear. Our work presented here reveals an evolutionarily conserved role for TOPBP1 in coordinating the balanced recruitment of the pro- and the anti-HR factors to ensure proper choice of repair pathways. Our results point to a model in which $TOPBP1^{Dpb11}$ is the crucial protein that establishes the competition between the pro- and the anti-HR repair factors via two mutually exclusive physical interactions, which serve as the molecular basis for the competition mechanism of DSB repair. Of importance, it has been recently reported that depletion of TOPBP1 abrogates RAD51 loading to chromatin and the formation of RAD51 foci, but does not impair DNA end resection and RPA loading (Moudry et al., 2016). This finding is consistent with our results and our proposed model for how TOPBP1 controls HR repair, as in the absence of TOPBP1 we predict that 53BP1 would not be able to be robustly stabilized at DNA breaks to block resection. This scenario is similar to what is observed in cells lacking both functional BRCA1 and 53BP1, in which case resection is restored as compared to cells lacking only BRCA1 (Bunting et al., 2010). In addition, we do not exclude that TOPBP1 plays roles in other subsequent steps in HR, such as the control of RAD51 loading by mediating PLK1-dependent RAD51 serine 14 phosphorylation, as proposed by Moudry et al. (Moudry et al., 2016). In fact, we also detected PLK1 in our proteomic analysis of TOPBP1 interactions and, interestingly, found that interaction of TOPBP1 with PLK1 appears to depend on

BRCA1 (Fig3.4b).

Our work suggests that both budding yeast and humans appear to utilize a highly analogous mechanism for the regulation of DNA repair pathway choice. First of all, in response to replication-induced lesions, ATR^{Mec1} signaling is key to promote the interactions between $TOPBP1^{Dpb11}$ and the pro-HR factors, BRCA1 in mammals and Slx4 in yeast. We reasoned that the functional relevance of this ATR^{Mec1}-induced interaction could be two fold. On the one hand, the formation of TOPBP1-$BRCA1^{Dpb11\text{-}Slx4}$ complex is important for facilitating HR in S phase. In yeast, this is supported by the previously published data that Slx4-S486A, which is a separation of function mutant of Slx4 with impaired interaction with Dpb11, displays a severe defect in the long range resection upon site-specific DSB induced by HO endonuclease (Dibitetto et al., 2016; Ohouo et al., 2013). However, to directly test this in humans, a separation of function mutant of BRCA1 with abrogated binding to TOPBP1 is needed. Since multiple pairs of BRCT domains are required for stabilizing TOPBP1-BRCA1 interaction, more than one phosphorylation motif might be involved. This is in line with the second potential function of this interaction. We argue that ATR^{Mec1} signaling promotes human BRCA1/yeast Slx4 phosphorylation, stabilizes their binding to $TOPBP1^{Dpb11}$ via simultaneous recognition by distinct sets of BRCT domains and ultimately sequesters $TOPBP1^{Dpb11}$ from interacting with the anti-HR factor $53BP1^{Rad9}$. The sequestration of $TOPBP1^{Dpb11}$ restrains the action of $53BP1^{Rad9}$ in promoting mutagenic NHEJ repair at the replication-associated lesions and reciprocally facilitates BRCA1-mediated HR repair. Again, this is congruent with the published results that deletion of yeast Slx4 results in enriched Dpb11-Rad9 interaction (Ohouo et al., 2013), increased recruitment

of Rad9 at HO endonuclease-induced DSB and consequently reduced resection rate in DSB response (Dibitetto et al., 2016). In humans, TOPBP1 sequestration model is also reinforced by the finding that the stabilized interaction of TOPBP1 with the pro-HR factor BRCA1 upon replication stress suppresses TOPBP1 interaction with the anti-HR factor 53BP1 (Figs 3.3b, c). Moreover, the absence of BRCA1 leads to increased interaction of TOPBP1 with 53BP1 (Figs 3.4b, c), which presumably leads to enhanced engagement of 53BP1 at the lesions sites, consequently inhibiting HR and promoting mutagenic NHEJ. This is also in keeping with the embryonic lethality of $BRCA1^{\Delta 11/\Delta 11}$ mouse and elevated genomic instability in $BRCA1^{\Delta 11/\Delta 11}$ MEFs (Bunting et al., 2010), supporting the central role of TOPBP1 in coordinating the competition between BRCA1 and 53BP1.

The second aspect that highlights the similarities in repair control between the yeast and humans is the common role of $TOPBP1^{Dpb11}$ in promoting $53BP1^{Rad9}$-mediated mutagenic repair. We rationally designed two engineered $53BP1^{Rad9}$ chimeras to bypass the $TOPBP1^{Dpb11}$ sequestration effect posed by Slx4 in yeast and BRCA1 in mammals to drive the balance toward the anti-HR branch and promote mutagenic repair. Congruent with the our reasoning, we showed compelling evidence that the expression of engineered $53BP1^{Rad9}$ chimeras in both model organisms result in much enhanced recruitment of $53BP1^{Rad9}$ and increased genomic instability suggesting that TOPBP1 mediates 53BP1-dependent mutagenic repair. Remarkably, in yeast cells expressing B3/4-Rad9 chimera, we observed severe impairment of resection, which in turn is rescued by the expression another chimera MBD that hyper-stabilizes Slx4 at the lesion sites (Cussiol et al., 2015). While the results in yeast clearly reveal a role of Dpb11-

Rad9 interaction in resection block, in mammals we were not able to show that resection is significantly impacted by monitoring RPA or Rad51 foci formation. We visualize two possible scenarios that may explain this discrepancy in mammals. First, since we utilized a system that cells are synchronized in S phase using HU, which causes widespread fork stalling and ssDNA accumulation, we suspect that RPA is not a good marker for resection in this case as RPA foci in HU may represent both ssDNA resulting from resected DSB caused by collapsed forks and ssDNA ensuing fork stalling. In the second scenario, which is more likely to be the case, we speculate that CTR-53BP1 chimera is recruited to and promotes the mutagenic repair at the DNA lesion sites that are not mature and reachable substrates for HR machinery and that may represent either DNA replication or repair intermediate structures.

It is noteworthy that in yeast the resection and recombination defects resulting from B3/4-Rad9 expression is accompanied by aberrant checkpoint hyper-activation as evidenced by Rad53 hyper-phosphorylation (Fig3.2e). It is tempting to speculate that the resection block imposed by Rad9 is an indirect result of its function in the facilitation of Rad53 signaling, given that Rad53 activation has been shown to inhibit resection. However, we cannot rule out the possibility that a Dpb11 plays a role in directly stabilizing Rad9 at the ssDNA::dsDNA junction to efficiently mask the 5' recessed end, therefore preventing access of the nucleases such as Exo1 and Dna2-Sgs1. Congruent with this hypothesis, recent findings have shown that Rad9 reduces the recruitment of Exo1 and Dna2-Sgs1 to DSBs (Bonetti et al., 2015; Ngo and Lydall, 2015). Differently from yeast, the function of TOPBP1-53BP1 interaction in promoting mutagenic repair appears to be independent of checkpoint signaling in mammals, as we did not observe

any aberrant checkpoint signaling induced by CTR-53BP1 chimera (data not shown). Instead, our results suggest that TOPBP1-53BP1 complex executes its anti-HR function via the recruitment of downstream effectors RIF1 and PTIP, likely by either acting as a physical block or remodeling chromatin architecture to restrict and limit resection.

Elucidating the regulatory basis of the recruitment of HR and NHEJ factors to DNA lesions has major implications for understanding the molecular mechanisms of tumorigenesis, especially in patients with germline mutations in HR repair genes, such as BRCA1 and BRCA2, which are among the most frequently mutated genes in hereditary breast and ovarian cancer (Venkitaraman, 2002). In addition, the knowledge generated from our studies will help in the design of better rationales for targeted cancer therapies. The unexpected role of TOPBP1 in promoting 53BP1-mediated mutagenic repair uncovered here may very well be exploited as another potential therapeutic target to further manipulate the DNA repair pathway choices in HR-deficient tumors beside the application of PARP inhibitors, reinforcing the concept of precision medicine. Our results also suggest that the mutagenic repair driven by TOPBP1-53BP1 appears to be synergistically lethal with defective HR exemplified by the use of ATR inhibitor in our studies, which again highlights its potential as a new therapeutic target to be applied in conjunction with olaparib or as an alternative to re-sensitize *brca1*$^{-/-}$ mutated tumors that have developed resistance to PARP inhibitors.

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CHAPTER 4

Conclusions and Future Directions

4.1 Conclusions

Maintenance of the genomic integrity in face of DNA damage relies on a highly integrated signaling system comprised of many interwoven biological pathways. In particular, DNA synthesis, DNA damage checkpoint signaling and DNA repair pathways need to be highly coordinated. However, the molecular mechanisms that regulate the orchestration of DNA damage checkpoint (DDC) signaling and DNA repair are not well understood. The work in this dissertation reveals an important and conserved role for the protein scaffold TOPBP1^{Dpb11}, previously known for its crucial functions in DNA replication initiation and DDC activation, in controlling DNA repair pathway choice via a competition-based mechanism, which exemplifies how DNA repair is closely monitored and tightly controlled by DDC signaling.

4.1.1 In budding yeast, Dpb11 modulates resection control and HR repair by fine-tuning checkpoint signaling via a competition-based mechanism.

In the last two decades, studies have shown that Dpb11, comprised of two pairs of BRCT domains, functions as a scaffolding protein by facilitating the assembly of ternary complexes via phospho-dependent interactions to promote both replication initiation and DDC signaling. While some previous work has hinted that Dpb11 is also implicated in DNA repair, its precise repair function remains elusive. In addition, it is unknown how the distinct functions of Dpb11 in different aspects of DNA metabolism are coordinated. The work in this dissertation addresses this question by uncovering a

role for Dpb11 as a modulator of both DDC signaling and HR-mediated repair. It is particularly interesting that our model points to a central role of Dpb11 in repair pathway choice via resection control. This ability of Dpb11 to regulate resection control is dependent and likely executed through its ability to fine-tune DDC signaling. This mode of regulation exemplifies how two aspects of Dpb11 function are tightly coordinated and closely monitored by each other.

In an integrated model, we propose that Dpb11 controls the recruitment of DDC adaptor Rad9 and repair scaffolds Slx4-Rtt107 to the lesions sites by forming two mutually exclusively complexes which ultimately results in a balanced control of the DDC activation level and resection via a competition-based mechanism. To briefly delineate the model, upon fork stalling or DSB resection, the apical checkpoint kinase Mec1 is recruited to the RPA-coated ssDNA and DNA damage sensor 9-1-1 complex (Ddc1-Mec3-Rad17) is independently loaded onto 5'ss-dsDNA junctions. A major role for 9-1-1 complex at the lesions is to directly stimulate Mec1 kinase activity via the C-terminal tail of Ddc1 to initiate DDC signaling. This checkpoint activation function of 9-1-1 is complemented by its ability to recruit a second Mec1 activator, Dpb11. Once Dpb11 is loaded at the lesion sites, it stimulates Mec1 activity via its C-terminal activation domain and simultaneously Dpb11 acts as a key regulatory point for coordinating the competition between Slx4-Rtt107 scaffolds and Rad9 to modulate checkpoint signaling. On the one side of the competition, Dpb11 mediates the stabilization of Rad9 via a physical interaction controlled by CDK to facilitate DDC signaling and downstream checkpoint kinase Rad53 activation. On the other side of this competition, Dpb11 forms a stable complex with Slx4-Rtt107 scaffold, which in turn

antagonizes Rad9 binding to Dpb11 and as a result 'dampens' DDC signaling. This physical interaction between Dpb11 and Slx4 is mediated by a key CDK-dependent phosphorylation site in Slx4 and this interaction is strongly enhanced by the activation of Mec1 upon DNA damage. We propose that this high affinity of Slx4-Rtt107 for Dpb11 sequesters Dpb11 from binding to Rad9 and therefore prevents Rad9-mediated DDC activation.

Collectively Dpb11 modulates DDC signaling by coordinating the competition between Slx4 and Rad9 to control the engagement of Rad9 at the lesions and therefore Rad53 activation level. In addition to the antagonistic roles of Dpb11 in checkpoint signaling, this Dpb11-mediated competition mechanism also regulates HR repair by controlling DNA end resection. In our working model, the recruitment and stabilization of Rad9 mediated by Dpb11 imposes a resection block and inhibits HR. On the other hand, the formation of the Dpb11-Slx4-Rtt107 complex plays a role in promoting resection and HR by antagonizing the binding of Rad9 to Dpb11 and restricting the engagement of Rad9 at the lesions. Despite the compelling experimental evidence showing that Dpb11-mediated competition controls Rad9 engagement in both DDC signaling and DNA end resection, it is not clear whether Rad9-mediated resection block is dependent on its DDC signaling function. Interestingly, the Rad53 FHA2 domain mutant (R605A), which ablates Rad9-mediated Rad53-dependent checkpoint signaling, fully rescues the resection defect induced by the hyper-stabilization of Rad9 (Fig4.2). This argues that the anti-resection function of Rad9 mostly requires its ability to transduce signal from Mec1 to Rad53. However, we cannot rule out a potential role for Rad9 as a physical barrier to mask 5' recessed end and counteract resection on its own

independent of Rad53 activation.

Taken together our work suggests that Dpb11 plays key roles in both fine-tuning DDC signaling and dictating DNA repair pathway choice by controlling the engagement of Rad9.

Figure 4.1

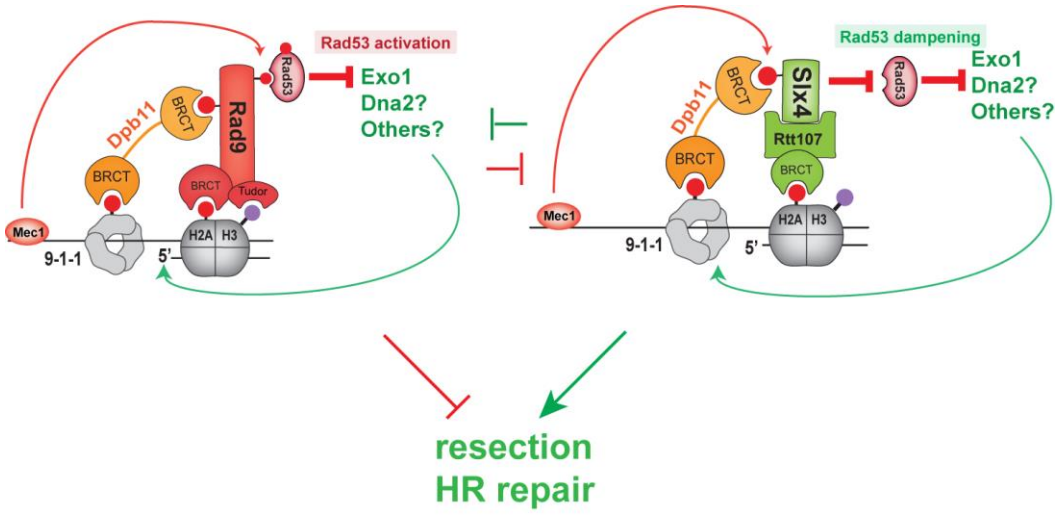

Figure 4.1 A model for the role of Dpb11 in the modulation of DDC signaling and HR-mediated repair. Upon replication fork stalling or DSB resection, 9-1-1 complex recruits Dpb11 to fully activate Mec1. Dpb11 mediates the stabilization of Rad9 to promote DDC signaling and inhibit extensive resection by suppressing the recruitment of nucleases. On the contrary, repair scaffolds Slx4-Rtt107 can form a stable complex with Dpb11 to sequester Dpb11 and counteract the engagement of Rad9 at the lesion, consequently resulting in the down-regulation of DDC signaling and the facilitation of long-range resection. Overall, Dpb11 plays antagonistic roles in both DDC signaling and resection via a competition-based mechanism.

Figure 4.2

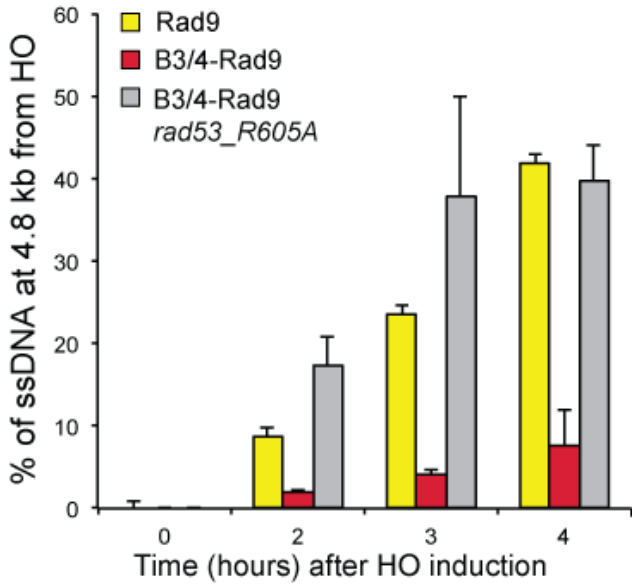

Figure 4.2 The resection block imposed by B3/4-Rad9 chimera is fully rescued by the Rad53 FHA2 domain mutation (R605A), which impairs Rad9-mediated Rad53-dependent checkpoint signaling.

4.1.2 In mammals, TOPBP1 controls DNA repair by balancing the engagement of the pro- and the anti-HR repair factors.

TOPBP1, comprised of a total of nine BRCT domains, has been shown to act as a scaffold in DNA replication initiation and checkpoint activation similar to its yeast counterpart. While it was found that TOPBP1 physically interacts with many DNA repair factors, the precise mechanism by which it regulates DNA repair remains unclear. The work in this dissertation suggests that TOPBP1 acts as a hub in DNA repair pathway choice by coordinating the balanced engagement of the pro- and the anti-HR repair factors. In an integrated model, we propose that the physical interactions with TOPBP1 license the stabilization and involvement of the pro-HR factor BRCA1 and the anti-HR factor 53BP1 at the DNA lesion sites (Fig4.3). Upon fork stalling or DSB resection, 9-1-1 complex is loaded at the 5' ss-dsDNA junction and recruits TOPBP1 to the sites of lesions to allow initiation of ATR signaling. Independently, the anti-HR factor 53BP1 is recruited to the chromatin via the recognition of histone marks H4K20Me2 and H2A-K15-Ub by its tudor and UDR domain, respectively. The pro-HR factor BRCA1 can also be recruited through several different molecular mechanisms involving its cofactors such as RAP80 or BARD1. Interestingly, both BRCA1 and 53BP1 can bind to TOPBP1, yet in a mutually exclusive manner. ATR signaling enhances and stabilizes BRCA1-TOPBP1 interaction and sequesters TOPBP1 from binding to 53BP1 which therefore restricts the actions of 53BP1 and inhibits 53BP1-dependent mutagenic repair at the DNA lesions. This model is supported by (1) the enhanced 53BP1-TOPBP1 interaction in the absence of BRCA1 (2) the same BRCT domains in TOPBP1 are required for its binding to BRCA1 or 53BP1 (3) The hyper-stabilized TOPBP1-53BP1 interaction

drives the increased recruitment of 53BP1 repair effectors RIF1/PTIP and chromosomal aberrations, suggesting enhanced engagement of 53BP1 in DNA repair. These results together place TOPBP1 at the center of DNA repair pathway choice as a key mediator for the competition between the pro-HR BRCA1 and the anti-HR 53BP1 at the lesion. However, it remains unclear if the stabilization of 53BP1-TOPBP1 interaction impacts resection and HR repair efficiency and if BRCA1-TOPBP1 interaction is indeed required for the proper function of BRCA1 in HR, which should be addressed in the future.

Collectively, the work in this dissertation suggests a highly conserved role for $TOPBP1^{Dpb11}$ in controlling the engagement of anti-resection factor $53BP1^{Rad9}$ at the DNA lesion and therefore ensure proper resection regulation and HR repair via a competition-based mechanism.

Figure 4.3

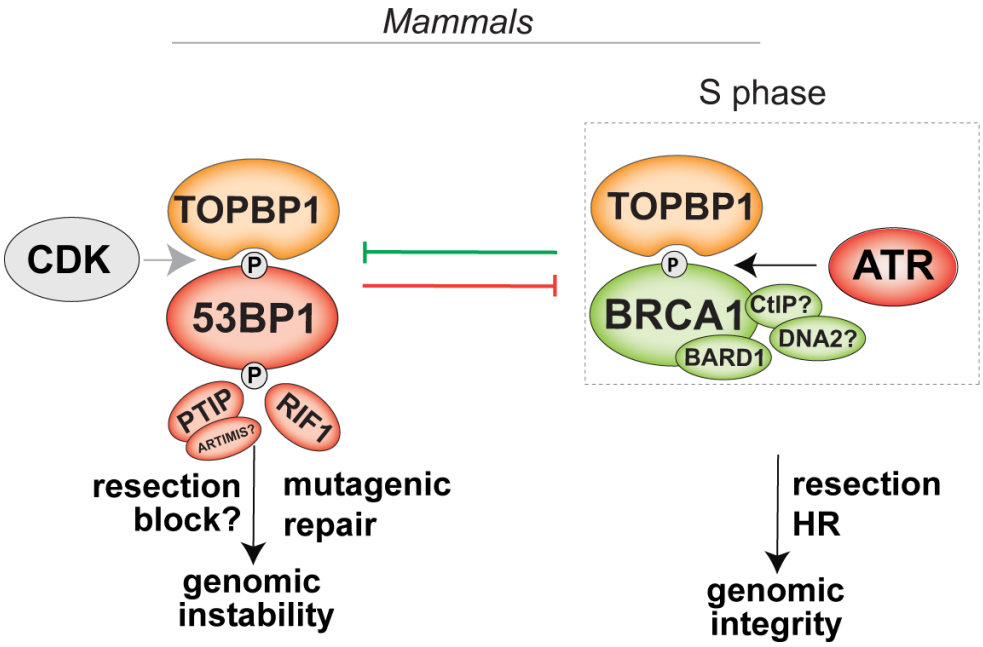

Figure 4.3 A working model for a conserved role of TOPBP1 in DNA repair pathway choice. Like in yeast, upon replication fork stalling or DSB resection, 9-1-1 complex is loaded and recruits TOPBP1 to initiate DDC signaling. At the lesion sites, TOPBP1 coordinates a highly regulated competition between the pro-HR factor BRCA1 and the anti-HR factor 53BP1 by establishing two mutually exclusive physical interactions. BRCA1 binding to TOPBP1 is stabilized upon the activation of ATR and presumably this enhanced interaction results in productive resection and HR repair through the action of nucleases such as CtIP and DNA2. We speculate that BRCA1 sequesters TOPBP1, therefore restricts 53BP1 from engaging at the lesions and prevents 53BP1/RIF1/PTIP-mediated mutagenic repair.

4.1.3 An evolutionarily conserved role for TOPBP1^{Dpb11} in DNA damage response.

The protein scaffold TOPBP1^{Dpb11} is an evolutionarily conserved multi-BRCT domain-containing protein. It has been well-established that TOPBP1^{Dpb11} plays crucial roles in DNA replication and DNA damage checkpoint activation in both budding yeast and higher eukaryotes. This dissertation work uncovers another conserved role for TOPBP1^{Dpb11} in controlling the engagement of the anti-resection factor 53BP1^{Rad9} and thereby proper regulation of HR-mediated DNA repair. In both budding yeast and mammals, we proposed that TOPBP1^{Dpb11} mediates a competition between the pro-resection factor scSlx4/hsBRCA1 and the anti-resection factor scRad9/hs53BP1 through mutually exclusive interactions with these two proteins. Upon DNA damage, the activation of ATR^{Mec1} signaling selectively enhances the interaction of TOPBP1^{Dpb11} with BRCA1/Slx4, sequestering TOPBP1^{Dpb11} from binding to 53BP1^{Rad9} and thereby restricting the engagement of 53BP1-medated mutagenic repair at the lesions.

Despite the highly conserved mechanism in HR-mediated repair control between budding yeast and mammals, there are several major differences in the regulation of this competition-based mechanism. First of all, in budding yeast, Dpb11-mediated recruitment and stabilization of Rad9 lead to a strong resection block. However, in mammals, the hyper-stabilization of the TOPBP1-53BP1 interaction does not appear to block resection even though we observed the enhanced engagement of the pro-NHEJ factors, RIF1 and PTIP, and increased chromosomal aberrations. We speculate that the hyper-stabilization of 53BP1 at the lesions allows NHEJ repair machineries to act on the replication fork and DNA repair intermediate structures, which might not be mature

substrates for resection and HR. A second major difference lies in how the proper control of HR repair is achieved through this competition mechanism. In budding yeast, Dpb11-mediated competition between Rad9 and Slx4 results in the modulation of both DDC signaling and HR-mediated DNA repair. The fact that the resection block imposed by B3/4-Rad9 chimera is fully rescued by the FHA2 mutation in Rad53 suggests that the function of Rad9 to block resection is likely executed through its role in promoting DDC signaling and thereby preventing the stabilization of the nucleases involving in resection. However in mammals, TOPBP1-mediated competition between BRCA1 and 53BP1 does not appear to play a major role in the regulation of DDC signaling. Instead, TOPBP1-53BP1 binding likely controls the engagement of 53BP1 and its downstream pro-NHEJ effector proteins, RIF1 and PTIP. Whether TOPBP-53BP1 hyper-stabilization impacts resection remains to be determined.

Besides the two major points of differences stated above, the pro-resection factors in yeast and mammals involving in this TOPBP1-mediated competition appear to be very different. While Rad9, the orthologue of the human anti-resection factor 53BP1, has also been shown to block resection, no clear structural or functional orthologue of BRCA1 has been identified in budding yeast. However, yeast Slx4 emerges to be a 'BRCA1-like' factor and counteracts the resection block imposed by $53BP1^{Rad9}$. Interestingly, human SLX4, an evolutionarily conserved repair scaffold with functions in coordinating the actions of the structure-specific endonucleases, has also been identified as an interactor with TOPBP1 in our screen. Yet unlike the yeast Dpb11-Slx4 interaction, the human TOPBP1-SLX4 interaction is not enhanced in response to replication stress or other forms of DNA damage, suggesting that the human SLX4-

TOPBP1 complex may not be the counterpart of the yeast Slx4-Dpb11 complex in the context of this competition-based mechanism. However, we cannot rule out a potential role for SLX4 in DDC and resection control.

Overall, TOPBP1^{Dpb11} plays a conserved role in the control of HR-mediated repair through a competition-based mechanism via regulated interactions with both pro-HR and anti-HR repair factors.

4.2 Future directions

The work in this dissertation together is summarized in a model whereby TOPBP1^{Dpb11} coordinates the balanced engagement of the pro- and the anti-HR repair factors at the DNA lesion sites via a phosphorylation-directed competition mechanism, placing TOPBP1/Dpb11 in the hub of DNA repair pathway choice regulation. In particular, yeast Dpb11 plays a decisive role in the regulation of DNA end resection and HR-mediated repair, presumably through the enforcement of DDC signaling. Despite the strong evidence supporting this competition model, there are many more interesting questions remaining to be answered in order to better elucidate the mechanistic functions of TOPBP1^{Dpb11} in DNA repair control, especially in mammals. First does enhanced TOPBP1-53BP1 interaction impact resection in mammals, and ultimately influence HR and NHEJ efficiency? Second, does strengthened TOPBP1-BRCA1 interaction avert chromosomal abnormalities and potential repair pathway defect induced by CTR-53BP1 expression? Third, what is the molecular basis of the phosphorylation-mediated TOPBP1-BRCA1 and TOPBP1-53BP1 interactions, and specifically what are the phosphorylation motifs in BRCA1 and 53BP1 that are recognized by TOPBP1 BRCT domains? With this information, we can interrogate the competition model using the separation of function mutants of 53BP1 or BRCA1 defective in binding to TOPBP1. Fourth, what is the molecular requirement for the anti-HR function of CTR-53BP1 and what are the downstream repair effectors for 53BP1-driven mutagenic repair in this competition model? Finally, in budding yeast, what is the molecular requirements for Rad9 to implement its anti-resection function in both replication stress and DSB?

4.2.1 How does enhanced TOPBP1-53BP1 interaction impact resection and HR/NHEJ repair pathway choices?

To answer this question, we integrated CTR-53BP1 chimera, which appears to be a TOPBP1 interaction 'stabilizer', using FLP-in T-Rex system and created stable cell lines with inducible expression of CTR-53BP1. Another stable cell line expressing CTR (T110A)-53BP1 chimera will also be generated as control. The resection speed in the stable cell lines expressing different 53BP1 variants can be assessed using a previously described assay (Cruz-Garcia et al., 2014). HR and NHEJ efficiency can also be directly measured using available reporter systems, including DR-GFP, SA-GFP and EJ5 reporter assays (in collaboration with Pablo Huertas lab). Since cells expressing CTR-53BP1 display enhanced recruitment of RIF1 (and also likely PTIP) to 53BP1 and results in increased chromosomal aberrations in response to replication-associated lesions, which all indicate hyper-utilization of NHEJ, it is tempting to speculate that CTR-53BP1 expression would result in reduced resection level and HR efficiency accompanied by increased NHEJ rate.

4.2.2 Characterize the role of TOPBP1 in BRCA1-mediated HR repair

While it has long been shown that TOPBP1-BRCA1 interaction is induced upon IR (Greenberg et al., 2006) (consistent with our result that BRCA1 is among the most highly induced TOPBP1-mediated interactions upon replication stress) the functional relevance of this interaction remains unknown. By far the most established function of BRCA1 is to mediate HR repair and lack of BRCA1 leads to HR defect.

Interestingly, recent work published by Moudry et al also showed that TOPBP1 knock-down results in defective HR repair. This together implies that TOPBP1-BRCA1 interaction may be involved in efficient execution of HR and required for proper repair of DSB. One way to test this is to use a separation of function mutant of BRCA1 defective in binding to TOPBP1, which we will address in the next section. Alternatively, we could take also advantage of the CTR system as described in Chapter3 to hyper-stabilize TOPBP1-BRCA1 interaction and examine its impact on HR. Based on our observation in yeast that MBD expression fully rescues the resection defect caused by B3/4-Rad9 chimera, I expect that CTR-BRCA1 chimera would outcompete CTR-53BP1 chimera, and rescue the chromosomal aberrations and avert the increased 53BP1/RIF1 foci formation induced by CTR-53BP1 chimera.

Understanding the precise function of TOPBP1 in BRCA1-mediated HR repair is crucial to affirm the central role of TOPBP1 in establishing the 53BP1-BRCA1 competition at DSB lesions, reinforcing the model whereby physically interactions with TOPBP1 license the engagement of repair factors and implementation of the according repair pathways.

4.2.3 Investigate the phospho-regulation of TOPBP1-BRCA1 and TOPBP1-53BP1 interactions

Our results suggest that TOPBP1 interacts with BRCA1 and 53BP1 in a phospho-dependent manner and both interactions may have implications in DNA repair control. Understanding the phospho-regulation of these interactions is important for the elucidation of molecular mechanisms that control the engagement of 53BP1 and

BRCA1 at the lesions and consequently the repair pathway choice. Since more than one pair of BRCT domains is required for the stabilization of the TOPBP1 binding to BRCA1 or 53BP1, we predict that multiple phosphorylation sites in BRCA1 and 53BP1 are involved. Identification of the phosphorylation sites in both BRCA1 and 53BP1 responsible for being recognized by TOPBP1 BRCT domains will provide us opportunities to generate separation of function mutants defective in binding to TOPBP1 and therefore directly annotate the functional relevance of TOPBP1-BRCA1 and TOPBP1-53BP1 interactions in repair control.

To identify a separation-of-function mutant for 53BP1 that has reduced binding to TOPBP1, we created a series of 53BP1 truncation mutants and assayed their ability to interact with TOPBP1. This allowed us to identify the N-terminal (1-650 amino acids) region, which appears to be sufficient for TOPBP1 binding. In yeast, the Dpb11-Rad9 interaction is regulated by CDK-targeted phosphorylation in Rad9. The analogous interaction between 53BP1 and TOPBP1 also seems to be cell cycle-dependent and we speculate that TOPBP1-53BP1 interaction is likely governed by the activity of CDK. With this rationale, we identified mutations at 6 canonical CDK target sites (S/TP to AP) in 53BP1 lead to a moderately reduced interaction with TOPBP1 (Fig4.4). However more phospho-mutations may be needed to completely abrogate the binding.

With the availability of separation of function mutants, it would be interesting to examine how the phospho-mutations in 53BP1 would impact the engagement of 53BP1 and ultimately repair pathway choice. Given that the physical interaction with TOPBP1 is required for 53BP1 to execute its function in mutagenic repair, I expect wildtype 53BP1 expression in *brca1*$^{-/-}$ *53bp1*$^{-/-}$ cells will inhibit HR, enforce NHEJ

mutagenic repair and re-sensitize them to PARP inhibitors while 53BP1 phospho-mutant would not be able to do so. HR/NHEJ repair efficiency can be assessed by monitoring 53BP1, RIF1, RPA or Rad51, which are typical markers for HR/NHEJ repair or alternatively by direct measurement of HR/NHEJ using reporter assays as previously described in the section 4.2.1.

A similar strategy can be taken to create separation of function mutants for BRCA1. Since ATR appears to play a major role inducing and stabilizing BRCA1-TOPBP1 interaction, I expect that phospho-mutations at canonical ATR/ATM target sites (S/TQ) in BRCA1 will be needed to abrogate TOPBP1-BRCA1 interaction. However, we cannot rule out a potential role of CDK here, which regulates the mirroring Dpb11-Slx4 interaction in budding yeast. Since we hypothesize that the interaction with TOPBP1 licenses the function of BRCA1 in HR repair, I expect that the BRCA1 separation of function mutant should at least partially, if not fully, mimic the *brca1*$^{-/-}$ cells and lead to defective HR repair.

4.2.4 Dissect the molecular requirements for the TOPBP1-mediated mutagenic repair function of 53BP1

While our results in mammals suggest that TOPBP1 drives 53BP1-dependent mutagenic repair at replication-induced lesions, careful delineation of this TOPBP1-53BP1-mediated repair pathway requires identification of upstream and downstream molecular components involved. First because both UDR and tudor domains in 53BP1 are known to be required for the recognition of chromatin marks and consequently its recruitment to the lesions, it would be interesting to test if mutations in these functional

domains impair the TOPBP1-mediated mutagenic repair function of 53BP1 in our system. Second, it would also be interesting to define the repair effectors required downstream of this pathway. RIF1 and PTIP-ARTEMIS (Wang et al., 2014) have been shown to be recruited via physical association with 53BP1 and are required for the anti-HR function of 53BP1, it would be interesting to test if the knockdown of RIF, PTIP or ARTEMIS would result in compromised function of 53BP1 in the mutagenic repair in our system, such as rescue of increased chromosomal aberrations induced by CTR-53BP1. Lastly what are the upstream molecular components involved? Given that TOPBP1 licenses the mutagenic repair function of 53BP1, how is TOPBP1 recruited in the first place? Because it has been shown that 9-1-1 serves to recruit TOPBP1 to the lesion sites during replication stress and the MRN complex contributes to TOPBP1 recruitment at DSB, it is tempting to speculate that these two DNA damage sensor complexes are required for proper function of TOPBP1 in promoting 53BP1-dependent mutagenic repair. Understanding the molecular requirements for the TOPBP1-mediated mutagenic repair function of 53BP1 is crucial to elucidate the mechanism of DNA repair pathway choice.

4.2.5 Define the molecular requirements for the anti-resection function of Rad9

While it is clear that Dpb11-mediated stabilization of Rad9 at the lesion sites plays a key role in resection control by counteracting the actions of nucleases and imposing a resection block, the exact molecular mechanism by which Rad9 inhibits resection is not clear. Therefore, like in mammals, it is important to define the molecular requirements that enable Rad9 to counteract resection. It would be interesting to

examine the importance of functional domains in Rad9 for its anti-resection function such as BRCT domain, Tudor domain, SQ/TQ cluster and Rad9 oligomerization etc. It is also important to find out whether certain molecular events are required in this pathway such as 9-1-1 mediated anchorage of Dpb11, Dpb11-mediated Rad9 stabilization, Ddc1 or Dpb11-dependent activation of Mec1 using the available separation of function mutants that have been characterized in the literature. Answers to these questions will certainly further our understanding of the molecular basis for the anti-resection function of Rad9 and its orthologue 53BP1 in mammals.

Figure 4.4

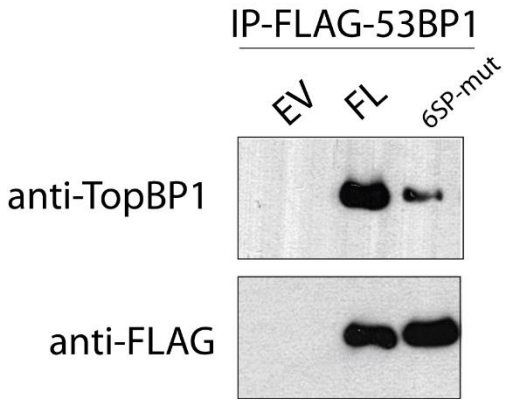

Figure 4.4 Co-IP experiment between TOPBP1 and 53BP1 showing that 6SP mutations in 53BP1 impair its physical interaction with TOPBP1. The serine/threonine to alanine mutations at 6 canonical CDK phosphorylation sites (S/TP) in 53BP1 impair its physical interaction with TOPBP1.

References

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Greenberg, R.A., B. Sobhian, S. Pathania, S.B. Cantor, Y. Nakatani, and D.M. Livingston. 2006. Multifactorial contributions to an acute DNA damage response by BRCA1/BARD1-containing complexes. *Genes & development*. 20:34-46.

Wang, J., A. Aroumougame, M. Lobrich, Y. Li, D. Chen, J. Chen, and Z. Gong. 2014. PTIP associates with Artemis to dictate DNA repair pathway choice. *Genes & development*. 28:2693-2698.

Appendix I
Supplementary tables for chapter 3

Table S1. Genotype of yeast strains used in this study.

Strain No.	Relevant Genotype	Reference
MBS164	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ::TRP1, bar1Δ::HIS3*	(Ohouo et al, 2010)
MBS448	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ::TRP1, bar1Δ::HIS3, DPB11-6HIS-3HA::LEU2*	(Ohouo et al, 2010)
MBS571	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ::TRP1, bar1Δ::HIS3, Dpb11-6HIS-3HA-LEU2, SLX4-6HIS-3FLAG::KANMX6*	(Ohouo et al, 2010)
MBS1050	*ura3-52 trp1Δ63, his3Δ200*	This study
MBS1053	*ura3-52 trp1Δ63, his3Δ200, rad9Δ::URA3*	This study
MBS1551	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ::TRP1, bar1Δ::HIS3, DPB11-6HIS-3HA::LEU2, RAD9-6HIS-3FLAG::KANMX6*	(Ohouo et al, 2013)
MBS2867	*ura3-52, trp1Δ63, his3Δ200, P_{RAD9}-Dpb11 (292-600aa)-Rad9-6xHIS-3xFLAG Chimera::KANMX6*	This study
MBS2869	*ura3-52, trp1Δ63, his3Δ200, PRAD9-dpb11-K544A (292-600aa)-Rad9-6xHIS-3xFLAG Chimera::KANMX6*	This study
Y1600 (JKM139)	MATa *ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG, ura3-52, lys5::ade3::GAL10::HO*	Pellicioli's lab
MBS2901 (JKM139)	MATa *ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG, ura3-52, lys5::ade3::GAL10::HO, rad9Δ::HPH*	Pellicioli's lab
MBS2931	*ura3-52, trp1Δ63, his3Δ200, P_{RAD9}-DPB11 (292-600aa)-Rad9-GFP Chimera::HIS3, RAD52-mRUBY2::KANMX6*	This study
MBS2933	*ura3-52, trp1Δ63, his3Δ200, P_{RAD9}-dpb11-K544A (292-600aa)-Rad9-GFP Chimera::HIS3, RAD52-mRUBY2::KANMX6*	This study
MBS2935	*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG, ura3-52, lys5::ade3::GAL10::HO, P_{RAD9}-DPB11 (292-600aa)-Rad9-3xFLAG Chimera::KANMX6*	This study
MBS2937	*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG, ura3-52, lys5::ade3::GAL10::HO, P_{RAD9}-dpb11-K544A (292-600aa)-Rad9-3xFLAG Chimera::KANMX6*	This study
MBS2939	*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG, ura3-52, lys5::ade3::GAL10::HO, P_{RAD9}-DPB11 (292-600aa)-Rad9-3xFLAG Chimera::NATMX*	
MBS2958	*ura3-52, trp1Δ63, his3Δ200, P_{RAD9}-DPB11 (292-600aa)-Rad9-GFP Chimera::HIS3, RFA1-mRUBY2::KANMX6*	This study
MBS2960	*ura3-52, trp1Δ63, his3Δ200, P_{RAD9}-dpb11-K544A (292-600aa)-Rad9-GFP Chimera::HIS3, RFA1-mRUBY2::KANMX6*	This study
MBS2986	MATa, *ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10,*	This study

	ade2Δ1, ade8, arg4Δ, sml1Δ::TRP1, bar1Δ::HIS3, Dpb11_6HIS3HA::LEU2, Slx4-6xHis-3xFlag::KANMX6, mec1Δ::NATMX	
MBS2988	MATa, *ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ::TRP1, bar1Δ::HIS3, Dpb11_6HIS3HA::LEU2, Rad9-6xHis-3xFlag::KANMX6, mec1Δ::NATMX*	This study
MBS2941	*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG, ura3-52, lys5::ade3::GAL10::HO, P_{RAD9}-dpb11-K544A (292-600aa)-Rad9-3xFLAG Chimera::NATMX*	This study
MBS2998	*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG, ura3-52, lys5::ade3::GAL10::HO, P_{RAD9}-DPB11 (292-600aa)-Rad9-3xFLAG Chimera::NATMX, P_{ADH1}-Dpb11(291-600aa)-Rtt107(742aa-end)-6xHIS-3xHA::KANMX6*	

Table S2. Plasmids used in this study.

Plasmid No.	Vector	Gene	Mutation	Tag	Reference
pMBS146	*pRS414*	-	-	-	Scott Emr lab
pMBS148	*pRS416*	-	-	-	Scott Emr lab
pMBS809	*pRS416*	P_{ADH1}*-MBD [dpb11(aa292-600)-rtt107(aa742-end) fusion]*	-	HA	Cussiol *et al., 2015*
pMBS825	*pFa6a*	*pFA6a-link-yomRuby2-KanMX6 (for mRuby2 epitope-tagging)*	-	-	Chris Fromme's lab
pMBS910	*pRS414*	P_{ADH1}*-dpb11(292-600aa)-rtt107(742aa-end)_6HIS-3HA*	-	HA	This study
pMBS912	*pRS416*	P_{RAD9}*-dpb11(292-600aa)-Rad9-6HIS-3FLAG Chimera*	-	FLAG	This study
pMBS913	*pRS416*	P_{RAD9}*-dpb11 K544A (292-600aa)-Rad9-6HIS-3FLAG Chimera*	K544A (Dpb11)	FLAG	This study
pMBS924	*pFa6a*	*Dpb11 (292-600aa)-Rad9-6HIS-3FLAG Chimera*	-	FLAG	This study
pMBS925	*pFa6a*	*Dpb11 K544A (292-600aa)-Rad9-6HIS-3FLAG Chimera*	K544A (Dpb11)	FLAG	This study
pMBS255	*pcDNA3*	-		HA	Qi Lab
pMBS252	*pcDNA3*	*Hs TopBP1 (amino acid 32-1522)*		HA	This study
pMBS946	*pEGFP-N1*	*H2B*			
AS159	*pHAGE-CMV-N-HA-FLAG-puro (gateway*				Smogorzewska Lab
pYL81	*pHAGE-CMV-N-HA-FLAG-puro (gateway)*	*53BP1*		FLAG-HA	This study
pYL105	*pHAGE-CMV-N-HA-FLAG-puro (gateway)*	*RFC1(amino acids 1-120)-53BP1*		FLAG-HA	This study

Table S3. Mass spectrometry analysis of TOPBP1 interactions in HU

HEK293T cells grown in 'light' and 'heavy' SILAC DMEM media were treated with 1mM for 24hrs to identify proteins that interact with TOPBP1 in response to replication stress using quantitative mass spectrometry analysis. In the first experiment, TOPBP1 was purified from 'light' HEK293T cells over-expressing HA-TOPBP1 using anti-HA agarose resin and 'heavy' HEK293T cells transfected with empty vector was used as control. 'light' and 'heavy' immunoprecipitated samples were then combined and subjected to mass spectrometry analysis. Proteins identified with 'light'/'heavy' ratios over 4 were considered as TOPBP1 interactor candidates, which were listed in the second column below. In a second experiment, endogenous TOPBP1 in 'heavy' cells were purified using antibodies against TOPBP1 and 'light' cells were immunoprecipitated using IgG. Similarly samples were analysis by mass spectrometry and proteins with a 'heavy'/'light' ratio over 4 were considered as TOPBP1 interactor candidates, listed in the third column below. Results from both experiment were then plotted on one scatter plot (Fig3.3a) and combined. Proteins identified with a ratio higher 4 in both experiments were designated as TOPBP1 interactors with high confidence.

protein	Log_2_gmean_HA (HA-TOPBP1/EV)	Log_2_gmean_antibody (anti-TOPBP1/IgG)	Sum of Log_2_gmean
FAM76B	9.1395	6.37412	15.51362
C12ORF32	7.06531	6.31639	13.3817
TOPBP1	5.2188	6.6847	11.9035
PARG	2.7971	7.93007	10.72717
C19ORF62	4.18129	5.77095	9.95224
BRCC3	5.50731	4.10167	9.60898
FAM175A	4.81326	4.69297	9.50623
USP34	3.60776	5.66083	9.26859
BRIP1	4.78406	4.45631	9.24037
BARD1	4.66464	4.49646	9.1611
BRE	4.19729	4.3122	8.50949
BRCA1	4.61669	3.49432	8.11101
UIMC1	3.44746	4.57485	8.02231
RFC1	4.28801	3.68687	7.97488
VPRBP	2.39743	4.731	7.12843

GINS2	4.66313	2.42857	7.0917
STUB1	4.30541	2.59218	6.89759
KIAA1524	4.05063	2.62183	6.67246
RBBP8	2.29078	4.10034	6.39112
GINS3	4.10811	2.03172	6.13983
RFC5	3.08786	3.03216	6.12002
RFC2	3.68102	2.41269	6.09371
HSPH1	3.29891	2.68872	5.98763
HUWE1	2.21355	2.91902	5.13257
APC	2.21562	2.81626	5.03188
HSPA1A,HSPA1B,HSPA6, HSPA7,HSPA1L	2.7535	2.15803	4.91153
ELP2	2.32399	2.49236	4.81635
GINS4	2.40012	2.19127	4.59139

Table S4. Mass spectrometry analysis of TOPBP1 interactions in nocodazole

Similar procedures are performed as described in Table S3 for cells treated with 100 ng/ml nocodazole for 14 hrs to define TOPBP1 interactions in G2/M.

protein	Log_2_gmean_HA (HA-TOPBP1/EV)	Log_2_gmean_antibody (anti-TOPBP1/IgG)	Sum of Log_2_gmean
TOPBP1	6.35269	7.51805	13.87074
RFC1	4.00039	4.89725	8.89764
TP53BP1	3.89157	4.42037	8.31194
SIRT1	3.8237	4.07372	7.89742
PLK1	2.55711	5.12736	7.68447
USP28	2.79535	4.86396	7.65931
BRCA1	4.32419	2.82965	7.15384
RFC3	3.06349	4.01449	7.07798
RFC5	3.30205	3.52079	6.82284
SLX4	2.47809	3.9555	6.43359
RFC4	2.93517	3.48164	6.41681
RFC2	2.6685	3.64168	6.31018
HSPH1,HSPA4,HSPA4L	3.10754	2.79045	5.89799
BARD1	2.89024	2.90297	5.79321
UIMC1	2.13456	3.46231	5.59687
RPS4Y1,RPS4XP21,RPS4Y2,RPS4X	2.89016	2.59294	5.4831
PHF8	2.59627	2.87815	5.47442
HSPA1A,HSPA1B,HSPA6, HSPA7,HSPA1L	3.13207	2.17286	5.30493
SMARCAD1	2.97361	2.33108	5.30469
HSPA1A,HSPA1B	2.85148	2.43277	5.28425
HSPA1A,HSPA1B,HSPA1L	2.82783	2.41327	5.2411
HSPA1A,HSPA1B,HSPA6,HSPA7	2.83975	2.35702	5.19677
HSPA1A,HSPA1B,HSPA6,HSPA1L	2.68452	2.39823	5.08275
HSPH1	2.56549	2.42604	4.99153
STUB1	2.51056	2.45653	4.96709
HSPA1A,HSPA1B,HSPA6	2.50868	2.08801	4.59669
HSPA2,HSPA6,HSPA8	2.28422	2.21381	4.49803
HTATSF1	2.10301	2.38395	4.48696
HSPA2,HSPA1A,HSPA1B,HSPA6, HSPA7,HSPA8,HSPA1L	2.0546	2.17001	4.22461

Table S5. Fold change analysis of TOPBP1 interactions in HU and nocodazole by mass spectrometry

HEK293T cells grown in 'heavy' and 'light' DMEM media were arrested with 1mM HU for 24hrs or with 100ng/ml nocodazole for 14hrs respectively. Endogenous TOPBP1 were purified using antibodies against TOPBP1 in both 'heavy' and 'light' lysates, which were combined and analyzed by mass spectrometer. TOPBP1 interactions defined in Fig3.3a (table S3, S4) were monitored for changes in the experiment and ratios are listed below.

protein	Log_2_gmean (HU/nocodazole)
BRIP1	3.40568
BRCA1	3.04482
BARD1	2.77511
BRE	1.04264
PARG	0.99916
C19ORF62	0.88487
BRCC3	0.63824
GINS2	0.61166
FAM175A	0.5862
UIMC1	0.53798
RBBP8	0.49421
RFC5	0.48059
ELP2	0.4713
SIRT1	0.43708
PHF8,PHF2	0.43109
C12ORF32	0.42731
RFC4	0.40272
RFC2	0.4002
RFC1	0.39062

RFC3	0.32742
STUB1	0.2183
GINS3	0.18872
GINS4	0.15367
HTATSF1	0.00876
TOPBP1	0.007
APC	-0.0568
PHF8	-0.06992
VPRBP	-0.09667
HSPA1A,HSPA1B	-0.19566
PLK1	-0.2281
HSPH1	-0.24604
HUWE1	-0.38979
SMARCAD1	-0.5129
KIAA1524	-0.85013
SLX4	-1.45355
USP28	-2.1004
TP53BP1	-2.20442

Appendix II

Supplementary results for Chapter 3

We found that the N-terminal region (1-120aa) of RFC1 (CTR) binds to the BRCT-1/2 in TOPBP1 and the mutation at threonine 110 to alanine in RFC1 abrogates its interaction with TOPBP1. Therefore we fused 53BP1 with CTR or CTR-T110A and assessed their ability to influence the recruitment of pro-NHEJ factors and genomic instability. We show that the engineered chimera CTR-53BP1 displays increased binding affinity for TOPBP1, promotes the recruitment of 53BP1/RIF1/PTIP to the lesion sites and induces chromosomal aberrations, compared with the mutated CTR-53BP1 (T110A). These results together show that the increased RIF1/PTIP recruitment to the lesion sites and the induced chromosomal aberrations indeed result from the hyper-stabilized 53BP1-TOPBP1 interaction, but not due to other unknown or indirect effects conferred by the presence of N-terminal of RFC1.

Figure S1

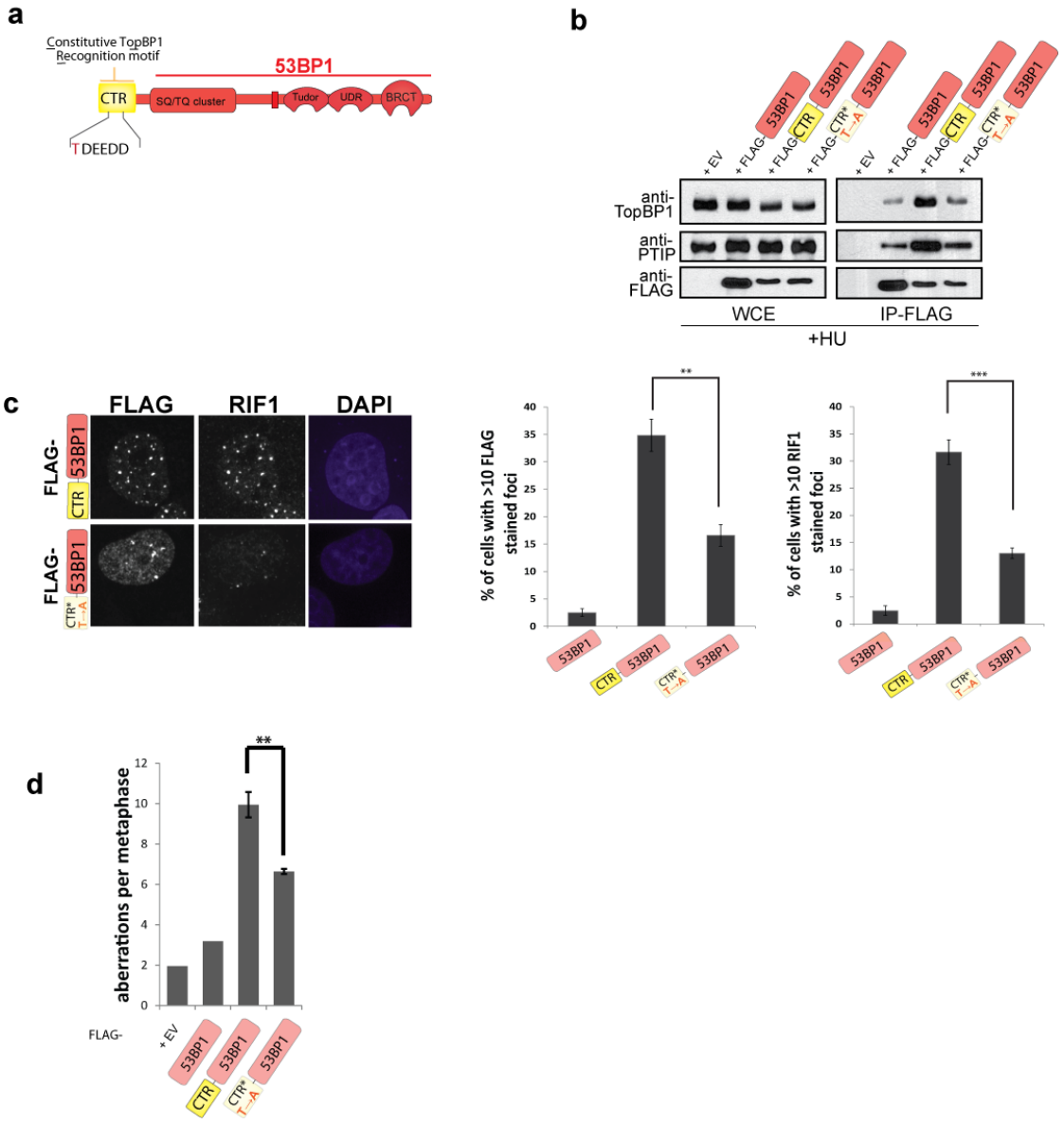

a
Constitutive TopBP1 Recognition motif
53BP1
CTR
SQ/TQ cluster
Tudor
UDR
BRCT
TDEEDD
b
+ EV
+ FLAG-53BP1
+ FLAG-53BP1 CTR
+ FLAG-53BP1 CTR* T→A
anti-TopBP1
anti-PTIP
anti-FLAG
WCE
IP-FLAG
+HU
c
FLAG
RIF1
DAPI
FLAG-53BP1 CTR
FLAG-53BP1 CTR* T→A
% of cells with >10 FLAG stained foci
% of cells with >10 RIF1 stained foci
**
***
d
aberrations per metaphase
FLAG-
+ EV
53BP1
CTR
CTR* T→A

Figure S1. The engineered chimera CTR-53BP1 displays increased binding affinity for TOPBP1, promotes the recruitment of 53BP1/RIF1/PTIP to the lesion sites and induces chromosomal aberrations, compared with the mutated CTR-53BP1 (T110A). (a) Schematic illustration of CTR-53BP1 fusion. (b) Co-IP experiment showing that CTR-53BP1 hyper-stabilizes the TOPBP1-53BP1 interaction and promotes the association of PTIP with 53BP1. (c) Immunofluorescence of the U2OS cells transiently transfected with CTR-53BP1 and CTR-53BP1 (T110A) after 3hrs release from a 24hrs HU arrest. 53BP1 and RIF1 are more efficiently recruited to the nuclear foci in response to the replication stress. (d) CTR-53BP1 chimera induces increased chromosomal aberrations, as compared to the mutated CTR-53BP1 (T110A).

Appendix III

The phospho-regulation of human SLX4 in DNA damage response

Human SLX4/FANCP is a DNA repair protein that has been shown to be a target of checkpoint signaling (Matsuoka et al., 2007). Mutations in the Slx4 gene were recently linked to a hereditary disease, Fanconi anemia, featured with bone marrow failure, congenital defects, developmental disabilities, and, in particular, a strong susceptibility to cancer development (Crossan et al., 2011; Kim et al., 2011; Stoepker et al., 2011). SLX4/FANCP is a conserved repair scaffold protein through yeast and vertebrate species (Fekairi et al., 2009) (Fig S2a). Mammalian SLX4 acts as a docking platform to coordinate the actions of several structure-specific endonucleases to resolve different damaged DNA intermediate structures (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). While the repair role of SLX4 is partially appreciated, the molecular mechanism by which SLX4 repair function is regulated is not yet clear. In the past few years, our lab has extensively studied the role of yeast Slx4 in DDR and uncovered an unexpected role of Slx4 in the modulation of checkpoint signaling and resection control, placing Slx4 clearly at the interface of checkpoint signaling and DNA repair. To expand our studies from yeast Slx4 to mammalian SLX4 and further understand whether the repair function of human SLX4 is regulated by the ATR/ATM-mediated checkpoint signaling, we used both the Slx4 gene-targeted mice and Fanconi anemia patient cells lacking functional SLX4 in conjunction with quantitative proteomics approach to study its role in DNA repair.

While in budding yeast, Slx4 forms a stable complex with Dpb11 to dampen checkpoint signaling and promote resection, we found that the interaction is highly conserved from yeast to humans. Human SLX4 is among the TOPBP1 interactors we identified and this interaction is further confirmed by western blot and also reciprocal

co-immunoprecipitation (Fig S2b-c). Since SLX4 has been shown to be a target of the ATR/ATM checkpoint kinases (Matsuoka et al., 2007), we hypothesized that ATR/ATM-dependent phosphorylation of SLX4 may regulate its binding to TOPBP1. In order to map the region in SLX4 that mediates the interaction with TOPBP1, we created a series of human SLX4 truncation mutants and assessed their ability to interact with TOPBP1. However we found that multiple regions in SLX4 are capable of binding to TOPBP1, and this renders it difficult to locate one specific TOPBP1-interacting motif in SLX4 (Fig S3).

To further understand the phospho-regulation of SLX4, we mapped the phosphorylation sites in mouse SLX4 using a quantitative proteomic approach (Fig S4a). Interestingly, we identified 6 canonical ATR/ATM target phosphorylation sites (S/TQ) in SLX4 being inducibly phosphorylated upon IR or MMC treatment (Fig S4b). In addition, we found that SLX4 is constitutively phosphorylated at many other serine or threonine residues including multiple canonical CDK target phosphorylation sites (S/TP).

In order to further understand the role of ATR/ATM-dependent phosphorylation of SLX4 in DDR, we created 3 human SLX4 phospho-mutants (SLX4-5mut, SLX4-7mut, SLX4-13mut) with mutations at conserved ATR/ATM target phosphorylation sites and assessed their ability to complement the MMC sensitivity in Fanconi anemia patient skin fibroblast cells lacking functional SLX4 (Fig S5a). We found that all these 3 phospho-mutants lead to sensitivity to MMC and, interestingly, a single point mutation at T860 to alanine results in a similar level of MMC sensitivity (Fig S5b-d), suggesting that T860 phosphorylation by ATR/ATM confers cellular resistance to MMC. Despite the MMC sensitivity caused by the phospho-mutations in SLX4, neither SLX4-13mut nor SLX4-5mut displays any changes in their ability to bind to TOPBP1 (Fig S5e).

Lastly we measured how the counterpart of human SLX4-5mut in mouse impacts the interaction network of SLX4 (Fig S6a). Interestingly, we found a slight increase in the interactions of SLX4-5mut with PLK1 and the nucleases including ERCC1, ERCC4, EME1, MUS81 and SLX1, as compared to wildtype SLX4 (Fig S6b). However whether the increased interaction with the nucleases is causative of the MMC sensitivity is yet to be determined.

Taken together, our results point to a model whereby TOPBP1 may play a role in the recruitment of SLX4 to the proximity of the checkpoint kinases ATR/ATM in order to promote SLX4 phosphorylation by ATR/ATM. The ATR/ATM-dependent phosphorylation of SLX4 then modulates the interactions of SLX4 with other structure-specific endonucleases and allows proper lesion processing and repair to take place (Fig S6c).

Figure S2

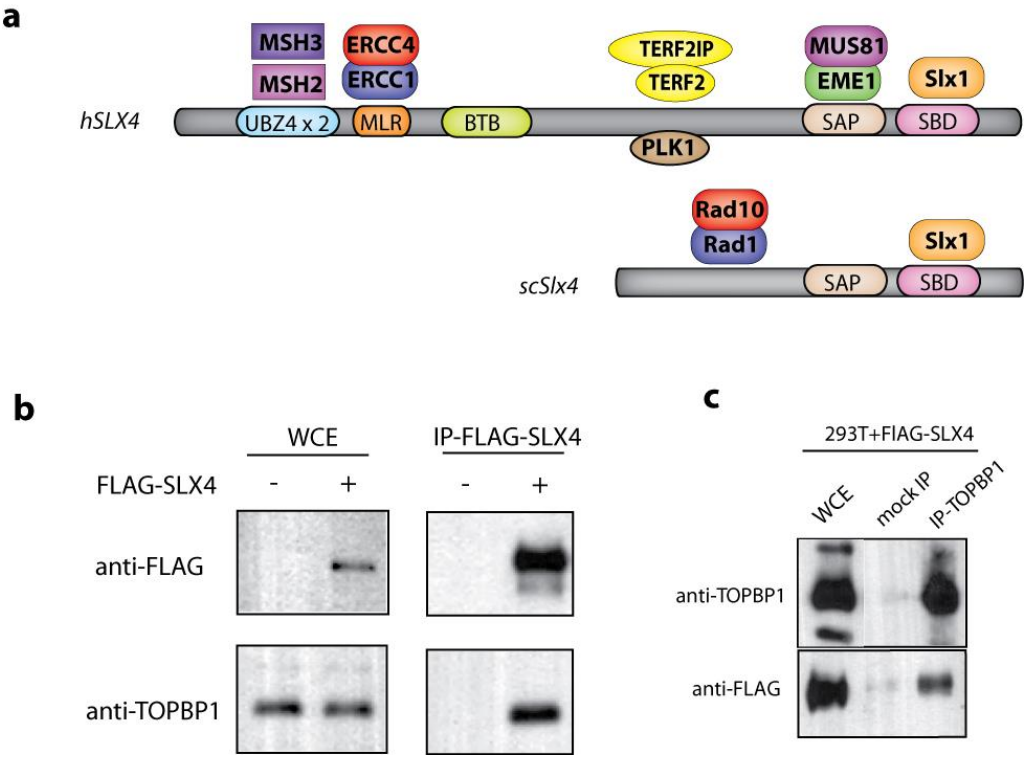


Figure S2. TOPBP1-SLX4 interaction is evolutionarily conserved in humans. (a) Schematic illustration of the human and yeast Slx4 domain structures with known interactions. (b)-(c) Co-IP experiment confirming the interaction of TOPBP1 with human SLX4.

Figure S3

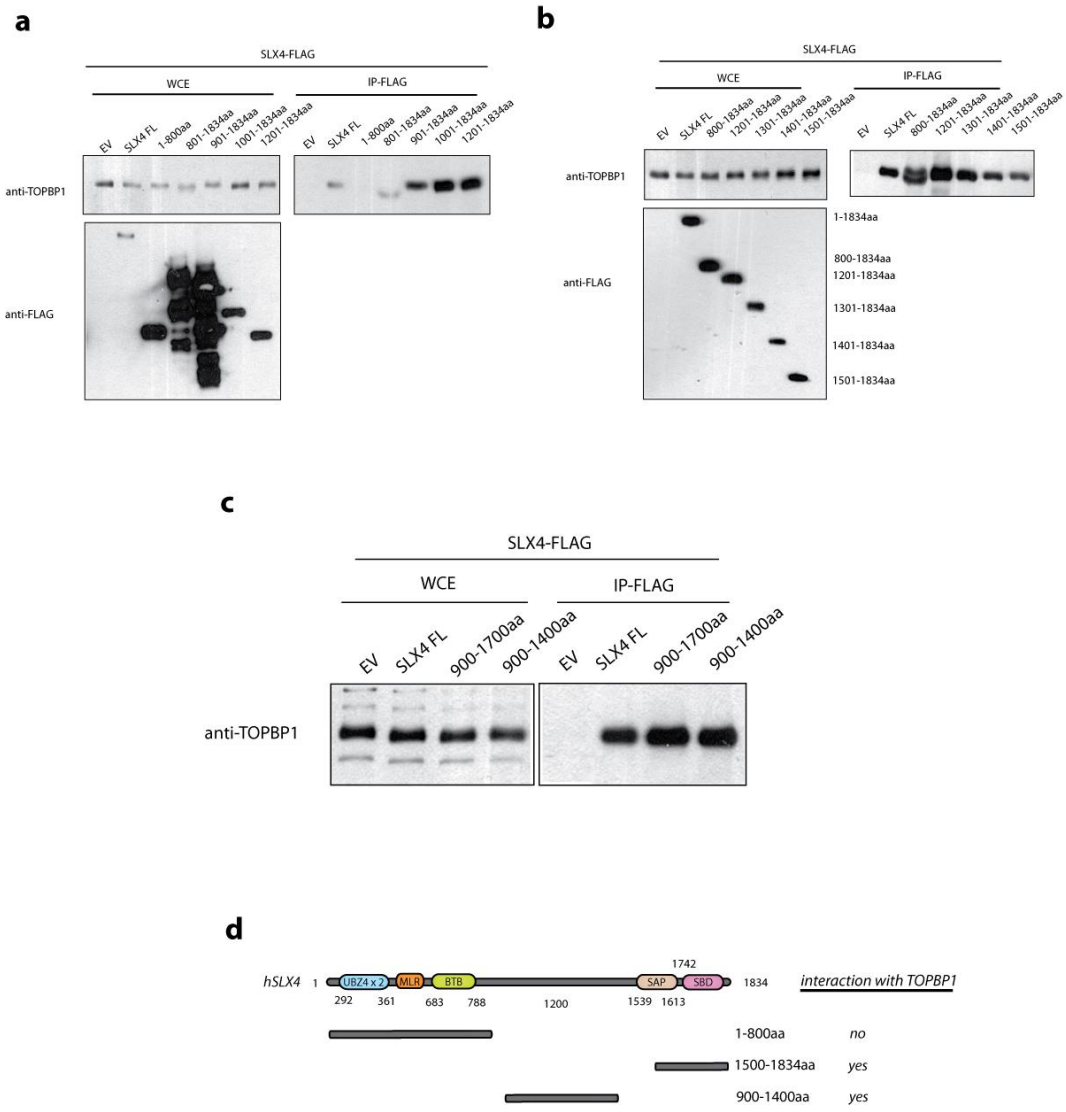


Figure S3 Multiple different domains in human SLX4 mediate its interaction with TOPBP1. (a) Co-IP experiment between TOPBP1 and different SLX4 truncation mutants showing that the C-terminus, but not the N-terminus, of SLX4 is required for its interaction with TOPBP1. (b)-(c) Co-IP between TOPBP1 and different SLX4 truncation mutants showing that multiple regions in the C-terminus of SLX4 are capable of binding to TOPBP1.

Figure S4

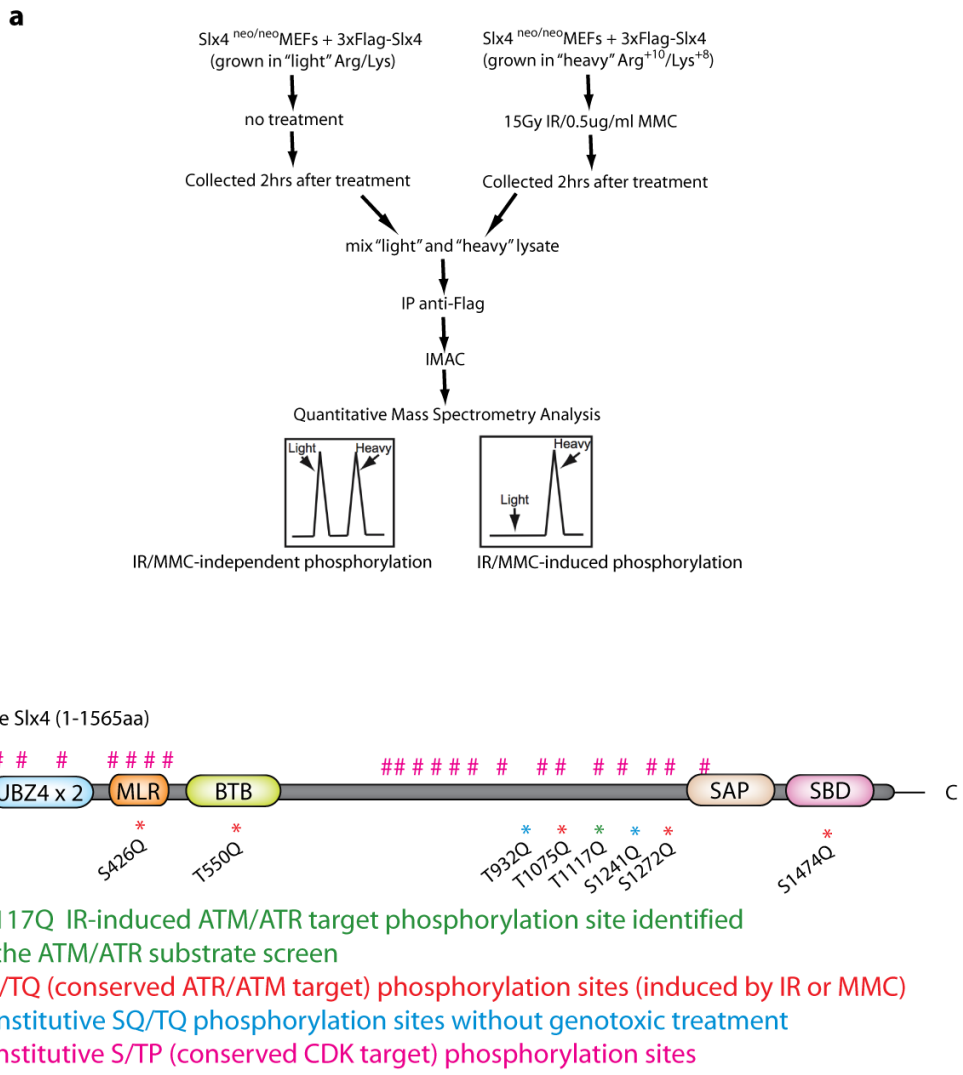


Figure S4 Mapping the phosphorylation sites in mouse SLX4. (a) Detailed experimental procedures for mapping the phosphorylation sites in mouse SLX4. (b) Schematic illustration of the phosphorylation sites in mouse SLX4 identified by mass spectrometry analysis.

Figure S5

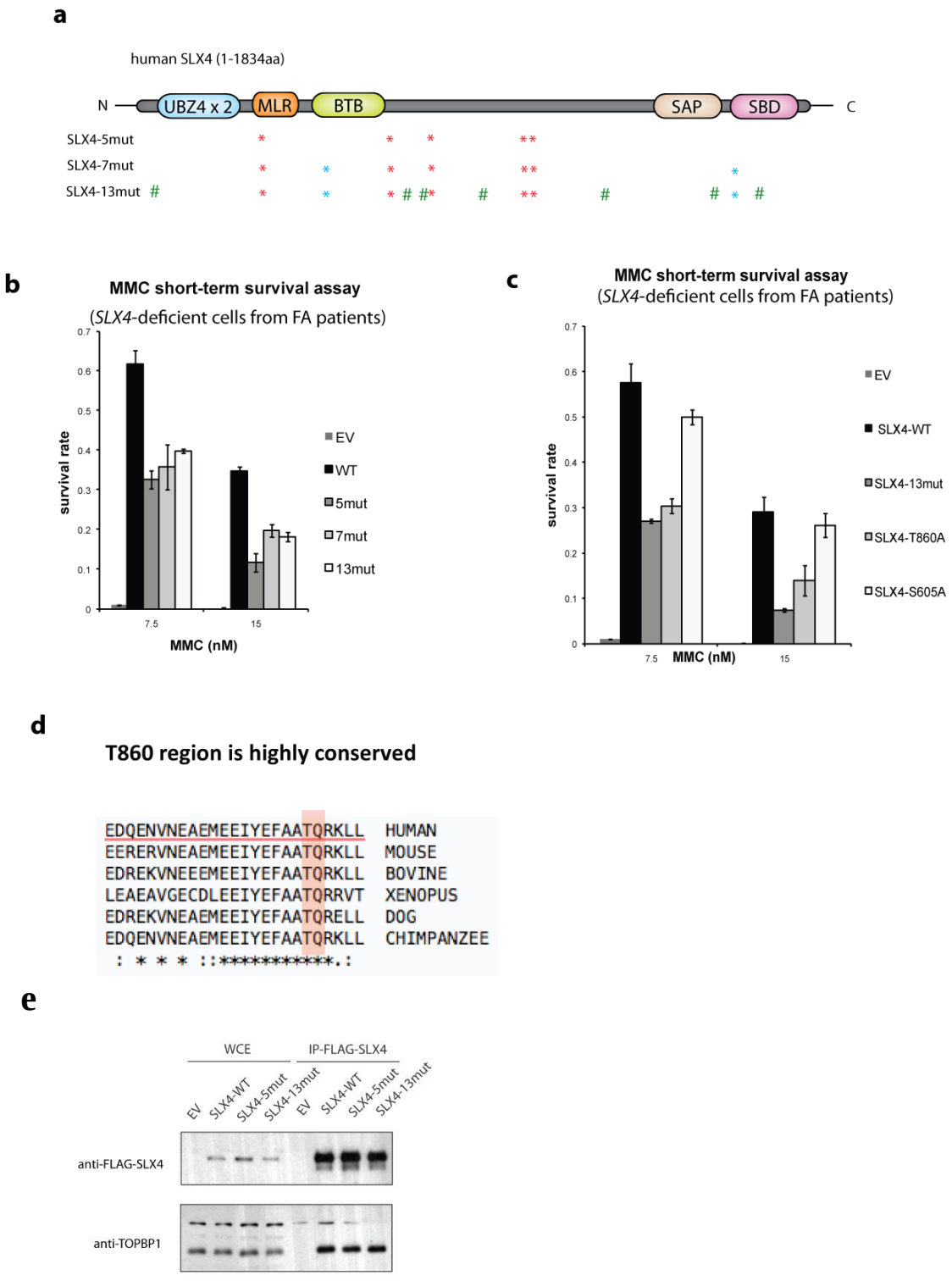

a
human SLX4 (1-1834aa)
N
UBZ4 x 2
MLR
BTB
SAP
SBD
C
SLX4-5mut
SLX4-7mut
SLX4-13mut
b
MMC short-term survival assay
(SLX4-deficient cells from FA patients)
survival rate
MMC (nM)
EV
WT
5mut
7mut
13mut
c
MMC short-term survival assay
(SLX4-deficient cells from FA patients)
survival rate
MMC (nM)
EV
SLX4-WT
SLX4-13mut
SLX4-T860A
SLX4-S605A
d
T860 region is highly conserved
EDQENVNEAEMEEIYEFAATQRKLL HUMAN
EERERVNEAEMEEIYEFAATQRKLL MOUSE
EDREKVNEEEMEEIYEFAATQRKLL BOVINE
LEAEAVGECDLEEIYEFAATQRRVT XENOPUS
EDREKVNEAEMEEIYEFAATQRELL DOG
EDQENVNEAEMEEIYEFAATQRKLL CHIMPANZEE
e
WCE
IP-FLAG-SLX4
EV
SLX4-WT
SLX4-5mut
SLX4-13mut
anti-FLAG-SLX4
anti-TOPBP1

Figure S5 The phosphorylation at a canonical ATR/ATM target site in human SLX4, threonine T860, is required for cellular resistance to MMC. (a) Schematic illustration of the human SLX4 phospho-mutants with mutations at the canonical ATR/ATM phosphorylation sites. (b)-(c) Short-term survival assays showing the ability of different SLX4-phosphomutants (SLX4-5mut, SLX4-7mut, SLX4-13mut, SLX4-T860A, SLX4-S605A) to complement the MMC sensitivity of human skin fibroblasts cells from Fanconi anemia patients with biallelic truncations in FANCP/SLX4. (d) The sequence alignment of SLX4 from different species showing the conservation flanking the T860 site, the mutation of which leads to MMC sensitivity. (e) Co-IP experiment determining the TOPBP1 interaction with SLX4-5mut and 13mut.

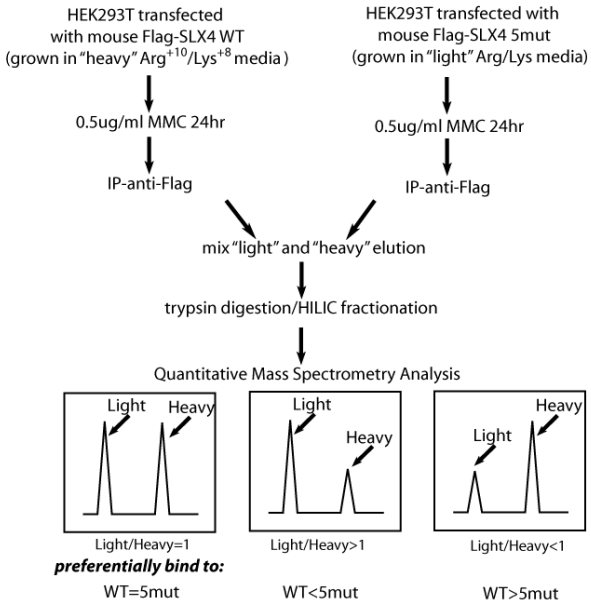
SLX4-5mut: SLX4_S605A_T860A_T1127A_T1271A_T1273A

SLX4-7mut: S605A_T735A_T860A_T1127A_S1271A_T1273A_S1699A

SLX4-13mut: SLX4-7mut+ S186A_T990A_S1204A_T1484A_S1617A_S1716A.

Figure S6

a


HEK293T transfected with mouse Flag-SLX4 WT (grown in "heavy" Arg+10/Lys+8 media)
HEK293T transfected with mouse Flag-SLX4 5mut (grown in "light" Arg/Lys media)
0.5ug/ml MMC 24hr
0.5ug/ml MMC 24hr
IP-anti-Flag
IP-anti-Flag
mix "light" and "heavy" elution
trypsin digestion/HILIC fractionation
Quantitative Mass Spectrometry Analysis
Light
Heavy
Light
Heavy
Heavy
Light
Light/Heavy=1
Light/Heavy>1
Light/Heavy<1
preferentially bind to:
WT=5mut
WT<5mut
WT>5mut

b

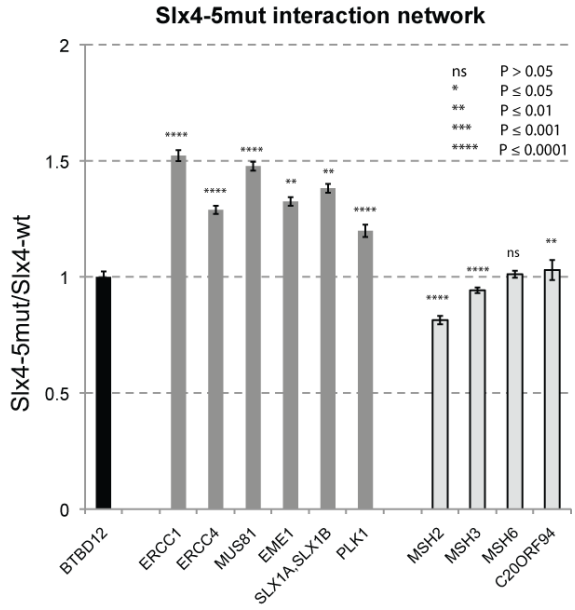

Slx4-5mut interaction network
Slx4-5mut/Slx4-wt
2
1.5
1
0.5
0
ns P > 0.05
* P ≤ 0.05
** P ≤ 0.01
*** P ≤ 0.001
**** P ≤ 0.0001
BTBD12
ERCC1
ERCC4
MUS81
EME1
SLX1A,SLX1B
PLK1
MSH2
MSH3
MSH6
C20ORF94

Figure S6

c

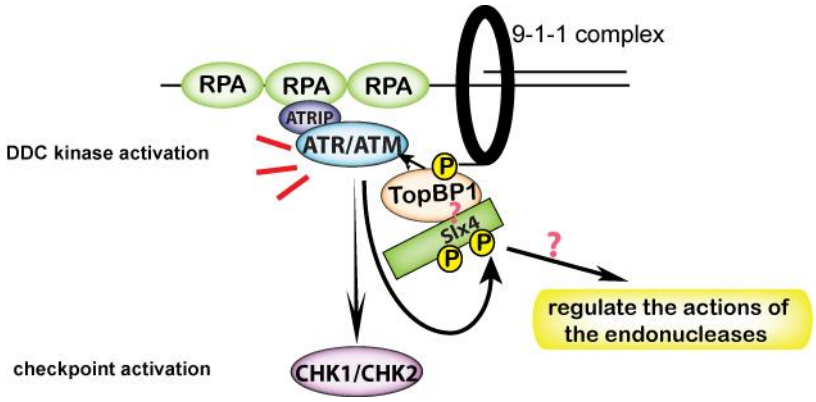

9-1-1 complex
RPA
RPA
RPA
ATRIP
ATR/ATM
DDC kinase activation
P
TopBP1
?
Six4
P
P
?
regulate the actions of the endonucleases
checkpoint activation
CHK1/CHK2

Figure S6 Mass spectrometry analysis comparing the interaction network of wildtype SLX4 and SLX4-5mut with 5 mutations at the canonical ATR/ATM target phosphorylation sites (S/TQ). (a) A flow chart showing the procedures of mass spectrometry analysis. (b) Mutations at the canonical ATR/ATM target phosphorylation sites (SLX4-5mut) result in slightly increased binding to the PLK1 and the nucleases including ERCC1, ERCC4, MUS81, EME1 and SLX1. (c) A working model for the role of Slx4 in the coordination of DDC signaling and DNA repair.

Mouse SLX4-5mut: SLX4_S426A_T674A_S932A_S1073A_T1075A

Reference

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Appendix IV

Investigating the functional relevance of the TOPBP1-RFC1 interaction

In our proteomics analysis, we found that RFC1, the largest subunit of the PCNA clamp loader RFC complex, binds TOPBP1 at nearly stoichiometric levels and is one of the most represented TOPBP1 interactors. This TOPBP1-RFC1 interaction is confirmed in both HEK293T and HeLa cell lines by co-immunoprecipitation and western blotting analysis (Fig S7a). We hypothesized that this interaction, like many other TOPBP1-mediated interactions, is regulated in a phosphorylation-dependent manner. Indeed, when we used recombinantly purified BRCT domains 1/2 and 4/5 of TOPBP1 to pull down RFC1 using lysates from HU-treated cells, we found that BRCT 1/2 but not BRCT4/5, specifically recognized RFC1 (Fig S7b). Consistently, mutations in BRCT1 (K154A, K155A) and BRCT2 (K250A) impair the binding of TOPBP1 to RFC1 (Fig S7c), suggesting that the TOPBP1-RFC1 interaction is mediated by the BRCT 1/2 in TOPBP1. Further, in order to identify the phosphorylation motif in RFC1 that is recognized by BRCT 1/2, we created a series of RFC1 truncation mutants and assessed their ability to bind to TOPBP1 by co-IP (Fig S7d-e, Fig S8). While 101aa-end truncation of RFC1 is fully competent in binding to TOPBP1, 116aa-end fragment barely pulls down any TOPBP1. This suggests that a phosphorylation motif in the 101-116aa region is likely being responsible for the binding of RFC1 to the BRCT1/2 of TOPBP1. Indeed, the sequence within the 101-116aa of RFC1, featured by a highly acidic region, highly resembles sequences in Rad9 that are known to be important for the TOPBP1-Rad9 interaction (Fig S7f). Interestingly, we find that the substitution of

threonine 110 for alanine abrogates the binding of RFC1 to TOPBP1 (Fig S7g). Collectively, these results suggest that TOPBP1-RFC1 interaction is mediated through the recognition of the phosphorylated T110 in RFC1 by the BRCT 1/2 of TOPBP1.

In order to understand the functional relevance of the TOPBP1-RFC1 interaction, we took advantage of this separation of function mutant, RFC1 T110A, which specifically disrupts the interaction with TOPBP1, and knocked in this point mutation RFC1 T109A in mouse by CRISPR. Considering the striking similarity of this interaction with TOPBP1-Rad9 binding, we hypothesized that RFC1-TOPBP1 interaction may function as a parallel mechanism to recruit TOPBP1 and promote ATR activation, independent of 9-1-1 complex. However, no striking growth or developmental defect was observed in the $RFC1^{T109A/T109A}$ mice and no significant checkpoint defect or hyper-sensitivity to genotoxins was detected in $RFC1^{T109A/T109A}$ MEFs as compared to wildtype (data not shown). Yet, this does not rule out the possibility that RFC1 and 9-1-1 complex may act in redundant pathways for TOPBP1 recruitment and ATR activation. Therefore, we crossed $RFC1^{T109A/T109A}$ mice with $HUS1^{neo/ex}$ mice, a subunit of 9-1-1, with the expectation that $HUS1^{neo/ex}$ provides a sensitized background to reveal the phenotype of $RFC1^{T109A/T109A}$. We performed the micronucleus assay to assess the genomic instability of the double mutant mice and monitored the growth by weekly weight measurement. However we could not detect any striking difference in weight or micronuclei level when we compare the $RFC1^{T109A/T109A}$ $HUS1^{neo/ex}$ mice with the $RFC1^{+/T109A}$ $HUS1^{neo/ex}$ mice (Fig S9-S10). Nevertheless, $RFC1^{T109A/T109A}$ $HUS1^{neo/ex}$ male mice show a very mild yet noticeable growth retardation and increased genomic instability as compared to the $RFC1^{+/T109A}$

HUS1$^{neo/ex}$ male mice (Fig S9-S10). The validity of this results needs to be further confirmed by repeating the experiment. We also challenged the mice with genotoxins and monitored their response to MMC treatment by measuring their body weight daily. However, no clear result was obtained due to the availability of the small number of animals and variation between different animals (Fig S11). Further repeat is needed in order to reach a conclusion here.

Taken together, our work revealed a novel phosphorylation-dependent interaction between TOPBP1 and RFC1. However, by using the mouse model, we could not clearly define the functional relevance of TOPBP1-RFC1 by far. Yet, we cannot rule out the possibility that RFC1 together with Rad9 and other TOPBP1-interacting factors may function redundantly to recruit TOPBP1 and activate ATR. In this case, simultaneous knockout of other factors in RFC1$^{T109A/T109A}$ mice may be required in order to reveal the importance of the TOPBP1-RFC1 interaction.

Figure S7

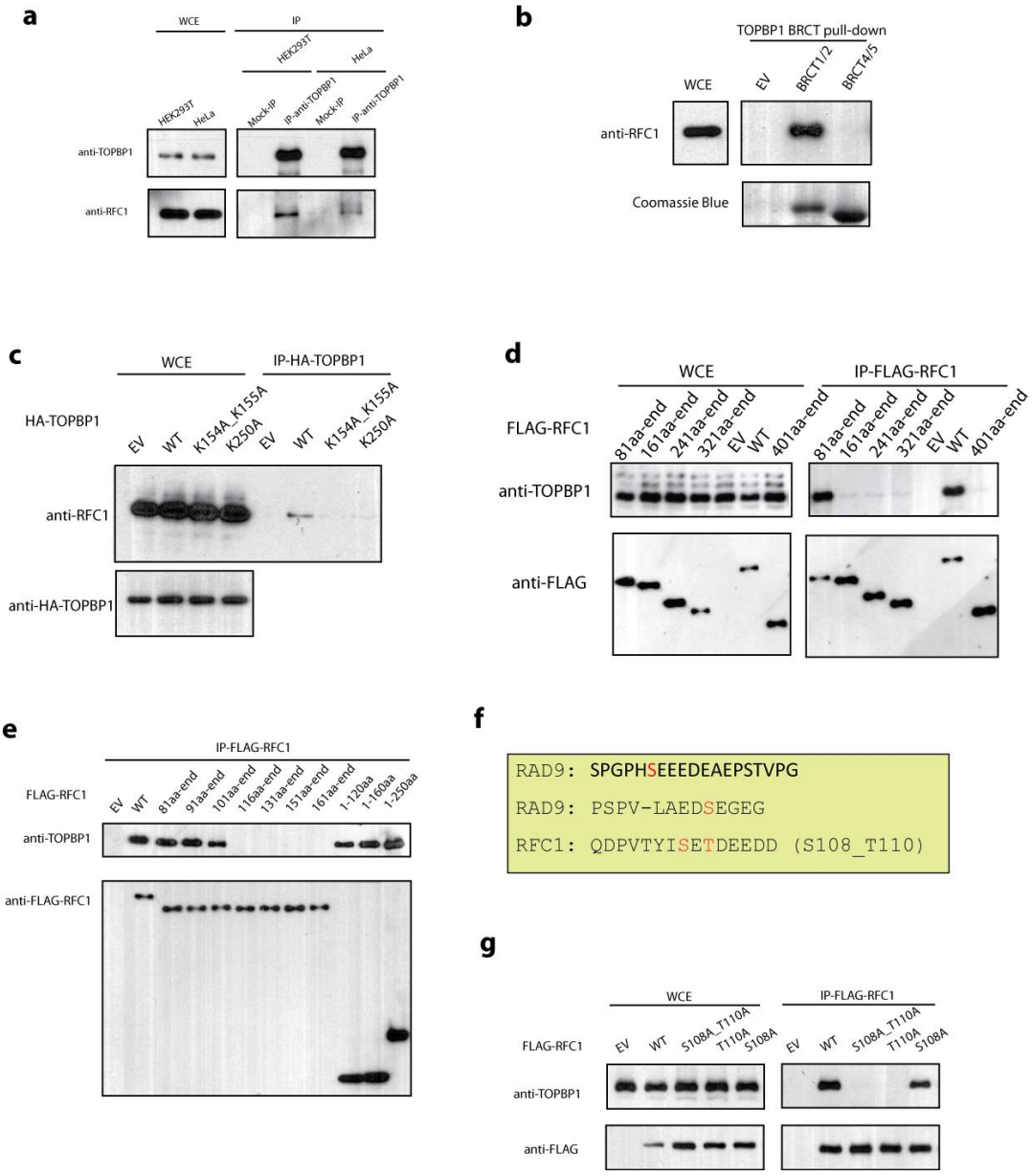

a
WCE
IP
HEK293T
HeLa
HEK293T
HeLa
Mock-IP
IP-anti-TOPBP1
Mock-IP
IP-anti-TOPBP1
anti-TOPBP1
anti-RFC1
b
TOPBP1 BRCT pull-down
WCE
EV
BRCT1/2
BRCT4/5
anti-RFC1
Coomassie Blue
c
WCE
IP-HA-TOPBP1
HA-TOPBP1
EV
WT
K154A_K155A
K250A
EV
WT
K154A_K155A
K250A
anti-RFC1
anti-HA-TOPBP1
d
WCE
IP-FLAG-RFC1
FLAG-RFC1
81aa-end
161aa-end
241aa-end
321aa-end
EV
WT
401aa-end
81aa-end
161aa-end
241aa-end
321aa-end
EV
WT
401aa-end
anti-TOPBP1
anti-FLAG
e
IP-FLAG-RFC1
FLAG-RFC1
EV
WT
81aa-end
91aa-end
101aa-end
116aa-end
131aa-end
151aa-end
161aa-end
1-120aa
1-160aa
1-250aa
anti-TOPBP1
anti-FLAG-RFC1
f
RAD9: SPGPHSEEEDEAEPSTVPG
RAD9: PSPV-LAEDSEGEG
RFC1: QDPVTYISETDEEDD (S108_T110)
g
WCE
IP-FLAG-RFC1
FLAG-RFC1
EV
WT
S108A_T110A
T110A
S108A
EV
WT
S108A_T110A
T110A
S108A
anti-TOPBP1
anti-FLAG

Figure S7 In humans, TOPBP1 stably interacts with RFC1 in a phosporylation-dependent manner. (a) Co-IP experiments between TOPBP1 and RFC1 in both HEK293T cells and HeLa cell lines. (b) Pull-down of RFC1 from human HEK293T lysates using recombinant BRCT1/2 and BRCT4/5 from TOPBP1; coomassie blue staining is also shown. RFC1 is specifically recognized by the BRCT1/2, but not the BRCT4/5 of TOPBP1. (c) Co-IP experiment determining the effects of TOPBP1 BRCT1 and BRCT2 mutations on TOPBP1-RFC1 interaction. (d)-(e) Co-IP experiments to map the region in RFC1 that mediates its interaction with TOPBP1 using different truncation mutants of RFC1. (f) Manual alignment of the sequneces in Rad9 and RFC1 that are important for binding to TOPBP1. (g) Co-IP experiment determining the importance of S108 and T110 in RFC1 for its binding to TOPBP1.

Figure S8

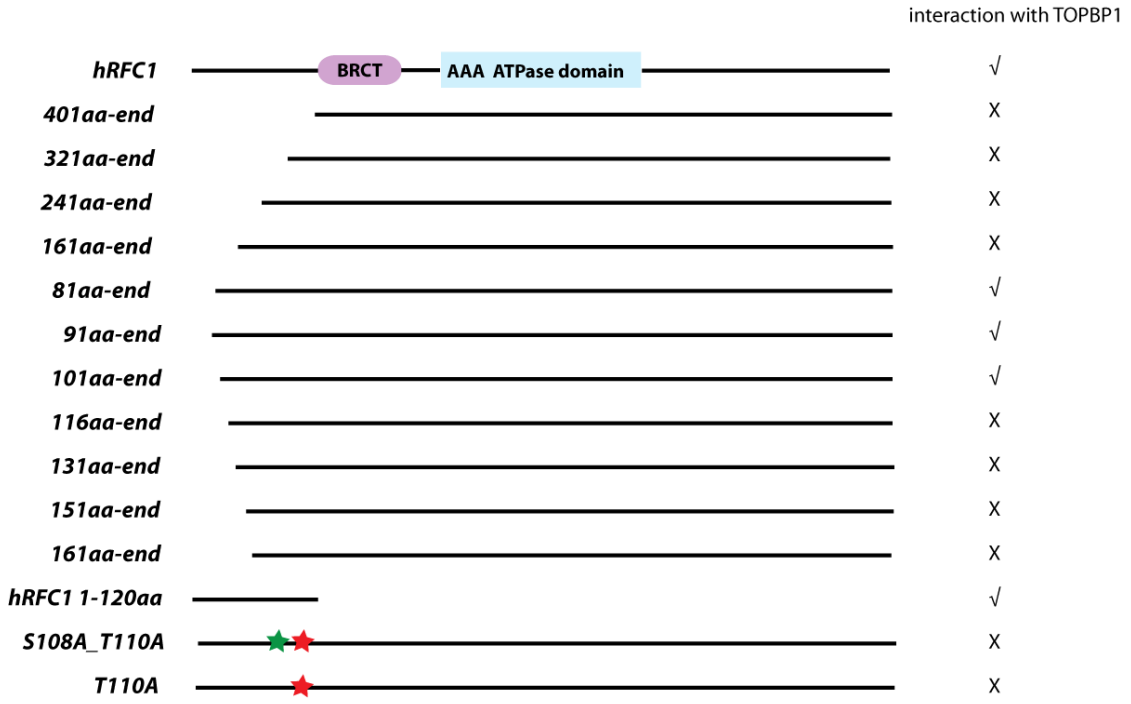


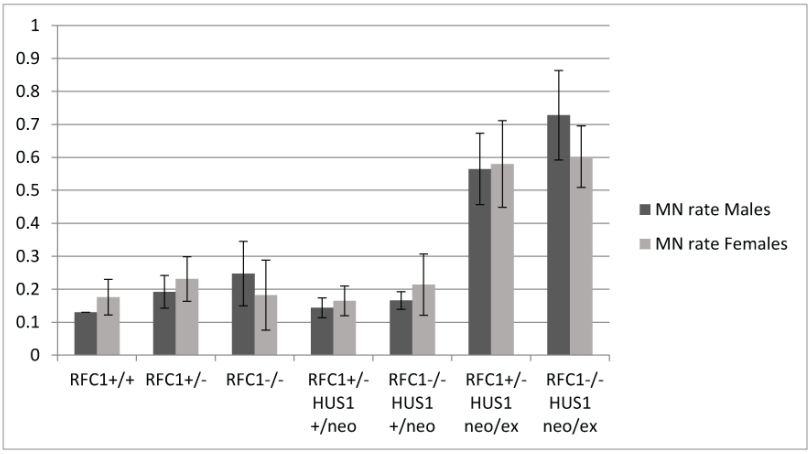
Figure S8 Summary of the interaction of TOPBP1 with different RFC1 truncation mutants.

Figure S9

a

		$RFC1^{+/+}$	$RFC1^{+/-}$	$RFC1^{-/-}$	$RFC1^{+/-}$ $HUS1^{+/neo}$	$RFC1^{-/-}$ $HUS1^{+/neo}$	$RFC1^{+/-}$ $HUS1^{neo/ex}$	$RFC1^{-/-}$ $HUS1^{neo/ex}$
MN rate	Males	0.130	0.192	0.248	0.144	0.166	0.565	0.728
stdev		0.000	0.050	0.098	0.030	0.027	0.108	0.135
n		1.000	5.000	4.000	5.000	5.000	4.000	5.000
		$RFC1^{+/+}$	$RFC1^{+/-}$	$RFC1^{-/-}$	$RFC1^{+/-}$ $HUS1^{+/neo}$	$RFC1^{-/-}$ $HUS1^{+/neo}$	$RFC1^{+/-}$ $HUS1^{neo/ex}$	$RFC1^{-/-}$ $HUS1^{neo/ex}$
MN rate	Females	0.176	0.231	0.182	0.165	0.214	0.580	0.602
stdev		0.054	0.068	0.106	0.045	0.093	0.132	0.094
n		5.000	8.000	5.000	2.000	5.000	4.000	5.000

b



c

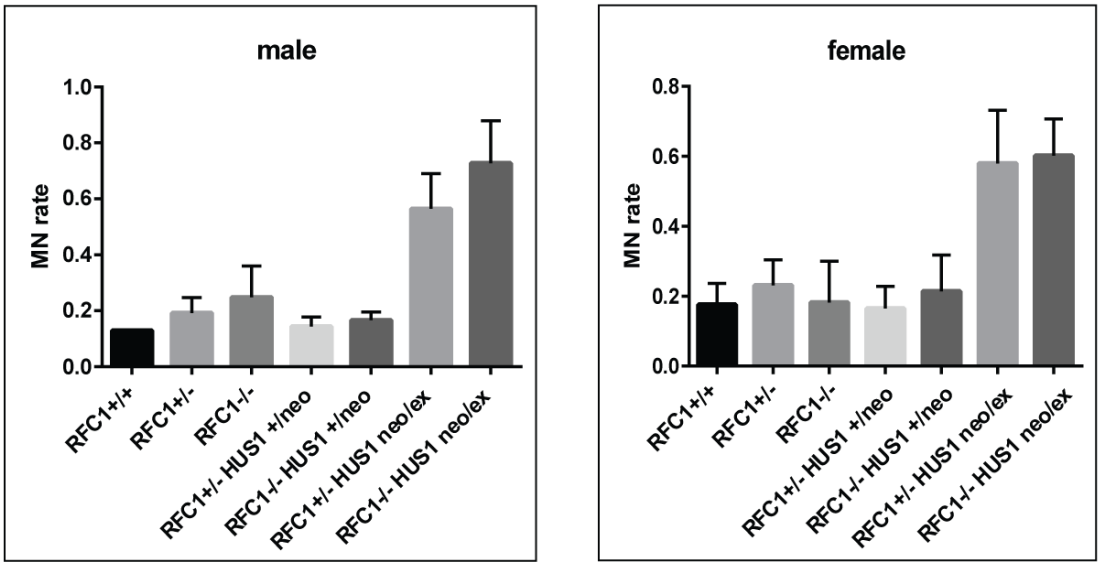
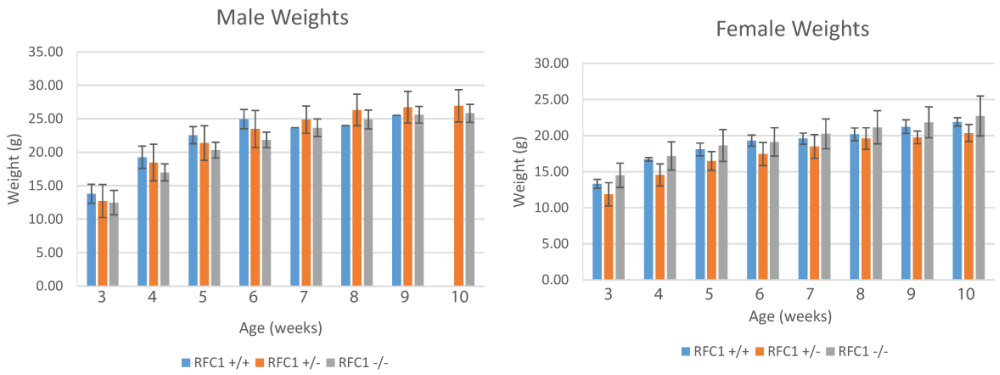


Figure S9 Summary of the micronuclei assay results. $RFC1^{+/+}$, wildtype; $RFC1^{+/-}$, $RFC1^{+/T109A}$; $RFC1^{-/-}$, $RFC1^{T109A/T109A}$.

Figure S10

a



b

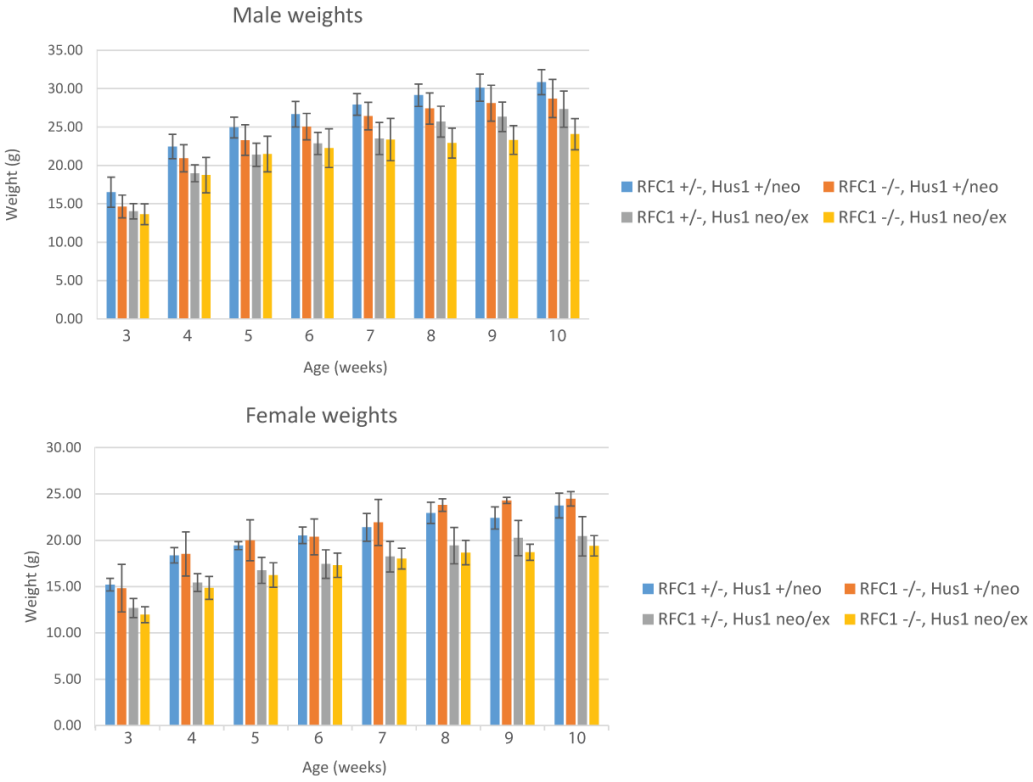
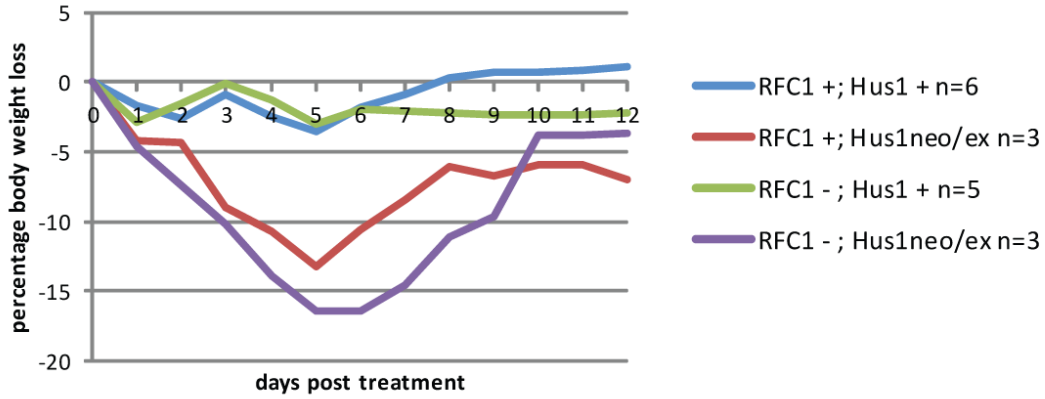


Figure S10 Weight results of the mice categorized by genotypes. $RFC1^{+/+}$, wildtype; $RFC1^{+/-}$, $RFC1^{+/T109A}$; $RFC1^{-/-}$, $RFC1^{T109A/T109A}$.

Figure S11

a



b

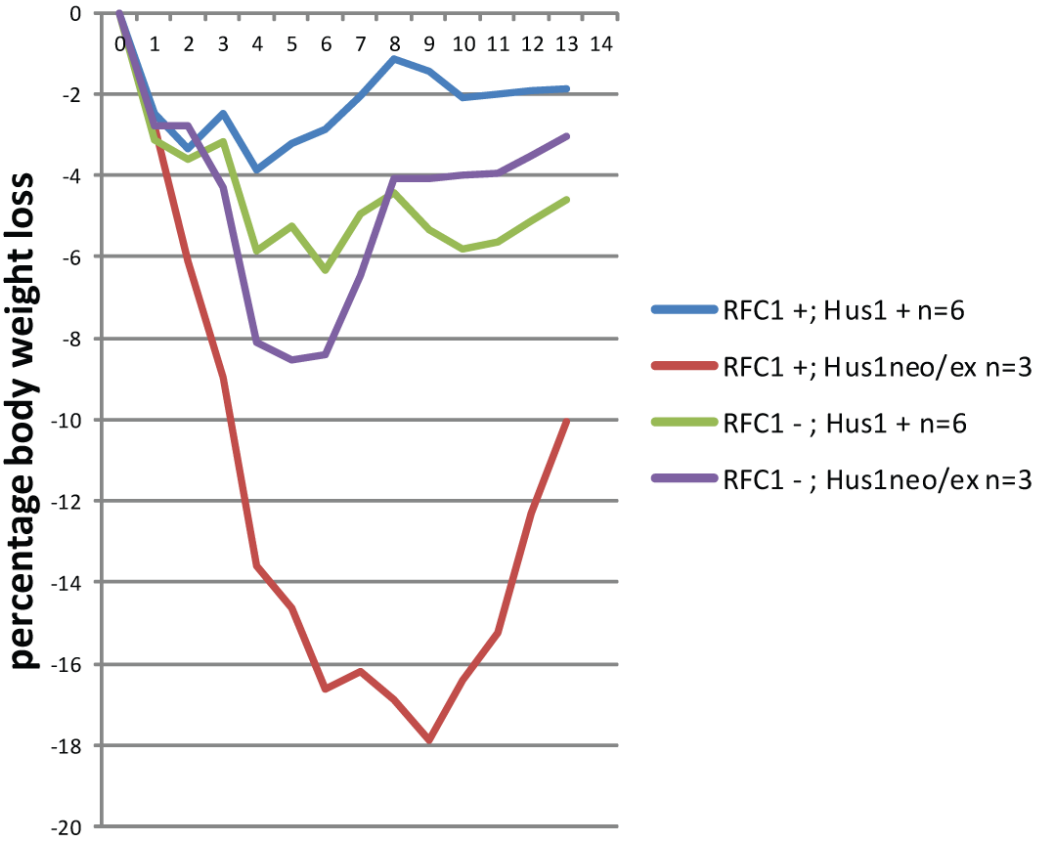


Figure S11 Weight loss curve after MMC injection categorized by genotypes.

Appendix V

TOPBP1 Takes RADical Command in Recombinational DNA Repair*

Yi Liu and Marcus B. Smolka

*This comment has been published as **Liu, Y.**, and Smolka, M.B. (2016). TOPBP1 takes RADical command in recombinational DNA repair. The Journal of cell biology 212, 263-266.

TOPBP1 is a key player in DNA replication and DNA damage signaling. Moudry et al. uncover a crucial role for TOPBP1 in DNA repair by revealing its requirement for RAD51 loading during repair of double strand breaks by homologous recombination.

Proper replication and maintenance of the eukaryotic genome requires the involvement of the scaffolding protein TOPBP1. Over the last 20 years, studies in yeast, frog, and mammals have revealed conserved roles for TOPBP1 in initiation of DNA replication and activation of DNA damage signaling. TOPBP1 has been shown to assemble ternary protein complexes necessary to "jump-start" DNA replication or to initiate DNA damage signaling events by recognizing distinct phosphoproteins via its multiple BRCA1 C-Terminus (BRCT) domains (Fig S12d) (Wardlaw et al., 2014). In this issue, Moudry et al. add to the list of crucial TOPBP1 roles in genome biology and reveal that TOPBP1 is also required for proper repair of double strand breaks by homologous recombination (HR).

Bartek and colleagues report that depletion of TOPBP1 makes cells highly sensitive to the PARP inhibitor olaparib, a drug known to sensitize cells with an already dysfunctional HR machinery. In particular, olaparib hyper-sensitizes cells that carry

mutations in the bona-fide HR factors and tumor suppressors *BRCA1* or *BRCA2*. In this work, the authors first identified TOPBP1 as a hit in a high-content RNAi screen for proteins whose depletion resulted in higher toxicity after olaparib treatment in osteosarcoma cells, which suggests that loss or inactivation of TOPBP1 predicts the response of cancer cells to this drug. Moudry et al. observed that RNAi-mediated knockdown of TOPBP1 in cancer cells treated with olaparib increased the level of DNA damage and induced DNA double-strand breaks (DSB) markers. The researchers subsequently examined whether olaparib sensitivity reflected defective HR in TOPBP1-depleted cells by measuring HR activity through several parameters and confirmed that TOPBP1-depleted cells showed reduced HR activity.

The HR process encompasses several phases, including end resection and chromatin loading of RPA and RAD51, which can be visualized by formation of microscopically detectable foci. Moudry et al. searched for which step of HR was compromised in cells depleted for TOPBP1 and found that DNA end resection, i.e. the processing of the 5' recessed end that exposes a 3' overhang used for homology search, seemed not to be affected, as evaluated by the amounts of ssDNA detected by BrdU incorporation under non-denaturing conditions. Interestingly, they found that the next key stage in HR, in which the RAD51 recombinase protein is loaded at these 3' overhangs (Fig S12a), was greatly impaired, based on the assessment of the formation of RAD51 foci by microscopy and of the biochemical analysis of RAD51 accumulation on chromatin. While the mechanism by which TOPBP1 promotes the loading of RAD51 remains unclear, the authors propose an interesting model in which TOPBP1 plays a scaffolding role to direct Polo-like Kinase 1 (PLK1), which phosphorylates RAD51 and

facilitates its loading to DNA damage sites (Fig S12a) (Yata et al., 2012). Consistent with this model, they show that TOPBP1 physically interacts with PLK1 and that depletion of TOPBP1 impairs PLK1-dependent RAD51 phosphorylation. While more work is needed to prove that the TOPBP1-PLK1 interaction is required for this phosphorylation event, the results are exciting as they suggest another important functional link between TOPBP1 and a kinase. During DNA damage signaling, TOPBP1 plays an established role in activating the ATR kinase (Kumagai et al., 2006) and is believed to direct ATR's action towards specific substrates. This latter function is best understood in yeast, in which TOPBP1/Dpb11 forms a ternary complex to direct yeast ATR action to phosphorylate the downstream kinase Rad53. Interestingly, recent data from fission yeast also suggest that TOPBP1 interacts with yet another kinase, CDK, and directs its kinase action (Qu et al., 2013). The emerging scenario is that TOPBP1 may function as a scaffolding hub for controlling the action of distinct kinases to ensure genome integrity (Fig S12b).

While Moudry et al.'s work is the first to show a clear role for TOPBP1 in RAD51 loading, studies in budding yeast have proposed links between the TOPBP1 ortholog Dpb11 and HR-mediated repair. It was shown that the temperature-sensitive *dpb11-1* mutant displays a sensitivity to DNA damage that is not further increased by deletion of *RAD51*, suggesting that Dpb11 functions in HR repair (Ogiwara et al., 2006). In addition, other groups showed that TOPBP1/Dpb11 is required for DSB-induced mating-type switching and also reached the conclusion that TOPBP1/Dpb11 is required for HR-mediated repair of a DSB (Germann et al., 2011; Hicks et al., 2011). These reports provided compelling evidence that the role for TOPBP1/Dpb11 in DSB repair

is independent of its roles in replication initiation and DNA damage signaling. In humans, there also is evidence pointing to potential roles for TOPBP1 in DNA repair, as depletion of TOPBP1 was found to increase sensitivity to ionizing radiation and lead to defective DSB repair by HR (Morishima et al., 2007).

The new set of results provided by Moudry et al. clearly place TOPBP1 at the center stage of HR-mediated repair, in what seems to be yet another key and evolutionarily conserved role for TOPBP1, in addition to replication initiation and DNA damage signaling. An intriguing and unanswered question relates to defining the evolutionary benefit conferred by maintaining these crucial roles in the same protein. It is tempting to speculate that having a single protein module in command of key licensing events helps ensure the ordered and mutually exclusive execution of distinct cellular processes (Fig S12c). This is a particularly attractive and well-suited idea for the established role of DNA damage signaling in inhibiting origin firing during DNA replication. Sequestration of TOPBP1 into a complex involved in DNA damage signaling would help ensure that replication initiation is inhibited. Consistent with this hypothesis, it is established in yeast that the same BRCT domains involved in replication initiation are also required for DNA damage signaling. In addition, it was recently shown that competition between DNA damage signaling proteins and DNA repair factors for binding to the BRCT domains of TOPBP1/Dpb11 is a mechanism to remove TOPBP1/Dpb11 from a pro-DNA damage signaling complex, resulting in dampening of DNA damage signaling (Cussiol et al., 2015; Ohouo et al., 2013). It will be exciting to further explore this competition-based regulatory mechanism in human cells, as well as in the coordination of DNA damage signaling with DNA repair. In this direction, it

is crucial that the precise molecular mechanism by which TOPBP1 promotes HR-repair is elucidated, including defining which TOPBP1 BRCT domains are required and which factors they are binding to favor RAD51 loading or other pro-HR functions (See Fig S12d). Through truncation mutation analyses, Moudry et al. show that the specific BRCT domains 7/8 of TOPBP1 are essential for TOPBP1's role in promoting HR. However, it remains unclear how this is accomplished mechanistically.

To make the scenario even more complicated, TOPBP1 is known to physically interact with an extensive network of repair factors, including, but not limited to, BRCA1, 53BP1, MRN, FANCJ and BLM (Wardlaw et al., 2014). This points to an extremely complex system by which TOPBP1 could be coordinating the action of a range of repair factors and repair pathways. It would not be surprising if TOPBP1 was found to be key for the regulation of other steps in HR-mediated repair as well as other repair pathways in response to varied types of genotoxic insults, including DNA replication stress. In yeast, the interaction between TOPBP1/Dpb11 and the repair scaffold Slx4 provides an additional example of the rich range of possibilities by which TOPBP1/Dpb11 functions in DNA repair. In addition to sequestering TOPBP1/Dpb11 and dampening the DNA damage signaling (Cussiol et al., 2015; Ohouo et al., 2013), the Slx4-TOPBP1/Dpb11 interaction was recently found to control DNA end resection (Dibitetto et al., 2015) and was proposed to affect the late step of resolution of repair intermediates (Gritenaite et al., 2014). The TOPBP1-SLX4 interaction is conserved in humans; however it remains unclear how this interaction impacts DNA repair in higher eukaryotes. Notably, in yeast it is possible to distinguish the pro-DNA damage signaling function from the pro-recombinational repair function of different TOPBP1/Dpb11-

mediated complexes (Fig S12d). In mammals however, the scenario is more complex and it is currently unclear the precise contribution of different TOPBP1 interactions for DNA damage signaling and recombinational repair. Finally, because the ATR kinase is expected to regulate several DNA repair factors, it is likely that the ATR-activating function of TOPBP1 plays important roles in DNA repair. A major experimental avenue to explore this possibility and improve our understanding of the other roles for TOPBP1 in DNA repair will be the generation of separation-of-function mutants that do not interfere with DNA replication or DNA damage signaling.

Following the findings reported by Moudry et al., it is interesting to speculate on the implications of understanding TOPBP1 roles in HR repair for cancer research and treatment. Little is known about the role of TOPBP1 in carcinogenesis. It was found that TOPBP1 expression and subcellular localization are altered in a subset of breast cancer samples (Forma et al., 2012; Going et al., 2007; Liu et al., 2009) and the Bartek group here also reports altered TOPBP1 protein expression in ovarian cancers, although at modest frequencies. Nonetheless, as we learn more about TOPBP1 mechanisms of action in HR, it is possible that it may become an important target for manipulating the HR response by using small molecules such as Calcein AM (CalAM), which targets BRCT 7/8 of TOPBP1 (Chowdhury et al., 2014) and was shown by Moudry et al. to impair HR. Concerning the finding that TOPBP1 plays a pro-HR function very much like *BRCA1* and *BRCA2*, which genes are most frequently mutated in ovarian and breast cancers, it is intriguing that while *TOPBP1* mutations have been found in cancers (Blaut et al., 2010; Forma et al., 2013; Rebbeck et al., 2009), they are relatively infrequent and are likely not driver mutations. If TOPBP1 plays a key role in RAD51 loading, which

is the step severely perturbed in *BRCA1* or *BRCA2*-mutated cancer cells, it is not clear how more cancer-driving mutations have not been identified in *TOPBP1*. One possibility is that *TOPBP1* mutations affecting TOPBP1's pro-HR function also affect DNA replication and DNA damage signaling and impair the replicative capacity of cancer cells. Disentangling potential antagonistic roles for TOPBP1 in both suppressing and supporting tumorigenesis could lead to exciting new directions to study this complex multifunctional protein and to potentially develop new therapeutic strategies.

Figure S12

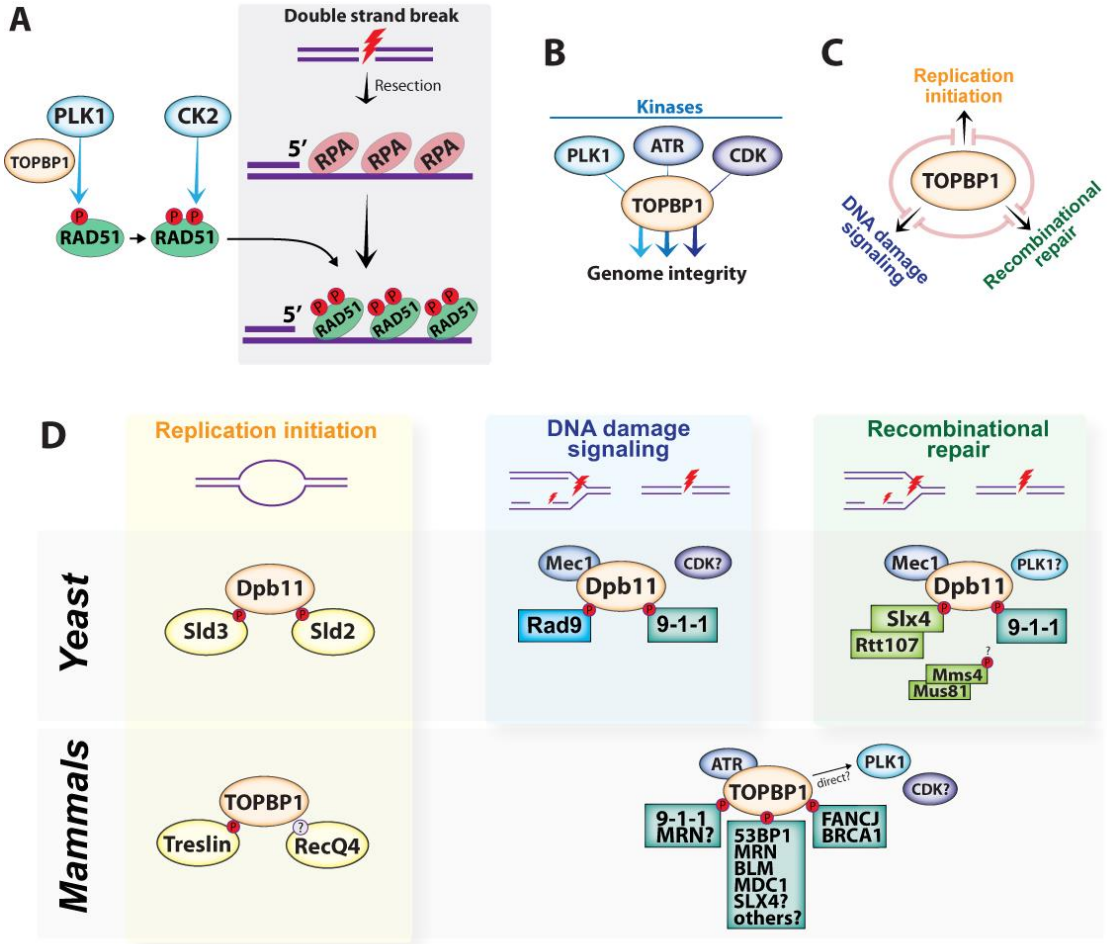

A
Double strand break
Resection
PLK1
CK2
TOPBP1
RAD51
RAD51
5′
RPA
RPA
RPA
5′
RAD51
RAD51
RAD51
B
Kinases
PLK1
ATR
CDK
TOPBP1
Genome integrity
C
Replication initiation
TOPBP1
DNA damage signaling
Recombinational repair
D
Replication initiation
DNA damage signaling
Recombinational repair
Yeast
Dpb11
Sld3
Sld2
Mec1
Dpb11
CDK?
Rad9
9-1-1
Mec1
Dpb11
PLK1?
Slx4
Rtt107
9-1-1
Mms4
Mus81
Mammals
TOPBP1
Treslin
RecQ4
ATR
TOPBP1
direct?
PLK1
CDK?
9-1-1
MRN?
53BP1
MRN
BLM
MDC1
SLX4?
others?
FANCJ
BRCA1

Figure S12 Involvement of TOPBP1 in homologous recombination (HR)-mediated repair. (a) The work by Moudry et al. (2016) supports a model in which TOPBP1 directs the action of PLK1 kinase towards RAD51, mediating a phosphorylation event that licenses a second phosphorylation event mediated by the casein kinase 2 (CK2) kinase. These phosphorylation events are believed to facilitate the loading of RAD51 at damage sites, which replaces RPA, and favor HR. (b) TOPBP1 as a hub for coordinating the action of multiple kinases towards genome maintenance. (c) Speculative model for the mutually exclusive engagement of TOPBP1 in distinct cellular processes as a strategy for coordinating genome replication and DNA damage responses (see text for details). (d) Current understanding of how TOPBP1 and its yeast ortholog Dpb11 mediate the formation of ternary complexes for replication initiation, DNA damage signaling and recombinational repair. In mammals, the indicated proteins have been shown to interact with TOPBP1, but the roles of most of those interactions in DNA repair remain unclear. For simplicity, certain TOPBP1 interactions and co-factors are not depicted.

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Appendix VI: Protocols

Protocol 1. PEI Transfection of HEK293T cells

Solutions:

-Transfection media:

500ml DMEM (without glutamine)
50ml Heat inactivated FBS (56°C water bath 30min)
5ml L-glutamine
5ml non-essential amino acid

-10% BCS DMEM

500ml DMEM (without glutamine)
50ml BCS
5ml Penicillin-Streptomycin
5ml L-glutamine
5ml non-essential amino acid

-PEI (1mg/ml)

Method:

1. Passage cells the day before transfection in 10cm dish 4*10^6 cells per plate.
2*. Change media in 10cm dish (~70% confluence) to transfection media 1hr before transfection: Aspirate off media; wash once with PBS; Add 5ml fresh transfection media
3. Bring everything to RT. To each microfuge tube, add 575ul 'DMEM only' (no supplements or serum) and 24ul PEI, mix and incubate for 5 min
3. Add 6ug DNA to the DMEM-PEI mix. Mix and incubate for 15min.
4. Add transfection mix to plates swirl plates to mix.
5*. Add 5ml additional transfection media 5hrs post transfection.
6. PEI is toxic to cells; change media on the second day of transfection to 10% BCS DMEM.
7. Harvest cells 48hrs post-transfection.
--aspirate off media; wash once with PBS; add 10ml PBS to the dish and scrape off the cells in PBS; add this 10ml cell suspension in PBS to a falcon tube; rinse the dish with an additional 5ml PBS to collect as many cells as possible and add the 5ml PBS/cell suspension to the falcon tube too; spin at 2000rpm 5min 4°C; freeze the pellets in -80°C.
*You can skip step 2 and 5 if you do not need maximum transfection efficiency.

	DMEM (ul)	PEI(ul)	DNA (ug)
6well	200	3	1
10cm	575	24	6
15cm	1500	50	15

Protocol 2. Sensitivity assay for mammalian cells

Solutions:
-Moxi Z Automated cell counter (Weiss lab)
-Moxi Z Cassettes, Type S, 3K–2.5M Cells/mL, Mean Cell Vol. (Mean Cell Dia.) 14–4200 fL (3–20 µm) MXC002
Or
-Hemacytometer

Solutions:
-PBS
-Trypsin
-Trypan blue
-10% BCS DMEM
500ml DMEM (without glutamine)
50ml BCS
5ml Penicillin-Streptomycin
5ml L-glutamine
5ml non-essential amino acid

Method:
1. Plate 2.5*10^4 cells in each well of a 6-well plate in triplicate.
2. 24hrs after plating the cells, replace the media with 10% BCS DMEM media containing desired genotoxins (eg. HU)
3. 24hrs after genotxoin treatment, replace the drug-containing media with the fresh 10% BCS DMEM media.
4. 3 days after changing the media, count cells for each sample
    a. aliquot media and trypsin into separate tubes
    b. aspirate off media
        - use the same glass pipette for all 6 wells
        - tilt the dish towards you when aspirating
    c. Wash the cells once with 3ml PBS;
        - expel PBS against side of well
        - swirl dish briefly to rinse cells thoroughly
        - aspirate off PBS
    d. Add 5 drops of trypsin per well
        - Take ~1.5ml trypsin into a 5ml serological
        - Slowly (drop by drop) expel trypsin onto center of each well
        - gently swirl to allow trypsin to spread thoroughly
    e. After cells are loose (a couple minutes), add 800ul media and resuspend.
        - Use the p1000 pipette
        - Add media to center of dish pick up dish and tilt towards you to take up media. Then expel (fairly vigorously) media onto all parts of dish (including the sides) to ensure efficient resuspension.
    f. Transfer cells into empty microfuge tubes

- Tilt the dish so all the cells aggregate closest to you
- Make sure you transfer all the media, including bubbles

g. Pellet 2k 5min
h. Aspirate off media (put a pipette tip on the end of the glass pipette to slow down suction)
i. Resuspend in 1ml media
   - Use p1000 and be precise. This is crucial for counting accuracy. Use filtered tips
   - Be careful not to resuspend too vigorously, or some media will fly out of the tube
   - When expelling the media, spray onto media already in the tube
j. Pipette 75ul of cells suspension onto the moxi Z counting cassette and use automatic cell counter to quantify.
k. (or mix 10 ul tryptophan blue with 100ul of the resuspended cells. Count the cells using hemacytometer.)

5. Calculate the cellular survival rate and plot the survival curve.

* Optimize the initial number of cells to seed for different cell lines.
* Optimize the drug dose for different cell lines and genotoxins
* Optimize the drug treatment time for different genotoxins

Protocol 3. SILAC DMEM recipe

DMEM

Prepare the following in a 2L beaker with a stir bar:

Powdered DMEM media…….13.5g
Sodium bicarbonate ($NaHCO_3$)…...3.7g
L-leucine……..105mg
L-proline……...200mg
ddH2O……..1L

Add ddH_2O upto 1L

Measure pH and adjust pH=7.4+/-0.2 using 1M HCl (Optional, pH is usually within the range)
Filter sterilize the media in 2*500ml corning filtration system bottles.

Prepare Light/Heavy amino acid stock:
Light aa:
Arginine 100mg
Lysine 100mg
Dissolve in 10ml PBS
Filter sterilize

Heavy aa:
Arginine 100mg
Lysine 100mg
Dissolve in 10ml PBS
Filter sterilize

For complete Heavy or Light DMEM:
500ml DMEM
50ml dialyzed FBS
5ml Penn/Strep
5ml Heavy or Light Arg+Lys

Protocol 4. Sodium orthovanadate stock preparation

Sodium orthovanadate (Na_3VO_4) is an inhibitor of several enzymes, including ATPase, alkaline phosphatase, and tyrosine phosphatase. During the purification of recombinantly expressed proteins in mammalian and insect cells, Na_3VO_4 is a common additive in buffers used to lyse these cells. Addition of this inhibitor to lysis buffers helps preserve the phosphorylation state of the recombinant protein of interest by inhibiting endogenous phosphatases.

However, the procedure described below must be performed in order to depolymerize the vanadate. Depolymerization renders Na_3VO_4 with significantly enhanced ability to inhibit phosphatases.

Method:

1. Prepare a solution of 200 mM sodium orthovanadate (Na_3VO_4, molecular weight = 183.91 g/mol) in ultrapure dH_2O. For a 100 mL solution, add 3.68 g Na_3VO_4 to 90 mL water and dissolve with stirring. Once dissolved, bring volume to 100 mL.

2. Depending on the pH of the solution, slowly add either 1 M NaOH or 1 M HCl with stirring to adjust pH to 10. Adding HCl will make the solution yellow.

3. Boil solution by heating in a microwave for 5 - 15 seconds. After boiling for 5 - 15 seconds, the solution will be clear and colorless.

4. Cool on ice until the Na_3VO_4 solution reaches room temperature.

5. At this point, the pH will be greater than 10. Add a small amount (several drops, with stirring) of 1 M HCl to adjust solution pH to 10.

6. Repeat steps 3-5 a total of 3-5 times. After several cycles of boiling, cooling, and adjusting pH, the solution should reach a point where the pH stabilizes at ~10. At this point, adding HCl should result in little, if any, appearance of yellow color in the solution.

7. Aliquot and store activated Na_3VO_4 at -20°C.

Protocol 4. IP-IMAC protocol for MEFs

Modified RIPA buffer for protein-purification:

	Final conc.	Stock conc	Volumn to add for 100ml
Tris-Cl pH7.5	50mM	1M	5ml
NaCl	150mM	3M	5ml
NP-40	1%	10%	10ml
Doc (Sodium deoxycholate)	0.25%	10%	2.5ml
EDTA	5mM	1M	0.5ml
NaF	50mM	powder	210mg
dH2O			77ml (up to 100ml)
Total volume			100ml

Urea/Tris solution (0.1ml, make fresh)
Urea……..48mg (8M urea)
1M Tris pH8.0……..5ul (50mM)
dH2O…….up to 100ul

Alkylating solution: (0.1ml)
Iodacetamide……..9.0mg (0.5M)
1M Tris pH8.0……0.1ml

1. Split the MEFs and seed at a density of 2.5*10^6/plate on 15cm dishes. (use 6 dishes for each IP)
2. Two days after the cell passage, cells are treated with desired genotoxins for two hours and cells are harvested two hours post-treatment.
   -Aspirate off media; add 10ml VERY COLD PBS to the plate; aspirate PBS; add another 10ml fresh cold PBS to the first plate, scrape off cells with cell scraper.
   -Pipette 10ml PBS/Cells into pre-chilled conical tubes on ice. Wash the plate with a 10ml fresh PBS and pipette into the second dish to collect cells.
   -Spin at 2000rpm for 5min at 4°C; Take off Supernatant; Spin 2 additional minutes and take out residual PBS. Cell pellets can be frozen at -80°C or can be lysed immediately
3. Resuspend the cell pellet in modified RIPA buffer supplemented with Roche complete protease inhibitor (100x) + home-made phosphotase inhibitor (100x) + PMSF (100x) + sodium orthovanadate (500x). Resuspend cell pellet from a single 15cm dish in100ul complete RIPA buffer, which gives a protein concentration=15mg/ml.
4. Sonicate until the lysate is clear. (For proteins extracted from three 15cm dishes, 3min @ power=90w is necessary to clear the lysate). Spin at max speed for 30min to precipitate DNA and chromosomes.

5. Bradford to measure protein concentration and dilute with complete modified RIPA buffer to protein concentration 10mg/ml. Add the lysate to 50ul pre-conditioned anti-Flag resin and let binding go for 3-4 hrs at 4°C on nutator.

Elution

6. Wash the anti-Flag resin 3-4 times with the modified RIPA buffer. Add 3 volumes of elution buffer (150ul SDS-Tris buffer+1.5ul 1M DTT) to the resin and boil @ 65°C for 5min.
7. Run the super and resin in a column. Apply air pressure gently and collect flow through as the elution.
8. Alkylate proteins for 15min at RT with idoacetamide solution (7.5ul of 0.5M stock). Save 2% for Western and add SB/DTT.

Protein precipitation and trypsin digestion

9. Dry samples until they reach 100ul total volume, which is required for efficient precipitation.
10. Precipitate proteins by adding 300ul of PPT solution and incubating on ice for 30min.
11. Centrifuge for 10min at maximum speed at RT and discard supernatant.
12. Wash well once with 200ul PPT (sonicate in water bath for 5-10sec before spinning)
13. Centrifuge once again (10min/max/RT). Remove liquid and keep the epper tube upside down for 5min to drain excess solution.
14. When the smell of acetone is gone, add 20ul of Urea/tris solution. Use micropipette to resuspend pellet. Add 60ul of ddH_2O and mix well.
15. Add 1ug of gold trypsin (1ug/ul) and digest ON at 37C on nutator.

Purification of phosphopeptides by IMAC-MS

16. Add acetic acid to a final conc of 1% HAC (~6ul of 20% HAC) (Compare the final solution pH with that of 1% HAC).
17. Apply 20mg of IMAC resin to the loading tip.
18. Apply sample to the fresh IMAC resin
19. Add 100ul of IMAC washing solution I, take 85ul (without disturbing the settled resin) and wash with the remaining 15ul
20. Add 100ul 1% HAC and take out 95ul, wash with the remaining 5ul.
21. Add 100ul Water, take out 90ul (without disturbing the settled resin) and wash with 10ul.
22. Elute with 60ul IMAC elution solution in a silanized vial.
23. Dry on speed vac for 30min until completely dried.
24. Resuspend the sample in 10ul 0.1p and shoot 2ul in mass spec.

Protocol 5. IP-MS protocol for mammalian cells

Modified RIPA buffer for protein-purification:

	Final conc.	Stock conc	Volumn to add for 100ml
Tris-Cl pH7.5	50mM	1M	5ml
NaCl	150mM	3M	5ml
NP-40	1%	10%	10ml
Doc (Sodium deoxycholate)	0.25%	10%	2.5ml
EDTA	5mM	1M	0.5ml
NaF	50mM	powder	210mg
dH2O			77ml (up to 100ml)
Total volume			100ml

Make sure check final buffer pH since pH may be important for Flag-IP affinity to the beads (which is more dependent on the charge).

Urea/Tris solution (0.1ml, make fresh)
Urea……..48mg (8M urea)
1M Tris pH8.0……..5ul (50mM)
dH2O…….up to 100ul

Alkylating solution: (0.1ml)
Iodacetamide……..9.0mg (0.5M)
1M Tris pH8.0……0.1ml

1. Passage the 293T cells (split cells lines light and heavy stable isotope media for at least 5 passages to guarantee efficient isotope incorporation) on 15cm dishes. (need 3 *15cm dishes for light and heavy each for IP-MS).
2. 2 days after the cell passage or post-transfection, cells are treated with desired genotoxins for two hours (or HU/MMC for longer time eg 12hrs to trap cells in S phase) and cells are harvested two hours or longer post-treatment.
   -Aspirate off media; add 10ml VERY COLD PBS to the plate; aspirate PBS; add another 10ml fresh cold PBS to the first plate, scrape off cells w/ cell scraper.
   -Pipette 10ml PBS/Cells into pre-chilled conical tubes. Rince the plate with a 10ml fresh PBS and pipette into the second dish to collect cells.
   -Spin at 2000rpm for 5min at 4°C; Take off supernatant; Spin 2 additional minutes and take out residual PBS. Cell pellets can be frozen in -80°C or lysed immediately.
3. Resuspend the cell pellet in modified RIPA buffer supplemented with Roche complete (100x), in-house phosphotase inhibitor (100x), PMSF (100x), sodium orthovanadate (500x). (double or triple the protease/phosphotase inhibtors if necessary.)

*Resuspend 293t cell pellet from 6*15cm dishes in 4ml complete RIPA buffer, which give a protein concentration=6-8mg/ml. MEFs from 40dishes gives around 100mg*

total proteins.

4. Sonicate until the lysate is clear. (I do sonication in Lammerding lab @ 50% power for 2*1min). Spin at max speed for 20min to precipitate DNA and chromosomes.
5. Bradford to measure protein concentration and dilute with complete modified RIPA buffer to protein concentration 10mg/ml/or to normalize light and heavy protein concentration. Take 50ul lysate and add in SB, boil 65°C for 10min. Add the light and heavy lysate to 2*100ul pre-conditioned anti-Flag resin (depending on how much bait you have) and let binding go for 3-4 hrs (I would do 3hrs for most of the experiment to maximize binding and minimize protein degradation) at 4°C on nutator.

Elution

6. Wash the anti-Flag resin 3-4 times with RIPA buffer (too many washes may lead to differences in background proteins, like chaperone proteins, ribosomal proteins, heat shock factors). Combine light and heavy resins in the last wash. Add 3 volumes of elution buffer 600ul SDS-Tris buffer) to the resin and boil at 65°C for 6-8min (tapping the tube every 2mins).
7. Run the sup and resin in a column. Apply air pressure gently and collect flow through as the elution.
8. Add 6ul of 1M (100x) DTT to a final concentration of 10mM. Reduce the elution at room temperature for 15min.
9. Alkylate proteins for 15min @ RT with idoacetamide solution (30 ul of 0.5M stock).

Protein precipitation and trypsin digestion

10. Dry samples until they reach 200ul-300ul total volume. (Do not dry down to 100ul, since SDS concentration would be too high).
11. Precipitate proteins by adding 3 elution volumes of PPT solution and incubating on ice for 30min.
12. Centrifuge for10min at max speed at RT and discard sup.
13. Wash well once with 200ul PPT (sonicate in water bath for 5-10sec before spinning)
14. Centrifuge once again (10min/max/RT). Remove liquid and keep the epper tube upside down for 5min to drain excess solution.
15. When acetone smell is gone, completely re-suspend in 50ul of Urea/tris solution and then add 150ul Tris 8.0/NaCl.
16. Add 1ug of gold trypsin (1ug/ul) and digest O/N at 37C on nutator.

Sample Clean-up

17. Acidify the samples by adding **10% TFA** (to a final concentration of 0.1%) using **glass syringe**.
18. Check pH (Should be acidic), spin (1 min/ 14,000 RPM/ RT) and collect supernatant. (May stop and store at -20C). (usu. I skipped this step)

C18 column: Binding and elution (WAT 0549-55)

19. Condition C18 column: Apply air pressure to the column for proper flow-through
    a. Condition with 1ml….80.0% ACN/ 0.1%HAc
    b. Equilibrate with 1ml….0.1% TFA
    c. Load your samples
    d. Wash:
        i. 1ml…..0.1%HAc
        ii. Fill up syringe with 0.1%HAc , invert a couple times and discard
        iii. 1ml…..0.1%HAc
    e. Flick residual volume off syringe tip.
    f. Elute in **silanized vial** with **200ul** of **80% ACN/0.1% HAc**
20. Pipette up and down to mix.
21. Collect a **20ul** aliquot (10% Input) for MS processing. (I did not collect 10% when IMAC mass spec run looks good in respect to the bait)
22. Dry both Input and remaining sample **completely** for 40min at 45C.
23. Re-suspend the **Input** in **7ul** of **0.1P** (0.1pmol angiotensin) and shoot 50% in MS. (Do a short 40min ms run to make sure the bait protein is there. At least 10 peptides should be identified in the search to be considered worthwhile for hilic fractionation).
24. To remaining sample add **15ul** of **dH_2O** to resuspend. (May stop and store at -20C or -80C).

HILIC Fractionation: (make sure that samples are at RT before proceeding)

1. Add **10ul** of **10% Formic Acid** and then Add **60u**l of **pure ACN using a glass microsyringe**.
2. Using a gel loader tip, pipette up and down to mix, then spin to remove insoluble material (1 min/ **gradually up to 8000RPM**/ RT Using a piece of kimwipes to stabilize the vial inside the tube).
3. Collect sup in a new small silanized vial.
4. Load into Hilic for fractionation after Blank and Angiotensin. (usu. for IP-MS fractionation, collection 10 fractions using so-called shallow...program) Prepare 1-10 labeled elution silanized vials in advance.
5. Dry fractions for 50 min at 45C.
6. Resuspend in **7.0ul** of 0.1P (8.0ul maximum)

7. Place silanized vial in holding vial and cap. May stop and store at -80C before loading into MS.
8. Print out hilic graph and estimate the average for each fraction. Calculate how much to load for 75unit…(vol to load in each fraction=525/average)

Protocol 6. Micronuclei assay

Reagent Preparation

1. Bicarbonate buffer: 0.9% NaCl, 5.3 mM sodium bicarbonate (84 g/mole), pH 7.5
    - To make 500 ml: 222.6 **mg** sodium bicarbonate plus 4.5 g NaCl, use less than total volume initially to mix
    - Balance pH: HCl to decrease, sodium hydroxide to increase
    - Add water to 500ml
    - Sterilize in autoclave for 30 min (place in bucket of water)
2. Heparin: 500 USP heparin/ml PBS
    - To make 25 ml: 25 ml sterile 1x PBS plus 69.4 **mg** heparin.
    - Filter using syringe, needle, 0.45 micron filter
3. Mitomycin C (MMC)
    - In tissue culture room draw up 10 ml sterile water in syringe
    - In hazardous chemical hood inject water in vial containing 2 mg MMC, mix under the hood
    - Bring back to tissue culture room
    - Aliquot 500 ul into 20 microcentrifuge tubes
4. RNAase A stock (10 mg/ml)
    - Work at tail bench to prevent contamination
    - Add 100 mg RNAase A to 9 ml 0.01M sodium acetate (concentration of RNAase A:10 mg/ml), total volume will be 10 ml once Tris-Cl is added
    - Transfer 450 ul to 20 microcentrifuge tubes
    - Heat in 100ºC dry bath for 15 minutes
    - Remove and let cool to RT
    - Make 1M Tris-Cl (pH 7.4): 7.5 ml 2 M Tris-Cl stock (pH 7.5) plus 7.5 ml water, invert to mix
    - Add 50 ul Tris-Cl to each of the 20 microcentrifuge tubes
5. RNAase A working (2.5 mg/ml)
    - 750 ul of 0.01M sodium acetate (left over from making stock RNAase solution) plus 250 ul of 10 mg/ml RNAase stock
6. 3M Sodium Acetate stock (82 g/mole, $NaC_2H_3O_2$), pH 5.2
    - To make 250 ml: 61.5 g of $NaC_2H_3O_2$
    - Balance pH: acetic acid to decrease
    - Measure final volume to be 250 ml
    - Sterilize in autoclave for 30 min (place in bucket of water)
7. 0.01M Sodium Acetate
    - To make 9 ml 0.01M sodium acetate, pH 5.2: 9 ml sterile water plus 33.3 ul 3M sodium acetate stock (total volume will be 10 ml once Tris-Cl is added for making RNAase stock)
8. Solution 1 (make up fresh solution for each use)
    - Work in the dark when using the antibody, keep antibody and bicarbonate buffer on ice

- Make antibody/buffer B cocktail for the number of samples used plus some extra, remember one sample is a PI only control. Mix by pipetting gently, do not introduce bubbles.
    i. (1.75 ul CD71:FITC) x (# of samples plus extra) = ul for cocktail
    ii. (75.75 ul bicarbonate buffer) x (# of samples plus extra) = ul for cocktail
    iii. (10.0 ul RNaseA) x (# of samples plus extra) = ul for cocktail
    iv. Mix by gently pipetting

9. Solution 2 (make up fresh solution for each use)
    - Make a cocktail of propidium iodide and bicarbonate buffer for the number of samples used, remember one is a CD71 only control
        i. (# of samples plus extra [ml]) x (1.25ug/ml final conc. PI) x (1 ul/ug stock PI) = ul for cocktail
        ii. (1 ml bicarbonate buffer) x (# of samples plus extra) = ml for cocktail

<u>Reagent locations</u>

- Bicarbonate: 4ºC, keep on ice when in use
- CD71:FITC: small 4ºC in secondary antibody box
- Heparin: in glass cabinet by tissue culture
- Mitomycin C (mutagen): in 4ºC by front end of lab in tissue culture refrigerator. Working solution in the -20ºC in Amy's RNAse A box.
- Propidium Iodide (mutagen): 4ºC in a white case in small 4ºC
- RNAase stock: -20ºC in "antibodies" box
- Sodium Acetate: shelf. Working solution in at bench 2.

<u>pH reader</u>

- Open collar when in use, close when done
- Store probe in electrode stopping bottle
- Rinse probe before, after and in between uses
- Standby turns on/off
- Follow sheet over counter
- Standards 4, 7 & 10. For sodium acetate and bicarbonate buffer can just use 4 & 7

<u>Mitomycin C Treatment</u>

- Bring MMC down to mouse room in a box
- Double glove using nitrile as outer layer
- Be careful not to contaminate hood (put down extra paper towels)
- IP injection using 26g 5/8 needle and 1 ml syringe
- Cull mice after 72 hours and put in freezer down in mouse common room
- After 72 hrs bedding goes back to lab into a bag then into biohazard, rinse cage with water and put away

<u>Bleeding Mice (mandibular vein)</u>

- Use 20g needle to forcefully puncture a spot caudal dorsal to the whisker spot on the cheek
- Place 1-2 drops of blood in microcentrifuge tubes filled with 200 ul heparin
- Invert to mix blood

Sample Preparation

1. The day prior to blood collection, place 2 ml methanol in 15 ml polypropylene conicals in -80^{o}C
2. Using cheek puncture techniques, place 3 drops of blood in 200 ul Heparin
3. Inject 200 ul of heparin-blood into 2 ml of methanol at -80^{o}C. Do not put the pipette tip in the methanol and do not let the blood touch the side of the tube. Hold the pipette tip 1cm away from methanol while injecting. Work directly out of the -80^{o}C freezer.
4. Strike the sample sharply 4-5x to prevent aggregation
5. Keep sample overnight at -80^{o}C
6. Wash samples with 12 ml bicarbonate buffer, invert tubes to mix
7. Centrifuge at 2000 rmp for 5 minutes
8. Dump off supernatant
9. Resuspend pellet in conical using left over pellet (run along a tube holder to mix)
10. Working in the dark, add 87.5 ul of Solution 1 to 5 ml round bottom tubes (can use one sample as a PI only control-for this sample just add 77.5 ul bicarbonate buffer).
11. Place 20 ul resuspended pellet into 5 ml round bottom tubes containing Solution 1 and RNAase. Place unused pellet on ice.
12. Mix by pipetting or gently flicking if necessary
13. Cover samples with foil, stain for 45 min in 4^{o}C
14. Work on ice from this point on
15. Wash with 2 ml Bicarbonate buffer
16. Centrifuge at 2000 rmp for 5 min
17. Dump supernatant
18. Resuspend pellets in 1 ml Solution 2, use one sample as CD71 only control-add 1 ml bicarbonate buffer only to this sample
19. Wait 5-10 min
20. Run through Flow cytometer

Flow Cytometer-Fluorescence activated cell sorting (Facs), 5th floor of VMC

1. Bring samples, covered on ice and Zip disk
2. Follow start-up procedure on machine
3. Amy > MN template
4. Acquire > connect to cytometer (windows >acquisition control)
5. Acquire > parameter description > folder > new > enter date > create > select > file >enter “001” into file count
6. Cytometer > status
7. Cytometer > instrument settings > open > pick protocol from Amy file > set > done

8. Acquire >counters
9. Test by checking “setup” > run, hi > vortex sample > acquire
10. To start run uncheck “setup” >run, hi > vortex sample >aquire
11. Follow clean up procedure on the machine
12. Save onto computer 1st then transfer to zip

Printing data in lab

- Zip > Mn template > edit > select all > plots > change data file > Amy (desktop) > open 1st file > print

Disposal of solution

- For left over flow solution add bleach then pour down the drain

Protocol 7. Immunofluorescence

1. Grow cells on a 6-well dish, with the coverslip in the well. (coverslip cat number: Fisher_12-545-81, 12CIR-1.5)

a. To autoclave the coverslips, dip the coverslips into 70% ethanol and autoclave

2. If you need to transfect the cells, do transfection with cells of 30%-40% confluence, stain after 24hr or 48hr transfection.
3. Wash with 1xPBS. Fix cells with 3.7% formaldehyde in PBS for 10-15mins RT
4. Wash with 1xPBS for 3 times (Never dry out the coverslips)
5. Permeabilized the cells with 0.2% TritonX-100 in PBS, 5min RT
6. Wash with PBS 3 times
7. Block with 5% BSA in 1xPBS for 30min
8. Primary antibody incubation (flag antibody M2 1:200) 1hr RT in 2% BSA. Use 50ul diluted primary antibody per coverslip.
9. Wash with PBS 3 times
10. Secondary incubation: 1:500 1hr RT. Wash with PBS 3 times.
11. (Stain with DAPI as needed)
12. Mount and seal with mounting solution and clear nail polish.

After fixation and wash with PBS, fixed cells can stay in 4C for days with sodium azide.

Protocol 8. FACS on mammalian cells (Machine model: BD Accuri C6**)**

Propidium iodide (or PI) is an intercalating agent and a fluorescent molecule with a molecular mass of 668.4 Da that can be used to stain cells. When excited with 488 nm wavelength light, it fluoresces red. Propidium iodide is used as a DNA **stain for both flow cytometry to evaluate cell viability or DNA content in cell cycle analysis** and microscopy to visualise the nucleus and other DNA containing organelles. **It can be used to differentiate necrotic, apoptotic and normal cells.** Propidium Iodide is the most commonly used dye to quantitatively assess DNA content. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining.

1. Collect cells by trypsinization. Make you trypsinize cells really well and have individual cells.
2. Rinse into cold PBS 1x. Spin down cells at 500g 5min 4C.
3. Resuspend cells well into 100ul cold PBS and add 100ul cell suspension in 900ul 77% EtOH drop by drop with vortexing. (This allows cells fixed singularly) Store at -20°C overnight
4. Spin down your cells and resuspend in 1ml PBS
5. Count cells (dilute 10 fold as needed) and spin down the same number of cells from all samples. Resuspend cell pellet again in 250ul of PBS/0.1%TritonX-100.
6. Add 2ul RNase A (100mg/ml)
7. Incubate for 1hrs at RT on nutator (shaking)
8. Spin down and resuspend cell pellet again 250ul PBS/0.1%TritonX-100.
9. Add 15ul of PI (protected from light) incubate 30min at RT
10. Spin and resuspend your cells in 500ul-1ml PBS, pipet and vortex to get cells back to solution.
11. Ready to be analyzed. (should expect a graph with y axis showing count and x axis showing absolute florescence. There should be higher peak showing G1 and lower peak showing G2 cell population).