

Functions of BRCA1, 53BP1 and SUMO isoforms in DNA double-strand break repair in mammalian cells

DISSERTATION

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Abstract

In this dissertation study, we have investigated the protein functions in DNA double-strand break (DSB) repair of three important factors, BRCA1, 53BP1 and SUMO isoforms, at levels of biochemical activity, protein dynamics and chromosomal DNA repair. Our work reveals novel mechanisms of these proteins functioning in response to DSB damage, hence providing insights of where and how they are actively involved in each subpathway of DSB repair.

In the first part of our work, we studied BRCA1, a tumor suppressor important for the maintenance of genomic stability including centrosome control and DSB repair, and found that a putative enzymatic mutant of BRCA1— BRCA1(I26A), which had been thought to disrupt its E3 ligase activity, was still functional in the cellular processes of regulating centrosome number and homologous recombination-dependent DSB repair, thereby raising a question of whether I26A mutant is indeed inert. Reevaluation of the ubiquitination activity of this BRCA1(I26A) mutant revealed that it is an active E3 ubiquitin ligase when associated with the appropriate E2 factor. We then think that conclusions about the dispensability of the BRCA1-dependent enzymatic activity in various cellular processes should be reconsidered.

Next we studied the unique function of 53BP1, a known NHEJ factor for DSB

repair. We found that 53BP1 specifically promotes the error-free conservative-NHEJ (C-NHEJ) mechanism, dependent on its upstream recruiters RNF8 and RNF168. 53BP1 has no effect on the highly mutagenic and deletional alternative-NHEJ (Alt-NHEJ) pathway or on homology-directed repair (HDR), but it suppresses single-strand annealing (SSA). We discovered that the localization of 53BP1 at sites of DSBs is accompanied by its bulk removal from the nucleus except at sites of DNA damage. And the degradation of bulk 53BP1 upon DNA damage is due to each action of RNF8 and RNF168. Further, we showed that failure to degrade bulk 53BP1 results in the failure for its downstream effector RIF1 to localize appropriately to DNA damage sites. These data provide a novel mechanism of 53BP1 responding to DSB damage.

In the third part of our study, we assessed SUMO isoforms in DSB repair. We identified that SUMO isoforms act differentially in DSB repair pathways: SUMO1 stimulates all four subpathways while SUMO2/3 is only required for C-NHEJ pathway. Strikingly, the single SUMO E2 enzyme, UBC9, was required for C-NHEJ but not for HR or Alt-NHEJ. And the conjugation-deficient SUMO1 mutant protein was competent for HR and Alt-NHEJ repair similar to the wild-type, but not for C-NHEJ. Our data together reveal a novel role of SUMO1 as a free protein, not a protein conjugate in homologous recombination and alternative-NHEJ.

Overall, we have identified biochemical steps at which these factors are required for DSB repair, as well as novel regulatory mechanisms controlling the process.

Dedication

This document is dedicated to my family and friends, my advisor Jeffrey Parvin, and my colleagues for their continuous support in everyway throughout my PhD study.

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Publications

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Ankur Shah, Vibhu Sachdev, Julian Taylor-Parker, Sohiya Yotsukura, Yara Hamade-Tohme, Yevgeniya Malinkovich, Cleo Rolle, **Yiheng Hu**, Ben Tran, Peter Gibbs, Susan Lees-Miller, Ravi Salgia, Forest White, Jeffrey Parvin, Al Ozonoff, and Ajit Bharti. (2014 submitted) DNA-PK dependent phosphorylation of topoI-S10 regulates its BRCA1 mediated ubiquitination: topoI-pS10 determines its degradation rate and CPT response.

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Fields of Study

Major Field: Molecular, Cellular and Developmental Biology

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Chapter 1: INTRODUCTION

1.1. DNA double-strand break repair

Cells are constantly exposed to DNA damaging conditions comprised of environmental and endogenous DNA damaging sources that cause an alteration in the chemical structure of DNA, such as DNA mismatches or DNA strand breaks, etc., thereby impairing DNA integrity and threatening genomic stability. If not repaired, the damage can lead to blockage of genome replication and transcription, thus resulting in mutations or wide-scale genomic aberrations, which threaten normal cellular homeostasis. Of the many types of DNA lesions, DNA double-strand breaks (DSBs) are considered the most harmful, because both strands are compromised and require a complex DNA repair pathway to fix the breaks. An unrepaired DSB is sufficient to trigger permanent growth arrest and apoptosis, and in multi-cellular organisms can promote genome rearrangements leading to cancer. Therefore an efficient and accurate repair of DSBs is critical for the maintenance of genome stability and prevention of carcinogenesis.

1.1.1. Single-strand breaks versus double-strand breaks

Another type of strand break in DNA double helix is single-strand breaks (SSBs), which are discontinuities in one strand of the DNA double helix structure and one of the common sources of SSBs is the endogenous oxidative attack by reactive oxygen species (ROS) in the cells. SSBs arise more frequently than double-strand breaks (DSBs)¹. If not repaired rapidly or appropriately, chromosomal SSBs pose a serious threat to genomic stability, and when two SSBs occur in close proximity, or the DNA replication machinery encounters a SSB which eventually causes the blockage or collapse of DNA replication forks during the S phase of the cell cycle, double-strand breaks are formed^{1,2}. Therefore, defective single-strand break repair (SSBR) can lead to an increased dependence on double-strand break repair (DSBR) machinery to repair the damage. One example is the PARP inhibitors (PARPi) that are used as a type of common anti-cancer drug to treat DSBR deficient cancer patients in that these DSBR-deficient tumor cells are more dependent on PARP, an important SSBR protein, than regular cell. Deficiency in SSBR process renders the genome stability susceptible. Consequently, cells have evolved rapid and efficient mechanisms for their repair. When one of the two strands of DNA double helix has a defect, the other strand can be used as a template to correct the damaged strand. In cells, there exist a number of excision repair mechanisms that remove and then replace the damaged nucleotide with a correct nucleotide complementary to the base found in the undamaged strand of DNA. Three major

repair mechanisms are involved in the cells for DNA (SSBR): Base excision repair (BER), which repairs a single base damage in the DNA strands; Nucleotide excision repair (NER), which recognizes and corrects bulky, helix-distorting DNA strand lesions such as pyrimidine dimers and 6,4-photoproducts; Mismatch repair (MMR), which corrects errors of mis-paired but undamaged nucleotides and insertion-deletion loops that arise during DNA replication and recombination. Defects in SSBR are associated with an increased risk of later development of cancer or hereditary neurodegenerative disease^{3,4}.

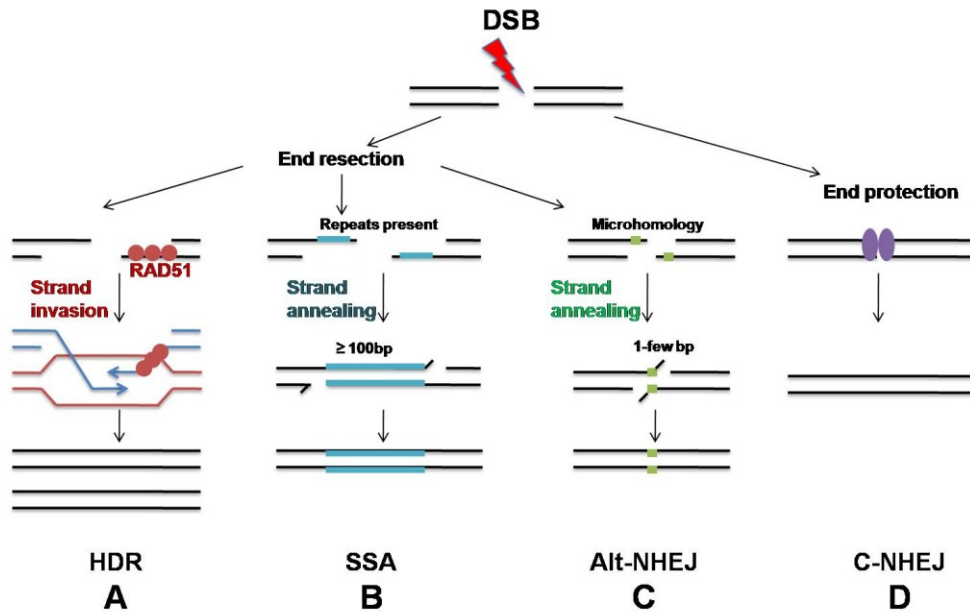
Comparatively, double-strand breaks (DSBs) are the worse kind of DNA damage arising in the genome induced by a variety of exogenous agents including ionizing radiation (IR) and a number of anti-cancer drugs (e.g. bleomycin and topoisomerase II inhibitors), or endogenous free radicals as byproducts of oxidative metabolism. Moreover, DSBs arise normally as intermediates in V(D)J recombination, a process of genetic recombination in the early stages of immunoglobulin (Ig) and T cell receptors (TCR) production of the immune system during mammalian lymphoid-cell development. Unrepaired DSBs potently induce gross chromosomal rearrangements such as deletions, translocations and amplifications, further triggering the activation of oncogenes and/or the loss of tumor suppressors, which in turn fuels malignant transformation⁵. Cells respond to DSBs by organizing a complex signaling network, which attunes in accordance of DNA damage checkpoint activation, chromatin reorganization and DNA repair reaction events. This signaling network is called the DNA damage response and

includes a cascade of signaling events to respond rapidly to DSB damage.

1.1.2. Double-strand break repair pathways

DNA double-strand breaks (DSB) present a major problem in genome maintenance since the repair machinery must bridge a gap of indeterminate composition. Two mechanistically distinct pathways are present for DSB repair in mammalian cells: homologous recombination (HR) and non-homologous end-joining (NHEJ)⁶. HR requires an early determinant process known as resection—the processing of the sequences adjacent to DSB ends (5') which undergo nucleolytic degradation and are processed to 3' single-strand tails, which are also important for the activation of DNA damage checkpoints. This early DNA end resection step is required for downstream events in HR but inhibits NHEJ. HR repairs DSBs during S and G2 phase of the cell cycle whereas NHEJ exists almost throughout the cell cycle⁷, though with NHEJ repair proteins reaching their peak abundance in G1 phase. There are two major mechanisms present in HR: the error-free Homology-Directed Repair (HDR) pathway and the error-prone Single-Strand Annealing (SSA). Both HDR and SSA utilize sequence homology from an intact sister chromatid as a template for repair during S and G2 phase when DNA synthesis and homologous recombination events carry out at a rate of high peak. The dominant mechanism of HR is the homology-directed repair (HDR) pathway, which is a relatively precise form of repair. The repair process starts with the early step of DSB end resection to generate single-strand DNA ends at the

damage sites and followed by strand invasion in which the resected ssDNA strands invade the duplex DNA in the sister chromatid template to find homologous sequence. Single-strand annealing (SSA), is the minor pathway in HR and this repair mechanism causes DNA resection until homology at repair junctions is revealed. However, SSA pathways does not involve strand invasion which is a critical and typical step for homologous recombination. SSA is mutagenic: it involves first the resection of DSB ends to yield long ssDNA overhangs and once homology is searched and revealed at the overhang ends, they are annealed and the protruding flaps are trimmed, leaving gaps filled by DNA polymerase, thereby resulting in deletions⁸. Similarly, to date two types of end-joining systems are defined in the NHEJ in eukaryotic cells: the first one is called conservative-NHEJ (C-NHEJ), in which DSB ends are ligated without homology and which protects DSB ends with minimal processing^{9,10}, hence predominately associated with precise joining of DSB ends without altering the DNA sequence¹¹; A second mechanism for NHEJ is alternative-NHEJ (Alt-NHEJ), which depends on minimal resection to generate single strand ends that are annealed and ligated via microhomology of sequences flanking the break site^{10,12,13}. This alternative pathway for NHEJ is highly mutagenic and deletional in that this alternative-NHEJ (Alt-NHEJ) catalyzes DNA resection and utilizes imperfect microhomology for end-joining partners and thus resulting in deletions at repair junctions¹⁴. Figure 1.1 demonstrates the simplified diagram of DSB repair summarizing four subpathways.



Modified from M. Jasin and R. Rothstein. Cold Spring Harb Perspect Biol 2013 Nov 01; Vol. 5 (11), pp. a012740.

Figure 1.1. The simplified diagram of summarized DSB repair pathways.

A. HDR: after DSB is induced and sensed by the cells, the initial step of DNA resection occurs by exonucleases to generate 3' ssDNA ends, followed by strand invasion, which is dependent on Rad51 or a related recombinase (red balls). When the homologous sequence has been recognized, the damage DNA strand (in blue) invades the undamaged DNA duplex (in red) and uses the homologous sequence as a template. The ssDNA is then extended with the formation of Holliday junctions. **B. SSA:** DNA resection takes place and ≥ 100 bp of homology is searched for and used for strand annealing, resulting in deletion of nucleotides. **C. Alt-NHEJ:** DSB ends are resected as in HDR and SSA. One to a few bp is used as microhomology sequences to anneal the gap, leading to deletions in the genome. **D. C-NHEJ:** DSB ends are protected by protein complex from processing. Then the ends at the break site are ligated by repair machinery.

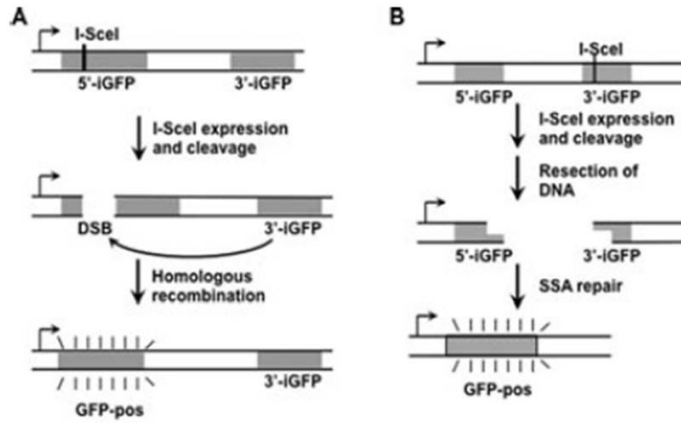
1.1.3. Current functional chromosomal DSB repair assays in vertebrate cells

To date, there are functional chromosomal DSB repair assays which have been developed as power tools by applying the use of reporter substrates for assaying the contributions of individual DSB repair pathway to DSB repair in mammalian cells including yeast and vertebrates. Here, we describe the current and commonly employed tools for functional repair assays in vertebrate cells because of the differed balance in choice of HR or NHEJ in vertebrates in comparison with yeast. The contribution of NHEJ is far greater in vertebrate cells than in yeast. Both conservative and alternative-NHEJ are robust in higher eukaryotic cells. HR plays a relatively minor role in DSB repair in many but not all types of higher eukaryotes in that DSB in packed chromatin structure hardly access a homologous template for repair¹⁵. These assays depend on the precise introduction of DSBs to the cell genome either by the rare-cutting mega-endonuclease I-SceI. The individual contributions of specific proteins to HDR, SSA, Alt-NHEJ and C-NHEJ can be quantified via GFP-based chromosomal reporters or polymerase chain reaction (PCR)-based methods¹⁶. These reporters are used to evaluate the effects of genetic background, dominant-negative constructs, or physiological conditions on DSB repair in a wide variety of mammalian cells, including cancer and normal cell lines. The following diagrams describe each individual functional assay with regard to the principle, the reporter construct and the practice.

Starting from HDR, a fluorescence-based assay using the DR-GFP reporter for

measuring the frequency of HR at a chromosomal DSB in living cells has been developed in various types of mammalian cells including murine embryonic stem (ES) cells, HEK293 cells and HeLa cells^{17,18}. The DR-GFP reporter substrate has been integrated into the cell genome as cells were stably transfected with the DR-GFP reporter construct, and a clone was selected that had no detectable background GFP signal, to generate an established cell line that stably expresses the recombination substrate. DR-GFP substrate contains an upstream green fluorescent protein (GFP) gene repeat (5'-iGFP), which is inactive due to the replacement of 11 bp of GFP sequence to create the 18-bp recognition site sequence for the I-SceI endonuclease. Transfection of an I-SceI expression vector (pCBASce)¹⁹ will generate a single site-specific DSB precisely at an intrachromosomal locus that can be repaired by HDR mechanism. A functional GFP gene can be restored and GFP protein can be expressed in the cells which will be rendered positive for green fluorescence if the downstream internal GFP repeat (3'-iGFP) is used as the repair template containing the identical sequence information (homology) (Figure 1.2.A) and the GFP positive (GFP+) cells are determined and quantified by a flow cytometer.

Similarly, cell lines that stably integrate the SSA recombination substrates have been established^{17,20}. The recombination substrate was based on similar design as the homologous recombination substrate (DR-GFP). However the restoration of GFP gene and expression of GFP protein occur when the two nonfunctional GFP genes are repaired by SSA (Figure 1.2.B).



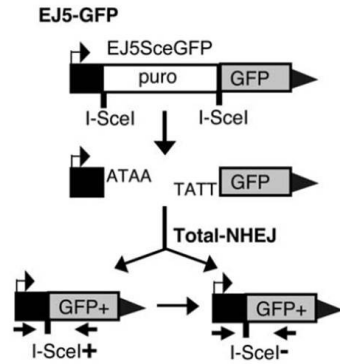
Adapted from Towler WI *et al.* Hum Mutat. 2013 Mar;34(3):439-45.

Figure 1.2. The chromosomal DSB repair assays in vertebrate cells: HDR and SSA.

For the DSB repair assays, the appropriate cell line was transfected with a plasmid expressing the I-SceI endonuclease, which initiates a DSB lesion. The amount of repair activity was then determined in each functional DSB repair assay. The repair substrates are diagrammed for HDR, SSA, respectively. **A.** HDR assay: when a DSB is induced by transfection of I-SceI in the sequence of the upstream defective GFP allele (5'-iGFP), the break can be repaired by the downstream GFP allele (3'-iGFP) which also is defective due to another lesion. This 3'-iGFP allele provides a donor sequence to repair via HDR the I-SceI-generated DSB. **B.** SSA assay: after a DSB is generated by I-SceI, DSB ends are resected until homology in the GFP encoding sequences is revealed, followed by annealing for repair.

To measure the total NHEJ repair events occurring in the cells, a system that is also based on I-SceI induction and GFP fluorescence quantification has been

developed¹⁷. EJ5-GFP measures repair between two tandem I-SceI endonuclease cut sites and detects multiple classes of NHEJ events. Therefore this GFP-based reporter can be considered an assay for total-NHEJ. Specifically, EJ5-GFP construct contains a promoter that is separated from a GFP coding cassette by a puro gene flanked by two I-SceI recognition sites that are in the same orientation. Once I-SceI is introduced and expresses in the cells, simultaneous cleavage at both I-SceI sites occur, hence resulting in loss, that is, pop-out, of the puro gene portion flanked by the two I-SceI sites. Then the promoter is joined to the rest of the expression cassette, leading to restoration of the GFP gene activity (Figure 1.3.). Since the two sites of I-SceI-induced DSBs have complementary 3' overhangs, such repair process could potentially restore an I-SceI site, mediated by a precise NHEJ mechanism as the conservative-NHEJ. Alternatively, NHEJ could fail to restore the I-SceI site, leading to an I-SceI-resistant site, and this process is mediated by a microhomology-dependent NHEJ mechanism as alternative-NHEJ in that deletion of nucleotides at I-SceI site at DNA junctions results from microhomology. Thus, NHEJ repair of the EJ5-GFP reporter results in either restoration of the I-SceI site or generation of deletion NHEJ events, corresponding to C-NHEJ and Alt-NHEJ, respectively. GFP fluorescence quantified by flow cytometry thus reveals the total events of NHEJ repair in the cells.



Adapted from Bennardo N *et al.* PLoS Genet. 2008 Jun 27;4(6):e1000110.

Figure 1.3. The chromosomal total-NHEJ repair assay.

Simultaneous cleavage of I-SceI results in two DSBs between the promoter and GFP encoding sequence with complementary overhangs which are then annealed either by an error-free NHEJ mechanism with restoration of I-SceI and/or by an error-prone pathway leading to deletion and loss of I-SceI site.

To explore one of the subset of total-NHEJ events that utilizes the alternative mechanism as Alt-NHEJ, a similar system as the EJ2-GFP has been developed. This reporter is designed so the GFP+ products would predominantly reflect a discrete Alt-NHEJ event among all NHEJ¹⁷. The EJ2-GFP reporter contains a single GFP expression cassette with a fused N-terminal tag, and an I-SceI site followed by stop codons (in all three reading frames) between the tag and GFP coding sequence. (Figure 1.4.). The I-SceI site and stop codons are flanked by 8 nucleotides of microhomology, which if annealed during Alt-NHEJ would gain active GFP by restoring the coding frame between the tag and GFP, and cause deletions ranged from 23-35 nt to 140-350 nt¹⁷.

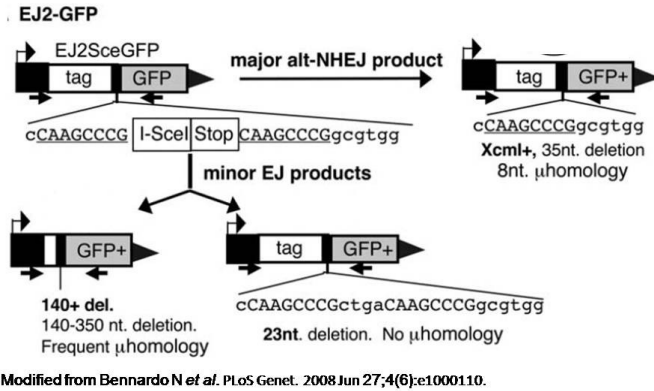


Figure 1.4. The chromosomal Alt-NHEJ repair assay.

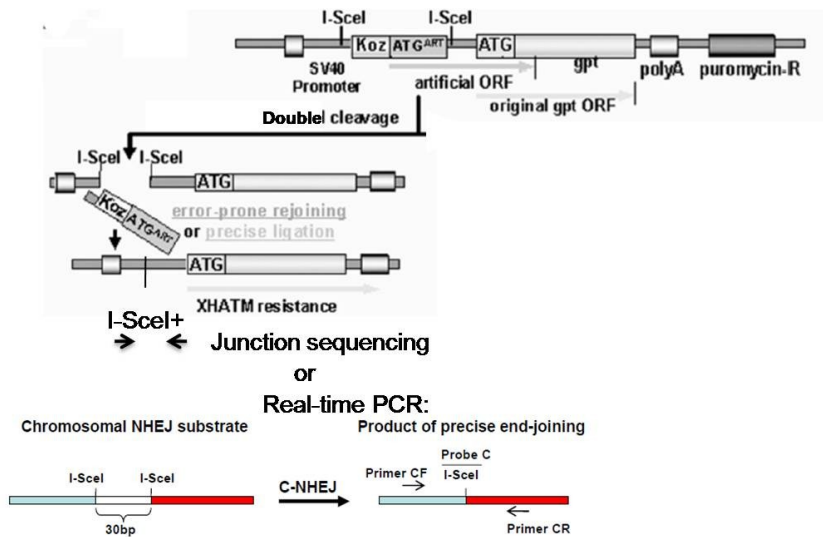
I-SceI generates a DSB followed by stop codons, flanked by 8 nt of microhomology. The break is repaired to give rise to functional GFP by a major Alt-NHEJ event or other minor events, resulting in deletions in a variable spectrum.

Finally, is the measurement of conservative-NHEJ events, which is not a GFP-based assay, but still depends on the I-SceI cleavage mechanism. pPHW1 stably integrated into the HEK293 genome is the construct that has been developed for this purpose^{21,22}. This substrate contains an artificial translational start sequence (ATG^{art}) inserted between an early SV40 promoter and the bacterial gpt gene. The corresponding artificial open reading frame (ORF) is shifted by 1 bp against the downstream gpt ORF and is dominant over the gpt start site (ATG^{gpt}), thus preventing gpt translation. Two I-SceI recognition sites flank the ATG^{art} site. Once the artificial ORF is excised by NHEJ repair of the two I-SceI-induced DSBs, the SV40 promoter is joined to the rest of the artificial ORF, leading to loss of the ATG^{art} with reconstituted translation of the original gpt ORF,

thereby allowing the detection of recombinants in HEK293 cells growing in XHATM selection medium (Figure 1.5.). The C-NHEJ frequency can be determined by detection of the repair junction after I-SceI cleavage using PCR with primers flanking the two I-SceI sites. For this purpose, the genomic DNA is extracted and PCR-amplified and the PCR products are subcloned into a vector for sequencing. Accurate end-joining would render no deletions whereas microhomology-dependent NHEJ causes nucleotide deletions at the annealed junction. The C-NHEJ frequency therefore is derived from the number of events of accurate end-joining divided by total sequences²². Alternatively, instead of the strategy of selective medium, a quantitative real-time PCR method has been developed to detect C-NHEJ events directly using primers flanking the dual I-SceI sites and a Taqman probe complementary to the sequence specific for precisely religated distal ends after double I-SceI cleavage²² shown in Figure 1.5.

Figure 1.5. The chromosomal C-NHEJ repair assay.

Double excision of I-SceI causes the pop-out of ATG^{art} sequence. The NHEJ mechanism anneals the overhangs and leads to restoration of I-SceI site, as well as the gain of XHATM resistance. Then either junction sequencing by a set of primers or real-time PCR amplification of the junction sequence can be used to determine the repair frequency.



Modified from Zhuang *et al.* J Biol Chem 2009 Oct 30;284(44):30565-73.
and Jiang *et al.* J Biol Chem 2013 Mar 29;288(13):8966-76.

1.1.4. DSB response regulators—the signaling cascade

Upon DNA double-strand break induction, a cascade of protein modification and relocalization is triggered: the Mre11-RAD50-NBS1 complex (MRN) binds to DSB ends and recruits the DNA damage sensor ATM. The key role of ATM is to recruit repair machinery into DNA damage sites, by phosphorylating H2AX (γ -H2AX) which results in the recruitment of downstream factors, such as the E3 ubiquitin ligases RNF8 and RNF168, leading to the formation of K63-linked polyubiquitin chains on histones at DSBs. This ubiquitination cascade regulated by RNF8 and RNF168 is responsible for the localization of repair mediators, including BRCA1 and 53BP1 to the DNA damage sites²³⁻³². BRCA1 accumulation to DSBs is through its interaction with the Abraxas-RAP80 complex which is already tethered to the damage sites by the ubiquitination signal mediated by RNF8 and RNF168³³.

Localization of 53BP1 involves its recognition of H2A ubiquitinated on Lys-15 (H2AK15ub), the latter being a product of RNF168 via its ubiquitination-dependent recruitment (UDR) motif binding to K63-linked ubiquitination on chromatin. 53BP1 binding to the chromatin also requires dimethylation of histone H4 on lysine 20 (H4K20me2) via the 53BP1 tandem tudor domain at the damage sites³⁴⁻³⁷.

While most DSB repair proteins appear to function exclusively in HR or NHEJ, a number of proteins influence both pathways, including ATM, the MRE11/RAD50/NBS1(XRS2) complex, histone H2AX, CtIP, RNF8, RNF168, BRCA1, PARP-1, RAD18, Ku70/Ku80 complex and DNA-dependent protein kinase catalytic subunit (DNA-PKcs)^{17,25-28,38}, suggesting that HR and NHEJ share repair proteins at the HR/NHEJ interface. Figure 1.6. demonstrates the repair protein machinery involved in each individual double-strand repair pathway.

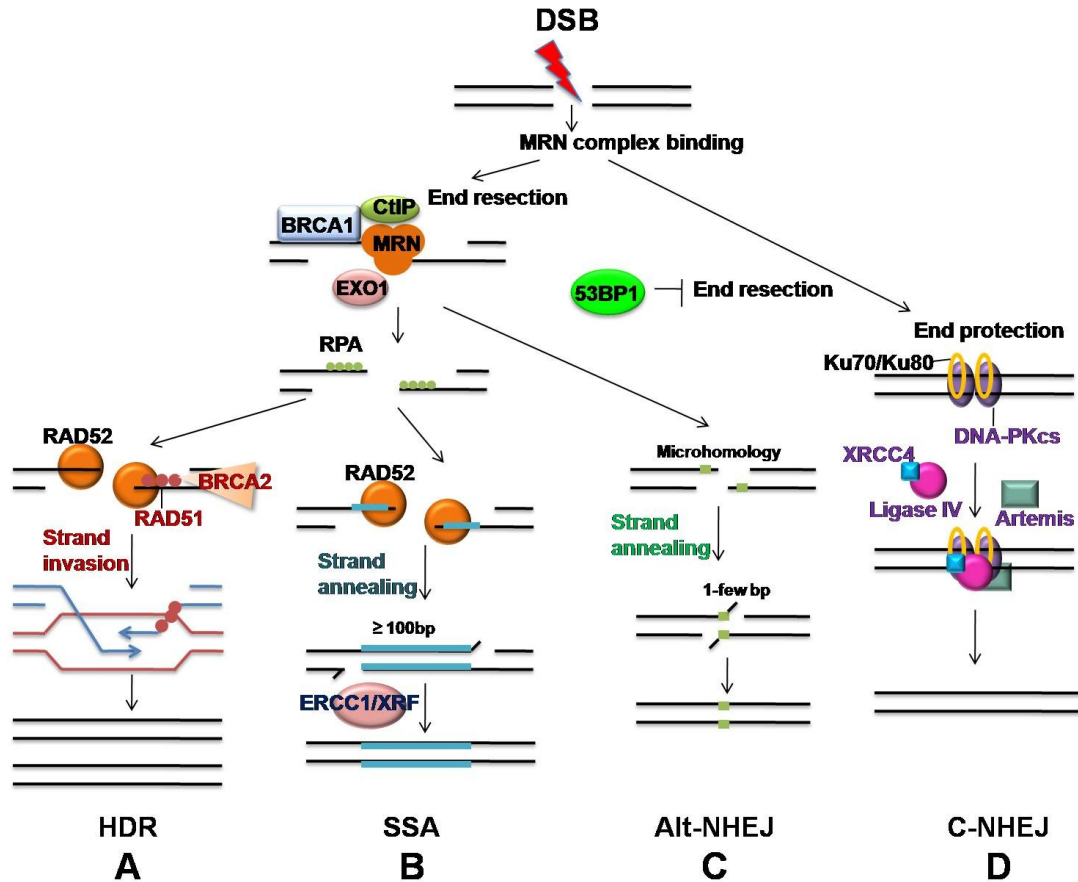


Figure 1.6. Repair protein apparatus in DSB repair pathways (simplified).

When DSB is induced, the damage sensor MRN (Mre11/RAD50/NBS1) complex quickly binds to the break site and recruits the downstream repair factors. DNA end resection initiates the process of HDR/SSA/Alt-NHEJ. The exposed single-strand DNA (ssDNA) is coated with the ssDNA-binding protein RPA subunits, which in turn activates HR repair. **A.** For HDR-mediated repair, the RPA-coated ssDNA is replaced with RAD51 or a related recombinase that mediates strand invasion, which is a defining step of HDR. This is followed by strand annealing completing accurate DSB repair. **B.** SSA can mediate repair if perfect repeats are unveiled by resection. Perfect repeats are uncommon and

therefore this pathway is non-conservative. **C.** For Alt-NHEJ repair, after the initial step of end resection, microhomology is revealed at DSB ends to repair the break. Mre11 can favor alternative NHEJ through its nuclease activity^{22,39}. **D.** 53BP1 inhibition of end resection might help cells channel the repair to C-NHEJ, which is initiated by DNA end-binding proteins Ku70/Ku80 complex, followed by the recruitment of DNA-PKcs. The Ku/DNA-PKcs complex ultimately recruits Artemis and XRCC4/ligase IV complex, which complete the repair of the break.

1.1.5. Choice between HR and NHEJ for DSB repair

Although NHEJ repair factors are recruited to the damage sites more rapidly than HR factors, and NHEJ and HR factors are independently recruited to DSBs, there is a significant period of time when both sets of factors are present at DSBs⁴⁰. Therefore the choice between HR and NHEJ in DSB repair pathway may be regulated by one or more proteins that function in both pathways.

The DSB repair pathways appear to compete for DSBs⁶, and the balance between them differs during different cell cycle phases—that is, HR occurs to repair DSBs during S and G2 phases when sister chromatids are available, while NHEJ is mostly predominant in the G1 phase of the cell cycle since it is active throughout the cell cycle⁷. The regulatory mechanism has been revealed that a cell cycle-regulated circuit underpinned by BRCA1 and RIF1, the critical effector of 53BP1, governs DSB repair pathway choice to ensure that NHEJ dominates in G1 and HR is favored from S phase onward. Remarkably, RIF1 accumulation at

DSB sites is strongly antagonized by BRCA1 and its interacting partner CtIP⁴¹, which is critical for DSB end resection, the initial step of HR and Alt-NHEJ¹⁷. Recruitment of BRCA1 then functions as a molecular switch to shift the balance of DSB repair from error-prone DNA end-joining to error-free homologous recombination⁴². Moreover, a recent study¹⁵ has indicated that NHEJ is the sole machinery for DSB repair in the G1 phase, while HR starts to be employed in addition to NHEJ in the late S to G2 phases. Collectively, the stage of the cell cycle is a decisive factor in the control of DSB repair that HR and NHEJ are differentially employed during the cell cycle.

Although the two DSB repair pathways could act in parallel, the initial binding of repair factors to DSBs may affect the choice of between HR and NHEJ since both HR and NHEJ are active in the S and G2 phases of the cell cycle and for a significant period of time both sets of factors are present at DSBs. In addition, as discussed above that both HR and NHEJ pathways share a number of repair factors binding to DSBs. NHEJ is initiated by DNA end-binding protein complex Ku70/Ku80, which rapidly accumulates at exposed DNA breaks⁴³. HR initiation involves strand resection of DSB—extensive 5' to 3' end-processing at broken ends and generating 3' single-strand overhangs which is regulated by Mre11/Rad50/NBS1 (MRN), Exo1 and another exonuclease CtIP³⁸. The choice between HR and NHEJ should consequently depend on either the affinity of the initiation factors for the DSB or the regulatory control of initial binding factors at DSBs.

Distinguishable from C-NHEJ, HR and Alt-NHEJ are initiated with resection regulated by CtIP^{17,44} and this exonuclease along with its binding partner BRCA1 (BRCA1-CtIP) antagonize NHEJ factors 53BP1-RIF1 to prevent NHEJ⁴¹. Therefore it is plausible that the early decisive event of DSB repair pathway choice is DNA end resection—the processing of DSB ends to form 3' single-strand tails, which is required for HR but prevents conservative-NHEJ in which DNA ends are protected with minimal processing before joining. Consequently, the resection of DSB appears to prevent cells from the choice towards NHEJ. End resection also provides an intermediate in SSA and Alt-NHEJ, as it gives rise to single strand overhangs, which can anneal at long (SSA) or short (Alt-NHEJ) complementary sequences. HR and SSA require more extensive end resection than Alt-NHEJ⁴⁵. The top portion of figure 1.6. illustrates that DNA end resection at DSBs is a determinant of the choice between HR and NHEJ.

1.1.6. Ionizing radiation induced foci (IRIF) upon DSB

As discussed above, DSB formation activates DNA damage response protein kinases ATM, ATR and DNA-PK which trigger histone H2AX phosphorylation and the accumulation of proteins to DSBs such as MDC1, RNF8, RNF168, BRCA1, 53BP1, CtIP, RPA and RAD51 into ionizing radiation induced foci (IRIF) that amplify DSB signaling and promote DSB repair^{46,47}. These DSB repair proteins participate in the formation of nuclear DNA damage foci, which can be defined as a definite place near damaged DNA where several DNA damage proteins come

together to cooperatively repair damaged DNA⁴⁸. Some of the DNA damage proteins form foci rapidly in response to DSB, and one of them is 53BP1, which accumulates at DNA damage tracts as early as 5 min after laser micro-irradiation for generation of localized damage in cellular DNA⁴⁹, mediated by RNF8 and RNF168²⁵⁻²⁸. Previous studies which identified that 53BP1 was transiently immobilized at the sites of damage by Fluorescence Recovery After Photobleaching (FRAP) experiment^{50,51}, however, also indicate dynamic molecule exchange of 53BP1 protein constantly occurs within the nucleus. The FRAP experiment showed that 53BP1 signal associated fluorescence recovered from photo bleaching in no irradiation and irradiation samples, indicating a dynamic exchange between unbleached 53BP1 molecules from the nucleoplasm and bleached 53BP1 at the photo bleaching spot. Further, 53BP1 was shown to be transiently immobilized at the DSB sites in that no irradiation sample had a markedly faster exchange kinetics than the irradiation sample in which photo bleaching was applied at IRIF 30 min post-IR to allow maximal steady-state level of accumulation of fluorophore-tagged 53BP1 at the DSB sites^{50,51}. Apart from being responsible for 53BP1 foci formation following DSB, RNF8 and RNF168 also recruits other repair factors including BRCA1 to DSBs to form foci, since these two DSB regulators are more upstream in the signaling cascade⁵¹, thereby being essential for efficient DSB repair to take place.

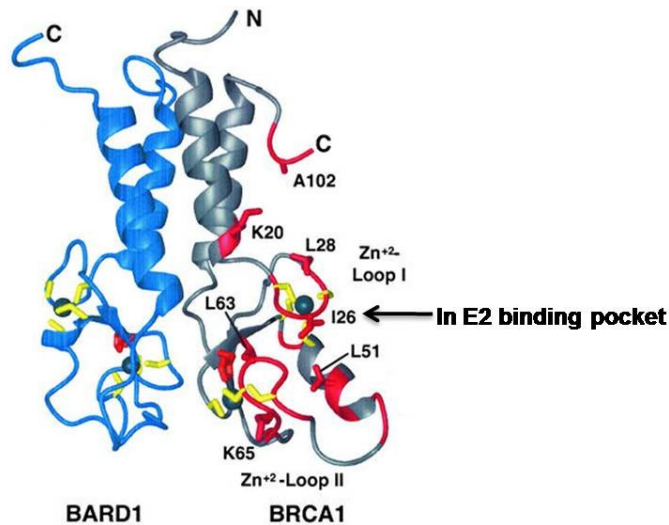
1.2. BRCA1: a key regulator of various DNA repair pathways

The breast and ovarian cancer specific tumor suppressor BRCA1 is involved in several important cellular pathways as caretaker in preserving genomic stability, including DNA damage repair, centrosome dynamics, chromatin remodeling, checkpoint activation and transcriptional regulation⁵²⁻⁵⁵. BRCA1 heterodimerizes with its binding partner BARD1 and exhibits an E3 ubiquitin ligase activity^{56,57}. The *BRCA1* gene is classified as a tumor suppressor since mutations in this gene can result in increased incidence of breast and ovarian cancer. The BRCA1 tumor suppression function has been attributed to its role in DNA double-strand break repair.

1.2.1. The BRCA1 protein

The *BRCA1* gene has 24 exons and encodes the BRCA1 protein of 1863 amino acids (aa). BRCA1 is such a large protein that it has multiple domains that numerous proteins bind on the several domains of BRCA1. The amino-terminal 109 residues of BRCA1 contain a RING domain, which is responsible for binding to the ubiquitin E2 enzyme thereby mediating the BRCA1 E3 ubiquitin ligase activity, and the residue isoleucine-26 is a critical amino acid within the E2 binding pocket⁵⁸ (Figure 1.7.). The binding partner BARD1 interacts with BRCA1 via its RING domain binding to the RING domain within BRCA1. The carboxyl terminus of BRCA1 protein encodes two repeats of BRCT domains (BRCA1 carboxyl terminal tandem repeats), that are responsible for the protein function in DNA

repair and interaction with other binding partners, i.e., CtIP and Abraxas/RAP80⁴⁸. BRCA1 has two nuclear localization signals (NLS) domains and only the first one is important for the nuclear localization of BRCA1.



Modified from Brzovic PS *et al.* Proc Natl Acad Sci 2003 May;100(10):5646-51.

Figure 1.7. BRCA1/BARD1 protein RING structure.

Residues which are responsible for binding of E2 factor are shown in red and labeled. I26 is important for E2 recognition and binding, hence it is critical for BRCA1 function as an E3, although mutation of I26 to alanine does not hinder heterodimerization with BARD1.

1.2.2. BRCA1 roles in DNA DSB repair pathways

Of all functions, the role of BRCA in DNA repair is attributed to explain its tumor suppression role⁵⁹. Indeed, BRCA1 is involved in several DNA repair pathways including DNA double-strand break repair and UV induced damage at stalled

replication forks⁶⁰. Here we emphasize on BRCA1 function as a tumor suppressor in double strand break repair pathways.

BRCA1 is essential for HR repair mechanism, hence protecting the individual's genome integrity, because a cell has to rely on more error-prone DNA repair pathways such as SSA and NHEJ repair in the absence of an intact HR repair mechanism. BRCA1 interactions with the exonuclease CtIP⁶¹ and the heteromeric protein complex Mre11/RAD50/NBS1 (MRN)⁶² are important for the processing of DSB. The phosphorylated form of CtIP binds to the BRCT domain of BRCA1 and is modified with non-canonical ubiquitin chains in a BRCA1-dependent manner. Interaction with BRCA1 mediates CtIP function in the 5' end resection of the DSB ends into ssDNA overhangs⁴². BRCA1 is important for both HR and SSA^{63,64}, as is CtIP^{17,65}, confirming that BRCA1 modulates the initial step of end resection and even further is involved in the regulation of choice of HR or NHEJ upon DSB damage⁴¹. BRCA1 also interacts via PALB2 with BRCA2 and RAD51 that regulate the exchange of genetic information between the damaged and the homolog DNA strands⁴⁸. Furthermore, studies indicated that BRCA1 regulates the ubiquitylation status of a range of proteins at DSBs⁶⁶, suggesting that the enzymatic activity of the ubiquitin E3 ligase protein, BRCA1, is essential for DSB repair^{26,54}. However, a recent study raised a conflict about this point that BRCA1 tumor suppression does not depend on its E3 ligase activity, but rather the BRCT phosphoprotein binding⁶⁷.

In addition to HR repair mechanism, BRCA1 enhances the fidelity of NHEJ repair

by promoting the precise C-NHEJ⁶⁸ while preventing mutagenic deletional Alt-NHEJ through interaction with conservative-NHEJ machinery during G1 phase⁶⁹. However different studies have reported conflicting results with regard to the involvement and function of BRCA1 in Alt-NHEJ mechanism, despite intense research over the years. For example, in addition to HR, Alt-NHEJ is the third DSB repair pathway that is also dependent on DNA resection where MRN complex is playing a regulatory role¹⁴, thereby is thought to rely on MRN complex. And BRCA1 interacts with MRN complex in response to DSB damage and regulates DNA resection as an initiation of DSB repair, thus might be involved in a positive modulation of Alt-NHEJ.

1.3. 53BP1: an important regulator of DSB signaling

p53 binding protein 1 (53BP1) is an important regulator of the cellular response to DSBs in that it is a central component of chromatin-based DSB signaling. Localization of 53BP1 to the sites of DNA damage requires the recognition of histone methylation, in particular H4K20me2³⁴ by its tandem Tudor domain^{35,36}. 53BP1 also binds H2A ubiquitinated on Lys-15 (H2AK15ub), a product of RNF168 ubiquitination on chromatin, via its ubiquitin-dependent recruitment (UDR) motif. 53BP1 has been implicated to prevent HR in that it acts as a competitor with BRCA1 in leading to opposite directions in the DSB repair process, NHEJ versus HR, respectively^{6,41,70,71}. The influence of 53BP1 on pathway choice of HR or NHEJ is mediated by mutual antagonism with BRCA1⁴¹. A recent study showed

that BRCA1 excludes 53BP1 to the irradiation-induced foci (IRIF) periphery, hence overcoming the inhibitory barrier posed by 53BP1 on HR⁷². Moreover, 53BP1 has been implicated in the repair of DSBs by NHEJ during G1 phase by antagonizing the resection of DSBs in G1, preserving DSB ends and thereby favoring repair by NHEJ over HR. By modulating 53BP1 localization (or retention) at damage sites, the cell can channel DSB repair towards either NHEJ (in G1) or HR (in S–G2), by controlling the extent to which a DSB is resected⁷³. However, a study recently suggested opposing roles of 53BP1 during HR-mediated repair. According to this study, 53BP1 is required for HR at two-ended DSBs in G2 phase; while it is dispensable for HR in S phase, when heterochromatin regions are likely relaxed during replication so that homologous sequences can be accessed at the sister chromatid⁷⁴.

Further, 53BP1 has been investigated to promote NHEJ-mediated DSB repair⁷⁵⁻⁸⁰. 53BP1 facilitates the end-joining of distal DNA ends, which is induced during V(D)J and class switch recombination as well as during the fusion of deprotected telomeres⁷³. 53BP1 deletion in mouse results in a severe defect in class-switch recombination, a process dependent on NHEJ and associated with increased DNA end resection at the IgH locus⁸¹⁻⁸³. Loss of 53BP1 restores homologous recombination in BRCA1-deficient murine cells, indicating that 53BP1 inhibits DNA resection in DSB repair, by the regulation of the downstream effector RIF1 to control 5' end resection^{6,70,84}. RIF1 is one of a few proteins identified to date that requires 53BP1 for its recruitment to DSBs⁸⁵ and is involved in

conservative-NHEJ⁴¹. While it is known that 53BP1 directly regulates efficient total NHEJ repair events in mammalian cells⁸⁶, the specific function of 53BP1 is not yet well-defined in terms of which subpathway in the NHEJ repair process 53BP1 functions, C-NHEJ versus Alt-NHEJ.

1.4. SUMO family proteins: modulators involved in DSB repair

In vertebrates, there are three functional forms of SUMO family proteins: SUMO1, SUMO2, and SUMO3. SUMO2 and SUMO3 share about 95% sequence identity but are only 45% identical in sequence to SUMO1, thus forming a distinct subfamily as SUMO2/3⁸⁷. The conjugation of SUMO isoforms onto target protein is designated as SUMOylation, an enzymatic cascade triggered by an E1 SUMO-activating enzyme (SAE1/SAE2), followed by a single specific E2-conjugating enzyme, UBC9, and several E3 SUMO ligases, resulting in a covalent isopeptide bond between the lysine of target protein and di-glycines at carboxyl-terminus of the activated SUMO^{88,89}.

The SUMO system has been shown to have strong ties to double-strand break repair. Abolition of activity of SUMO E3 enzymes in human cells impairs DSB repair^{51,68,90}. Mutation of the single SUMO E2 enzyme, UBC9 in yeast or human cells results in defects in DNA repair, including recombination abnormality and impaired DSB repair⁹¹⁻⁹³. Furthermore, many HR proteins are modified by SUMO^{51,68,92,94-97}. For example, during DNA end resection (the initial step of HR and Alt-NHEJ), which requires 12 proteins⁹⁸, significantly 7 of them are

SUMOylated in yeast⁹⁷, including the subunits of MRX complex (Mre11, Rad50, and Xrs2), the nuclease Sae2, the helicase Sgs1, and 2 subunits of RPA. The SUMOylation of these proteins appears or increases after DNA damage, suggesting that induced SUMOylation might be involved in DNA end resection^{99,100}. Moreover, an important recombinase RAD52 required for HR, is SUMOylated when the DSB is induced, and SUMOylation may shelter RAD52 from accelerated degradation, perhaps by sequestering the protein into repair foci⁹⁴. Nonetheless, cells expressing individual SUMOylation-defective mutant substrates often lack notable phenotypes^{100,101}. SUMO modification of individual NHEJ proteins however, regulates their function in DSB repair^{92,102,103}.

SUMO1 has been discovered as a non-covalent binding partner for human HR proteins RAD51, RAD52 and RPA via SUMO-interacting motifs (SIMs) as well as other DNA repair proteins¹⁰⁴⁻¹⁰⁸. The SIM-dependent non-covalent binding to SUMO is important for loading of the recombinase RAD51 onto resected DSB ends for HR-mediated repair^{97,109}. However, it has not been elucidated which specific subpathways of DSB repair the SUMO isoforms are playing a role in. Figure 1.8. shows the involvement of SUMO proteins in DSB repair pathways.

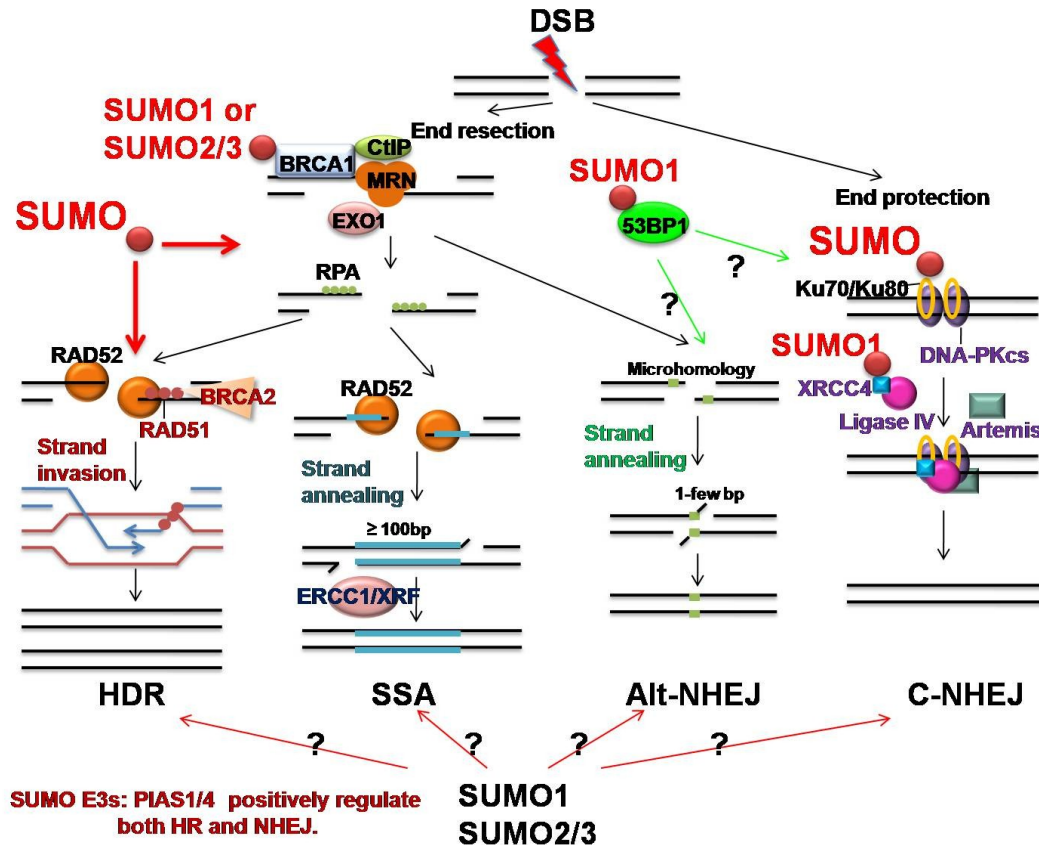


Figure 1.8. The involvement of SUMO proteins in DSB repair pathways.

The SUMO E3 ligases are implicated in both HR and NHEJ whereas SUMO isoforms have not been directly tested in specific DSB repair subpathways. BRCA1 and 53BP1 have been reported to be conjugated by SUMO proteins: BRCA1 is modified by SUMO1 or SUMO2/3 while 53BP1 is only SUMOylated by SUMO1. Yeast studies show that SUMO is conjugated onto many HR proteins including the resection factors, the ssDNA coating complex RPA and the recombinase RAD52 which mediates strand invasion. Almost all of these proteins were predicted or identified to have SIM binding to SUMO and SUMO interaction with RAD51 non-covalently via SIM is important for RAD51 loading onto DSB

ends. On the other hand, SUMOylation of some NHEJ factors is important for regulation of their repair function including the example of SUMOylation of the yeast Ku70/Ku80 complex and SUMO1 conjugation of human XRCC4.

Chapter 2: Rationale

Double-strand break repair pathways have been studied for years and still have remaining questions to be answered, i.e., what are the detailed mechanisms of how each step of repair is initiated and proceeds? What are the repair proteins involved in each stage? How do cells coordinate the proteins in the signaling cascade for efficient DSB repair? What are the molecular and biochemical functions of the repair factors involved in DSB repair. Among all these unsolved questions, 53BP1, a DSB repair protein previously identified to influence the NHEJ process^{6,86}, has not yet been elucidated about its specific function in this pathway (Figure 1.8. green arrows and question marks). Furthermore, the SUMO system has been implicated in the DSB repair pathway since many repair factors are SUMOylated. However, the corresponding SUMOylation-deficient mutants often barely exhibit deleterious phenotypes^{94,101}. It is then unclear whether SUMOylation is dispensable or required for DSB repair. In addition, the fact that SUMO has different isoforms adds to complexity.

In this study, we hypothesized that BRCA1, 53BP1, and the three SUMO isoforms execute unique functions in double-strand break repair (DSBR). We investigated the specific functions of 53BP1 and SUMO proteins in DSB repair subpathways to

answer question marked in Figure 1.8. We used established DSB repair assays in which a specific recombination substrate is integrated into the genome of a cell at a single site. In order to maintain a flexible analytic approach, the cell line was competent for double-strand break repair, and we depleted single proteins or multiple proteins in combination via RNAi. In this way, we could identify subpathways that depended on specific factors and whether different factors had epistatic relationships. We tested 53BP1 and the SUMO isoforms in each chromosomal functional DSB repair assays in human cells discussed in Figure 1.2-1.5. This method directly reflected the contribution of the protein in a specific subpathway of DSB repair and helped us to categorize the pathways in which the tested protein is required or is being stimulatory.

We also tested whether BRCA1 ubiquitination activity was required for its functions in DSBR and in centrosome control. To our surprise, we found that a mutant BRCA1(I26A), which is inert in ubiquitin ligase activity, functioned in our cell based assays. We instead hypothesized that the reported enzymatic mutant was in fact active. We tested this hypothesis using biochemically purified full length of mutant BRCA1/BARD1 protein, and we found that under a certain set of biochemical conditions the I26A mutant was in fact a functional E3 ubiquitin ligase.

Overall, these experiments identify the specific pathways in which these factors are required for DSBR, and we identified novel regulatory mechanisms controlling the process.

Chapter 3: Reevaluation of a putative BRCA1 enzymatic mutant

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Manuscript ready to submit

Author contributions:

- Hu, Y & Parvin JD designed the experiments.
- Hu, Y performed the experiments.
- Kais K and Ransburgh D performed the centrosome and HDR experiments, respectively.
- Meyer L purified the E2 enzyme proteins.
- Parvin JD provided mentorship, advice, and financial support.

3.1. Abstract

The breast and ovarian cancer specific tumor suppressor, BRCA1 in complex with BARD1, is an E3 ubiquitin ligase, and this BRCA1-dependent activity has been shown to be important in the regulation of several processes including control of centrosome function. A laboratory-derived BRCA1 mutant, BRCA1(I26A), has been thought to be inert as an E3 ubiquitin ligase. In this study, we tested the BRCA1(I26A) variant in two tissue culture based functional assays and found this mutant to be about as active as the wild-type BRCA1 in control of centrosome duplication and homologous recombination repair. We thus questioned whether the BRCA1(I26A) mutant protein is in fact enzymatically inert. *In vitro* ubiquitination assays with purified full length BRCA1/BARD1 revealed that BRCA1(I26A)/BARD1 is indeed active as an E3 ubiquitin ligase when in the presence of the heterodimeric E2, Ubc13/Mms2. We found that BRCA1(I26A)/BARD1 monoubiquitinates γ -tubulin *in vitro* similarly as does the wild type in reactions containing the E2 factor Ubc13/Mms2. These results redefine the enzymatic activity of the BRCA1(I26A) variant and indicate that in the cell this variant may yet function as an E3 ubiquitin ligase in the regulation of the processes of homologous recombination repair and centrosome duplication.

3.2. Introduction

The breast and ovarian cancer specific tumor suppressor BRCA1 has multiple functions in the cell as a caretaker in preserving genomic stability including DNA

damage repair, centrosome dynamics, cell cycle checkpoint control and transcriptional regulation⁵²⁻⁵⁵. BRCA1 heterodimerizes with BARD1 and exhibits an E3 ubiquitin ligase activity^{56,57}. Composed of 1863 amino acids, the amino-terminal 109 residues of BRCA1 contain a RING domain, which is responsible for binding to the E2 factor, and the residue isoleucine-26 is a critical amino acid within the E2 binding pocket⁵⁸.

BRCA1 has been tested in many functional assays. Previous studies have shown that BRCA1/BARD1 heterodimer is required for homology-directed recombination repair (HDR) of chromosomal double-strand breaks (DSBs)⁶³. In addition, BRCA1-specific ubiquitin linkages have been detected at the sites of DSBs⁶⁶, and SUMO modification of BRCA1, which potently stimulates its E3 ubiquitin ligase activity, was tightly correlated with its function in DSB repair⁶⁸. These results would be consistent with a requirement for the BRCA1-dependent ubiquitin ligase activity in the DNA repair process.

In addition to its multiple functions in nucleus, BRCA1 has been shown to control centrosome dynamics. In breast cells, the regulation of centrosome duplication and function requires BRCA1-dependent E3 ubiquitin ligase activity, mediated by the monoubiquitination of γ -tubulin by full length BRCA1/BARD1^{54,110,111}.

Structural considerations had identified isoleucine-26 as critical to binding to the E2 factors⁵⁸. Mutation of this residue to alanine in BRCA1(1–772) maintained the overall structure of the RING domain and resulted in a significant reduction in BRCA1-dependent ubiquitination activity when reactions included the E2 factor

UbcH5c⁵⁸.

In this study, we tested the BRCA1(I26A) variant function in the regulation of centrosome duplication and homologous recombination repair of DNA damage by two tissue culture based assays. Intriguingly, the BRCA1(I26A) variant functioned similarly as did the wild-type BRCA1. These results triggered us to test whether the BRCA(I26A) mutant is indeed enzymatically inert as an E3 ubiquitin ligase. We found that while the BRCA1(I26A) mutant has very low activity with the E2 factor UbcH5c, it is nearly as active as the wild type when reactions include the E2 factor Ubc13/Mms2.

3.3. Materials and Methods

In vitro ubiquitination.

Full length BRCA1/BARD1 wild type as well as the mutant BRCA1(I26A)/BARD1 (110 nM) were purified from baculovirus-infected insect cells⁵⁴. Polyubiquitination assays⁵³ contained 10 mM HEPES (pH 7.9), 0.5 mM EDTA, 5% glycerin, 60 mM KCl, 2 mM ATP, 5 mM MgCl₂, 30 nM E1-his, 38 μM bovine ubiquitin (Sigma) and 35 nM full length BRCA1/BARD1 wild type or the mutant. Reactions contained the E2 factor UbcH5c (10 μM) or Ubc13/Mms2 (1.6 μM), as indicated. Plasmids for the expression of Ubc13 and Mms2 in bacteria were the gift of P. Brzovic and R. Klevit (U. of Washington, Seattle). In reactions containing Ubc13/Mms2 the MgCl₂ concentration was 3 mM. Reactions were incubated at 37C for 30 min followed by SDS-PAGE and immunoblotted for ubiquitin.

The purified centrosome fraction (2 µg) was added to reactions as described above¹¹⁰ and immunoblotted with anti-γ-tubulin (Sigma).

Tissue culture-based assays.

The homologous recombination assay was done using published methods¹⁸. For the centrosome assay, Hs578T cells were transfected with either the control GL2 siRNA or BRCA1-3'UTR-specific siRNA with cotransfected plasmid expressing BRCA1 wild type or the I26A mutant using Lipofectamine 2000 (Invitrogen). The GFP-centrin plasmid was co-transfected and cells with abnormal centrosomes were counted by fluorescence microscopy¹¹¹.

3.4. Results

3.4.1. In vivo analysis of the BRCA1(I26A) Mutant Protein.

We tested whether the BRCA1(I26A) variant would affect two tissue culture based assays for BRCA1 function. In each assay, the endogenous BRCA1 protein is depleted by RNAi specific for the 3' UTR of the *BRCA1* mRNA, and mutant proteins are re-expressed in these cells using a co-transfected plasmid. We had previously shown that the BRCA1-dependent ubiquitin ligase activity was required for centrosome function in Hs578T cells^{110,111}. We were, thus, surprised that depletion of the endogenous BRCA1 protein and re-expression of the putative BRCA1(I26A) enzymatic mutant fully restored control of centrosome duplication (Figure 3.1.A). Depletion of BRCA1 resulted in the number of cells with aberrant

centrosomes changing from about 2% in the case of the control siRNA to 14% when treated with the BRCA1 3'-UTR specific siRNA, and re-expression of the BRCA1(I26A) mutant fully restored control of centrosome number.

We tested the mutant BRCA1 for BRCA1-dependent control of homologous recombination^{18,112}. In this assay, homology directed recombination that occurs at the cut-site of a rare-cutting enzyme, I-SceI, results in the gene conversion of an inactive GFP³ allele into an active allele. Active homologous recombination results in green fluorescing cells¹¹², and depletion of BRCA1 blocks this process^{18,63}. Consistent with an earlier observation¹¹³, we found the re-expression of the BRCA1(I26A) mutant restored about 75% of the activity of the wild type in this assay (Figure 3.1.B). Western blot for BRCA1 showed similar expression levels of wild type and I26A mutant of BRCA1 in the cells (Figure 3.1.C). Thus, the BRCA1(I26A) mutant protein functioned normally in regulating centrosomes and homologous recombination.

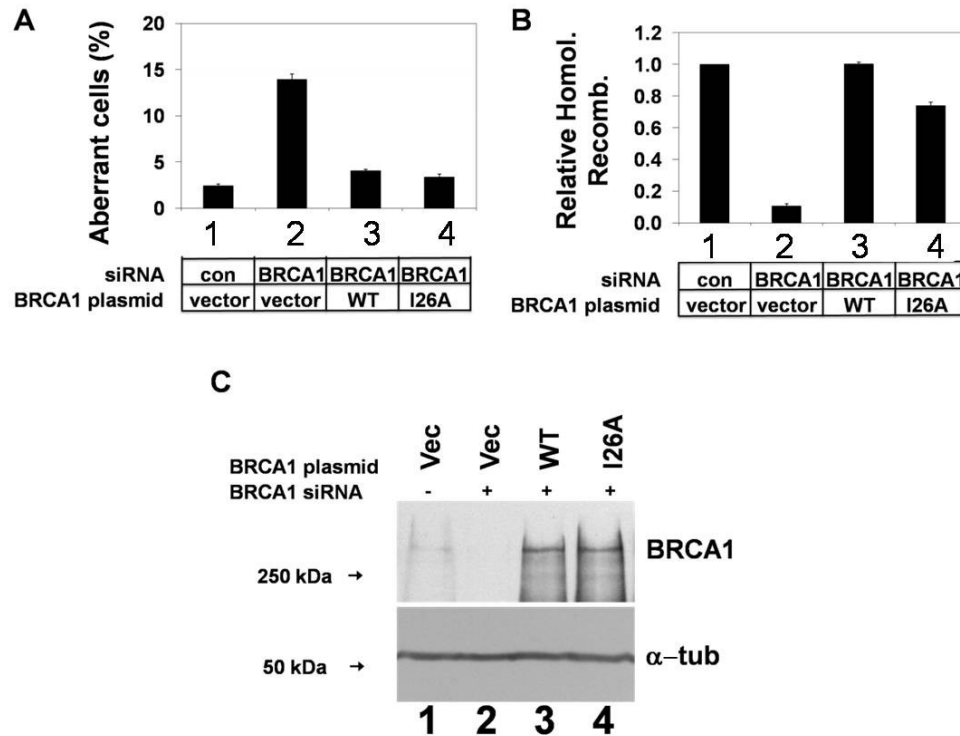


Figure 3.1. The activity of the BRCA1(I26A) mutant in tissue culture based functional assays.

A. Centrosome duplication regulation was tested using a BRCA1 depletion and add-back strategy where endogenous BRCA1 was depleted from the breast cancer cell line Hs578T by a BRCA1-3'UTR specific siRNA (lanes 2-4) and exogenous wild type (lane 3) or mutant (lane 4) proteins were re-expressed by a co-transfected plasmid. The percentage of cells with supernumerary centrosomes was quantified. **B.** BRCA1-dependent control of homologous recombination was tested under the same BRCA1 depletion and add-back strategy as in panel A and using a cell line that expresses GFP following active homologous recombination¹⁸. The results following control siRNA transfection (lane 1) were normalized to 1.0. The level of homologous recombination after depletion of BRCA1 (lanes 2-4) and

re-expression of the wild type (lane 3) or BRCA1(I26A) mutant (lane 4) are shown.

C. Immunoblot analysis of BRCA1 content of cells in panel B transfected as above.

The loading control was α -tubulin.

3.4.2. Active E3 ubiquitin ligase of BRCA1(I26A)/BARD1 mutant protein with the E2 factor Ubc13/Mms2.

Since the BRCA1(I26A) protein was fully functional in controlling centrosome duplication, we wondered whether this mutant might in fact be enzymatically active. It had been shown that this mutant does not bind to the E2 factor UbcH5c⁵⁸, and it had been shown that the ubiquitination of some substrates was diminished in the presence of this mutant^{55,114}, and a variety of E2 factors were found not to bind to this mutant in a yeast two-hybrid assay¹¹⁵. Nevertheless, we tested whether the BRCA1(I26A) mutant was in fact enzymatically inert as an E3 ubiquitin ligase. We purified full length wild-type and BRCA1(I26A)/BARD1 from baculovirus infected insect cells (Figure 3.2.A) and tested it in a polyubiquitination assay.

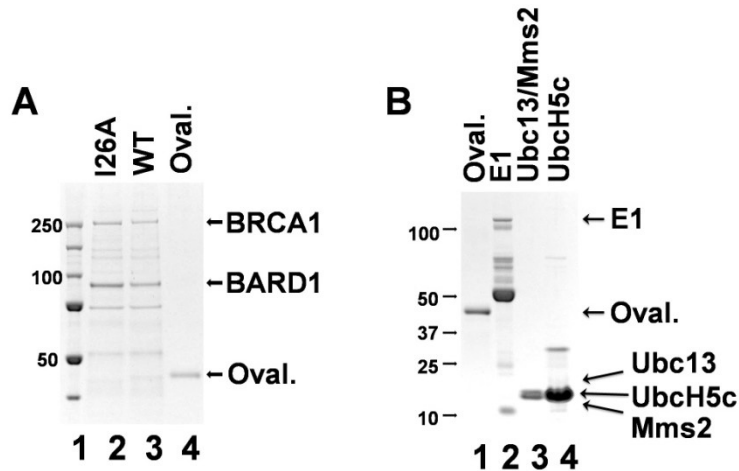


Figure 3.2. Protein content analysis for BRCA1/BARD1, E1 and E2 preps.

A. full length wild type (lane 3) and BRCA1(I26A) mutant (lane 2) proteins were co-purified with BARD1 from baculovirus infected High Five cells. Purified products were analyzed by SDS-PAGE and stained with Coomassie blue. As a quantitation control, 0.5 μ g of ovalbumin was analyzed in lane 4. **B.** Ubiquitination factors expressed and purified from bacteria were analyzed by SDS-PAGE and stained with Coomassie blue: His₆-E1 (110 kDa, lane 2), Ubc13/Mms2 (each subunit at 16 kDa, lane 3) and UbcH5c (16 kDa, lane 4). As a quantitation control, 2 μ g of ovalbumin were analyzed in lane 1.

We tested the E2 factor UbcH5c, which binds to BRCA1/BARD1^{56,116} and functions in a variety of assays^{54,55,110,117}. In addition, we tested the heterodimeric E2 factor, Ubc13/Mms2, which can function with BRCA1/BARD1, though a two-hybrid assay suggested that the Ubc13 does not bind to the BRCA1(I26A) variant¹¹⁵. The purified E2 factors are shown in Figure 3.2.B.

In the *in vitro* polyubiquitination assay, E1, UbcH5c, ubiquitin, and BRCA1/BARD1 (3-35 nM) are incubated together, and reaction products are evaluated by immunoblot analysis using a polyclonal antibody specific for ubiquitin⁵³. Ubiquitin monomer migrates below the 10 kDa marker and a smear of bands from 25-250 kDa indicates positive ubiquitin ligase activity. Consistent with previous studies^{58,66,117,118}, in the presence of the UbcH5c E2 factor the abundance of polyubiquitin chain products were significantly reduced when comparing the BRCA1(I26A)/BARD1 mutant relative to the wild-type BRCA1/BARD1 (Figure 3.3.A). The wild-type BRCA1/BARD1 polymerizes ubiquitin monomers into chains that migrate on gels with mass consistent with 25 kDa to over 250 kDa and when titrated to higher concentrations, a dose-dependent increase in polymer products of ubiquitin was detected (Figure 3.3.A, lanes 6-8).

The heterodimeric E2 factor Ubc13/Mms2 is a good candidate for activity with the mutant BRCA1 since it is required for the repair of double-strand DNA breaks^{119,120}, a process in which BRCA1/BARD1 also function and for which the I26A mutant is not defective¹¹³. This E2 factor (1.6 μ M Ubc13/Mms2) generates short oligomers of ubiquitin independent of the presence of an E3 (Figure 3.3.B, lanes 1 and 5), consistent with previous studies¹¹⁵. We had found that when in the presence of 3 mM MgCl₂, as opposed to 5 mM MgCl₂ typically used with UbcH5c, that BRCA1/BARD1 and Ubc13/Mms2 effectively polymerized long chains of ubiquitin (Figure 3.3.B, lanes 6-8). Strikingly, the BRCA1(I26A) mutant also

produced long ubiquitin polymers. With both, BRCA1 and BRCA1(I26A), the abundance of ubiquitin polymer product increased consistent with the concentration of the BRCA1. Though the BRCA1(I26A) was moderately less active as an ubiquitin ligase than the wild-type protein, in reactions containing 35 nM BRCA1(I26A), the abundance of product was higher than the concentration of polyubiquitin in reactions containing 10 nM BRCA1 wild-type. We interpret this result to indicate that when in the presence of Ubc13/Mms2, the BRCA1(I26A) enzyme is less than three-fold impaired relative to the wild-type (Figure 3.3.B). We conclude that the BRCA1(I26A)/BARD1 protein complex is indeed active as an ubiquitin ligase.

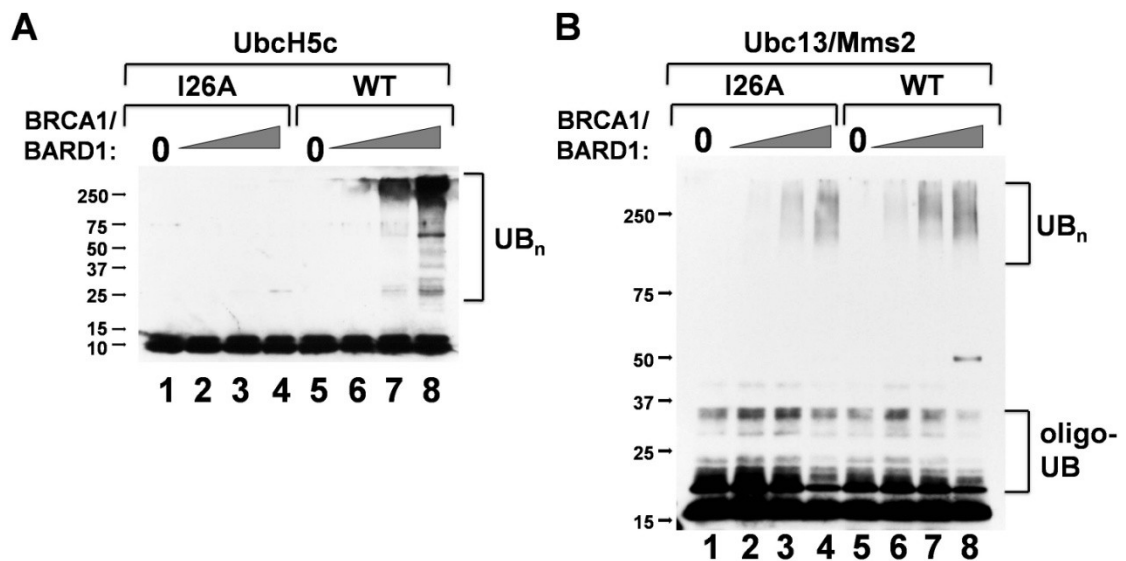


Figure 3.3. BRCA1(I26A)/BARD1 is an active E3 ubiquitin ligase when in the presence of Ubc13/Mms2.

A. *In vitro* BRCA1-dependent polyubiquitination assay including the E2 UbcH5c

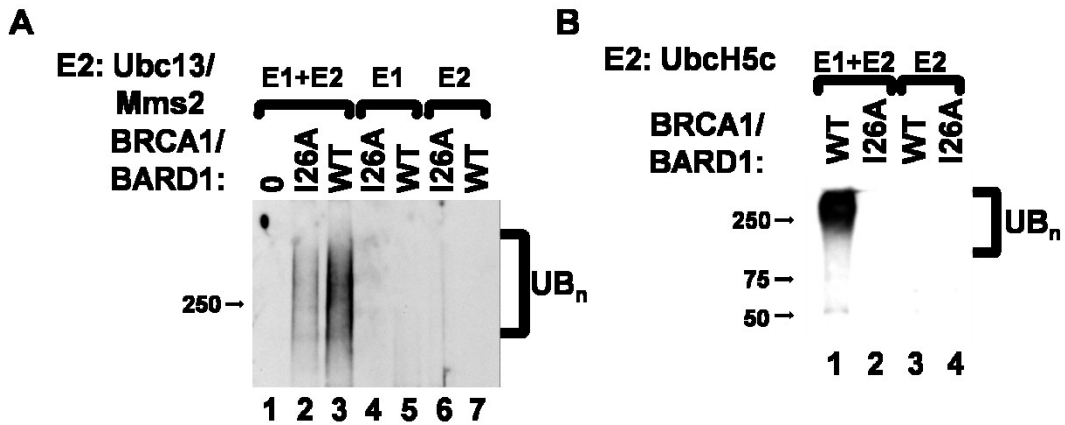
and BRCA1/BARD1 wild type (lanes 6-8) or I26A mutant (lanes 2-4) proteins were tested at concentrations of 3 nM (lanes 2, 6), 10 nM (lanes 3, 7), and 35 nM (lanes 4, 8). Reaction products were analyzed by immunoblotting with ubiquitin-specific antibody. Ubiquitin polymers are indicated (UB_n). **B.** *In vitro* polyubiquitination assays, as in panel A, were repeated except including the E2 Ubc13/Mms2 in reactions. E3-dependent ubiquitin polymers (UB_n) and oligoubiquitin (oligo-UB) were detected.

In order to rule out a trivial explanation for the positive E3 ubiquitin ligase activity of the BRCA1(I26A)/BARD1, we tested whether the production of polyubiquitin in the presence of the BRCA1(I26A)/BARD1 preparation was dependent on E1 and E2 factors. The generation of long polymers of ubiquitin by BRCA1(I26A)/BARD1, E1, and Ubc13/Mms2 was dependent on the presence of all three factors (Figure 3.4.A). Further, the similar reaction but including Ubch5c as the E2 factor was dependent on the wild-type BRCA1/BARD1 plus the E1 and E2 (Figure 3.4.B). We thus conclude that BRCA1(I26A)/BARD1 is indeed an active E3 ubiquitin ligase.

Figure 3.4. Generation of polyubiquitin by BRCA1(I26A)/BARD1 requires both E1 and E2.

A. Polyubiquitination assays were tested for the requirement of E1 or E2. Reactions were analyzed by immunoblots using an ubiquitin-specific antibody.

Reactions containing both, the E1 and Ubc13/Mms2 (lanes 1-3), just E1 (lanes 4, 5), or Ubc13/Mms2 (lanes 6, 7) also included BRCA1(I26A)/BARD1 (lanes 2, 4, 6) or wild-type BRCA1/BARD1 (lanes 3, 5, 7). **B.** Reactions were analyzed as in panel A, except reactions included the E2 factor, UbcH5c as indicated.



3.4.3. BRCA1(I26A)/BARD1 monoubiquitinates γ -tubulin.

BRCA1-dependent ubiquitination of γ -tubulin has been shown to be important in the regulation of centrosome function¹¹⁰. Since the I26A mutant functioned similarly as did the wild-type BRCA1 in controlling centrosome number (Figure 3.1.A), we tested whether BRCA1(I26A)/BARD1 could monoubiquitinate γ -tubulin. Wild-type BRCA1/BARD1 monoubiquitinated γ -tubulin in the presence of either E2 factor (Figure 3.5., lanes 3 and 6). Consistent with our results for active BRCA1(I26A)/BARD1 in the polyubiquitination assay, we find that BRCA1(I26A)/BARD1 and Ubc13/Mms2 monoubiquitinates γ -tubulin (Figure 3.5.,

lane 2). Surprisingly, a low level of monoubiquitination was evident when the E2 factor included in the reaction was UbcH5c (Figure 3.5., lane 5). We infer from the result in lane 5 that the centrosome preparation contains some contaminating E2 factor, such as Ubc13/Mms2.

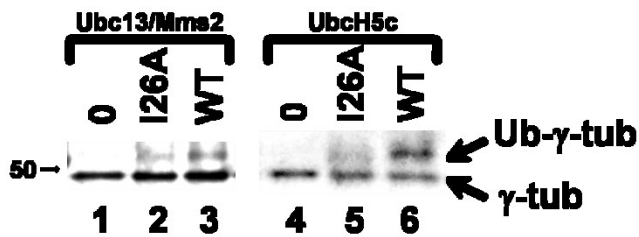


Figure 3.5. BRCA1(I26A)/BARD1 monoubiquitinates γ -tubulin *in vitro*.

The products of ubiquitination assays containing centrosomes were immunoblotted for γ -tubulin. Reactions included Ubc13/Mms2 (lanes 1-3), UbcH5c (lanes 4-6), BRCA1(I26A)/BARD1 (lanes 2 and 5), and BRCA1/BARD1 (lanes 3 and 6).

3.5. Discussion

Taken together, these results indicate that the putative enzymatic mutant BRCA1(I26A) still functions in the processes of centrosome control and of DNA repair (Figure 3.1.). It cannot, however, be concluded that the role of BRCA1 is independent of its E3 ubiquitin ligase activity since we have found that this mutant retains enzymatic activity when associated with the appropriate E2 factor. Using purified BRCA1(I26A)/BARD1 full length protein plus ubiquitination factors

purified from bacteria tested *in vitro*, we found conditions in which the mutant BRCA1 protein was about as active an E3 ubiquitin ligase as the wild type protein. Indeed, the BRCA1-dependent control of centrosome duplication in the breast cancer cell line is thought to be regulated by monoubiquitination of γ -tubulin^{54,110}, and we found that the BRCA1(I26A)/BARD1 mutant can catalyze the ubiquitination of this substrate.

Continuing studies are targeted toward the identification of the Ubc13/Mms2 binding surface on BRCA1(I26A)/BARD1. We speculate that the binding surface for the E2 factor is complex in the context of the full length proteins and may have reduced dependence on the isoleucine-26 residue.

In summary, the reevaluation of the E3 ubiquitin ligase activity of the putative BRCA1(I26A) enzymatic mutant reveals that it is indeed an active E3 ubiquitin ligase. From these results we think that conclusions about the dispensability of the BRCA1-dependent enzymatic activity in various cellular processes should be reconsidered.

Chapter 4: Regulation of 53BP1 protein stability by RNF8 and RNF168 is important for efficient DNA double-strand break repair

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Submitted

Author contributions:

- Hu, Y, Parvin JD and Mondal N designed the experiments.
- Hu, Y performed the experiments.
- Wang C & Huang K performed bioinformatic image analysis.
- Xia F provided the technique and cell line for C-NHEJ experiment.
- Parvin JD & Mondal N provided mentorship, advice, and financial support.

4.1. Abstract

53BP1 regulates DNA double-strand break (DSB) repair. In functional assays for specific DSB repair pathways, we found that 53BP1 was important in the conservative non-homologous end-joining (C-NHEJ) pathway, and this activity was dependent upon RNF8 and RNF168. We observed that 53BP1 protein was diffusely abundant in nuclei, and upon ionizing radiation, 53BP1 was everywhere degraded except at DNA damage sites. Depletion of RNF8 or RNF168 blocked the degradation of the diffusely localized nuclear 53BP1, and ionizing radiation induced foci (IRIF) did not form. While prior observations had suggested that 53BP1 is recruited to damage sites, our results indicate that the 53BP1 pool is degraded everywhere with the exception of DNA damage sites where 53BP1 is bound and stabilized. Furthermore, when 53BP1 degradation was inhibited in the nuclei, localization of its downstream effector RIF1 at DSBs was abolished. In conclusion, our data suggest a novel mechanism for responding to DSB that upon ionizing irradiation, 53BP1 was divided into two populations, ensuring functional DSB repair: damage site-bound 53BP1 whose binding signal is known to be generated by RNF8 and RNF168; and unbound bulk 53BP1 whose ensuing degradation is regulated by RNF8 and RNF168.

4.2. Introduction

DNA double-strand break (DSB) repair involves two major pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ). HR has a major homology-directed repair (HDR) pathway, which is a relatively precise form of repair and a minor subpathway called single-strand annealing (SSA), which causes DNA resection until homology at repair junctions is revealed¹²¹. To date, two types of end-joining systems are defined in the NHEJ: the first one is the conservative NHEJ (C-NHEJ), which is predominantly associated with precise joining of DSB ends without altering the DNA sequence¹¹. The alternative pathway for NHEJ (Alt-NHEJ) is highly mutagenic and deletional since it catalyzes DNA resection and utilizes imperfect microhomology for end-joining partners and thus resulting in deletions at repair junctions¹⁴. 53BP1 is known to promote the repair of DSBs by NHEJ⁷⁵⁻⁸⁰. 53BP1 deletion in mouse results in a severe defect in class-switch recombination, a process dependent on NHEJ and associated with increased DNA end resection at the IgH locus⁸¹⁻⁸³. Loss of 53BP1 restores homologous recombination in BRCA1-deficient murine cells, indicating that 53BP1 inhibits DNA resection in DSB repair, by the regulation of the downstream effector RIF1 to control 5' end resection^{6,70,84}. While it is known that 53BP1 directly regulates efficient total NHEJ repair events in mammalian cells⁸⁶, the specific function of 53BP1 is not yet well-defined in terms of which subpathway in the NHEJ repair process 53BP1 functions, C-NHEJ versus Alt-NHEJ. Upon DNA DSB induction, a cascade of protein modification and relocalization is triggered: phosphorylation of H2AX (γ -H2AX) results in the recruitment of downstream

factors, such as the E3 ubiquitin ligases RNF8 and RNF168, leading to the formation of K63-linked polyubiquitin chains on histones at DSBs. This ubiquitination cascade regulated by RNF8 and RNF168 is responsible for the localization of repair mediators, including BRCA1 and 53BP1 to the DNA damage sites^{23-27,29-32,122}. Localization of 53BP1 involves its recognition of H2A ubiquitinated on Lys-15 (H2AK15ub), the latter being a product of RNF168 via its ubiquitination-dependent recruitment (UDR) motif binding to K63-linked ubiquitination on chromatin. 53BP1 binding to the chromatin also requires dimethylation of histone H4 on lysine 20 (H4K20me2) via the 53BP1 tandem Tudor domain at the damage sites³⁴⁻³⁷. In this study, we identified that 53BP1 acts specifically to promote conservative-NHEJ in a RNF8- and RNF168-dependent manner. We found that RNF8 and RNF168 not only mark histones at the break site to create a 53BP1 binding site, but these ubiquitin ligases also regulate the proteasome-mediated degradation of 53BP1. Failure to degrade 53BP1 protein not at DSBs leads to mislocalization of a downstream factor RIF1, thus impairing DSB repair.

4.3. Materials and Methods

HR and end-joining assays

HDR, SSA and Alt-EJ were performed as previously described^{17,18,20}, respectively. The HeLa-derived cell lines that stably integrate the HDR and SSA recombination substrates have been described²⁰. The Alt-EJ substrate¹⁷ was stably integrated

into the HeLa genome. For the DSB repair assays, the appropriate cell line was transfected with siRNAs and/or expression plasmid on day 1. On day 3 the cells were re-transfected plus a plasmid expressing the I-SceI endonuclease, which initiates a DSB lesion. The amount of repair activity was determined by counting the percentage of GFP-positive cells using flow cytometry. The C-NHEJ assay utilized quantitative real-time PCR and was carried out as described in²² with the following modification. The genomic DNA isolated 3 days after transfection of the I-SceI plasmid was treated with the restriction enzyme XhoI and purified by Qiagen PCR purification kit before real-time PCR was applied. RPS17 probe (Hs00734303_g1, Applied Biosystems) was used as an internal control and quantitative $\Delta\Delta C_T$ method was used to analyze the data.

RNA interference and plasmids

We used the following siRNAs produced by Sigma: siControl targeting the luciferase gene: 5'-CGUACGCGGAAUACUUCGA-3'¹⁸;
si53BP1:5'-GAAGGACGGAGUACUAAUA-3'⁵¹; si53BP1-2 starting at nucleotide 6051: 5'-UACUUGGUCUACUGGUUU-3';
siRNF8:5'-GGACAAUUAUGGACAACAA-3'¹²³; siRNF168:
5'-GGCGAAGAGCGAUGGAGGA-3'⁵¹; siLigase IV:
5'-AGGAAGUAUUCUCAGGAAUUA-3'⁵¹; siBRCA1:
5'-GCUCCUCUCACUCUUCAGU-3'¹⁸; siBRCA2:
5'-UAAAUUUGGACAUAAGGAGUCCUCC- 3'¹⁸. Wild-type 53BP1 expression

plasmid was a kind gift from Kuniyoshi Iwabuchi (Kanazawa Medical University). I-SceI expression plasmid has been previously described¹⁸ and was a kind gift from Maria Jasin (Memorial Sloan-Kettering Cancer Institute). The total siRNA amount was adjusted to be the same in each sample by adding siControl. All RNAi transfections were carried out using Oligofectamine (Life Technologies) and plasmid transfections were using Lipofectamine2000 (Life Technologies).

Preparation of Whole Cell lysates

Whole cell extracts (if not indicated) were prepared by lysing cells in cell lysis buffer (50 mM Tris, pH 7.9, 300 mM NaCl, 0.5% Nonidet-40, 1 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1X complete protease inhibitor cocktail from Sigma). Alternatively, when indicated the whole cell extracts were prepared by direct boiling in sodium dodecyl sulfate (SDS) containing buffer (2% SDS in phosphate-buffered saline).

Immunofluorescence microscopy

Cells were fixed with cold 4% paraformaldehyde for 15 min and permeabilized with cold 70% ethanol for 5 min before blocking in 8% bovine serum albumin/phosphate-buffered saline for 1 h. Primary antibodies were diluted at 1:500 (the rabbit γ -H2AX antibody was used at 1:1000 dilution) for incubation at room temperature for 2 h. Cells were washed with phosphate-buffered saline and stained with secondary antibodies. DAPI was then added at 1: 10,000 for 5 min to

stain the nucleus. For experiments in which the cells were extracted with NP40 prior to fixation, the extraction buffer (same as the cell lysis buffer above) was applied to cells that had been washed in PBS, followed by the above protocol for fixation and staining. Images were viewed and acquired using the 60X oil objective lens with a Zeiss Axiovert 200 M microscope. Signals for the same indicated protein were obtained in the same exposing time for all samples. The captured 12-bit microscopic images in zvi format were exported to 8-bit RGB tif images by software AxioVision 4.8. This process does not affect the downstream quantitative image analysis.

Image analysis

Image analysis of 53BP1 protein signal in the nuclei was carried out using MATLAB R2013a. 100 cells in each sample were randomly selected. The distributions of pixel intensity values of 53BP1 protein signal in different samples were then compared. Specifically, DAPI stain was used to segment the outlines of nuclei and masked on the 53BP1 stain to measure the 53BP1 signal within the nuclei mask area. Distribution of pixel intensity in each cohort in 100 nuclei was examined using histogram with the x-axis being for intensities of {1, 5, 9, ..., 253}, (bin size = 4). To analyze the relative 53BP1 density in different samples, total signal of 53BP1 was measured within the mask and normalized to the size of the nuclei measured by DAPI in each sample. The above normalized value of 53BP1 signal in irradiation sample was further normalized according to the no irradiation

sample in each experimental repeat.

Antibodies and Reagents

We used the following primary antibodies: 53BP1 (Santa Cruz, H-300, for immunoblot and immunofluorescence), RAD51 (Santa Cruz, H-92), γ -H2AX (Millipore, clone JBW301, for immunoblot and Immunofluorescence), RIF1 (Santa Cruz, N-20, for immunoblot and Immunofluorescence), RNF8 (Abnova), RNF168 (Abcam), α -tubulin (Sigma), H4 (Millipore), β -actin (Cell signaling), and RHA (purified from rabbit serum). MG132 (Enzo life Sciences, dissolved in DMSO, treated 30 min prior to irradiation), caffeine (Sigma, dissolved in ddH₂O, treated 1 h prior to irradiation), cycloheximide (Fluka Analytical, dissolved in ethanol, treated 15 min prior to irradiation).

Statistical Analysis

Data were objectively compared between different groups for each sample using unpaired and two-tailed Student's t test (*, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

4.4. Results

4.4.1. 53BP1 functions in conservative-NHEJ dependent on RNF8 and RNF168.

53BP1 regulates DNA double-strand break (DSB) repair in the NHEJ pathway,

but its specific function is unclear. To explore the role of 53BP1 in a specific pathway of DSB, we used cell lines that contain integrated into their genomes recombination substrates that specifically probe the conservative-NHEJ, alternative-NHEJ, homology directed recombination (HDR), and single-strand annealing (SSA) repair pathways^{17,18,20,22}. The general strategy is to deplete by siRNA transfection 53BP1 or another factor, followed by transfection of a plasmid that expresses the rare-cutting restriction endonuclease I-SceI, which simulates a DSB at a specific site. 293/HW1 cells²² contain a DNA substrate with two neighboring I-SceI sites in the genome for which repair by the conservative-NHEJ pathway can be measured by the precise joining of the DNA ends following I-SceI expression. The concentration of DNAs repaired by conservative-NHEJ was measured by real-time PCR using an oligonucleotide probe that spans the break site. Depletion of ligase IV, which is known to affect the NHEJ repair frequency, reduced the conservative-NHEJ repair to 18% relative to the control siRNA. To test 53BP1 in the conservative-NHEJ pathway, 53BP1 was depleted in 293/HW1 cells, and we found that repair efficiency decreased to approximately 41% relative to the control siRNA (Figure 4.1.A). Transfection of another siRNA targeting the *TP53BP1* 3' untranslated region (3'UTR) sequence to deplete endogenous mRNA resulted in a decrease of conservative-NHEJ to 70% relative to the control. This siRNA reproducibly yielded less inhibition of the conservative-NHEJ than did the siRNA targeting the 53BP1 coding region. When this 3'-UTR specific siRNA was co-transfected with a plasmid expressing wild-type 53BP1 resistant to this siRNA,

conservative-NHEJ repair efficiency was restored to 98% relative to the control, demonstrating the specificity of the siRNA depletions (Figure 4.1.B). Depletion of BRCA1, a protein that regulates multiple DSB repair pathways, reduced conservative-NHEJ repair efficiency to about 49%. Co-depletion of 53BP1 and BRCA1 depressed the ratio further to about 19.4%, indicating that in this repair pathway BRCA1 and 53BP1 function independently. The E3 ubiquitin ligases RNF8 and RNF168 have been demonstrated to be required for 53BP1 and BRCA1 localization at DSBs in an ubiquitination-dependent manner²³. We depleted RNF8 or RNF168 by siRNA in 293/HW1 cells, resulting in a decrease in the conservative-NHEJ to 14% and 31%, respectively (Figure 4.1.A, lanes 6, 7). Co-depletion of both RNF8 and RNF168 did not have any additive effect compared to either single depletion (Figure 4.1.A, lane 10), indicating the epistatic role of RNF8 with RNF168 in the conservative-NHEJ pathway. We then tested whether RNF8 or RNF168 also regulates 53BP1 function in the C-NHEJ process. Co-depletion of RNF8 or RNF168 with 53BP1 had no additive effect relative to single depletion, consistent with the concept that RNF8 and RNF168 function in the same NHEJ pathway as 53BP1. These results along with prior observations^{25,26,28} indicate that RNF8 and RNF168 are epistatic with 53BP1, which functions in C-NHEJ in a RNF8/ RNF168-dependent manner. We next tested 53BP1 function in the Alt-NHEJ pathway using a cell line, HeLa-EJ2, which has integrated in its genome a recombination substrate that is repaired by Alt-NHEJ to generate a functional GFP gene¹⁷. Ligase IV served as a positive

control, and depletion of ligase IV resulted in a decrease in Alt-NHEJ to 12%. BRCA1 depletion caused a decrease to 55% relative to the control. Depletion of 53BP1, RNF8, or RNF168 each had minimal effect on Alt-NHEJ, which was not statistically significant (Figure 4.1.C). Co-depletion of 53BP1 with RNF8 or RNF168 had little impact on Alt-NHEJ though co-depletion of both RNF8 and RNF168 did impair the Alt-NHEJ repair efficiency compared to control (Figure 4.1.C, lanes 8-10). This last result suggests that RNF8 and RNF168 have redundant function in the Alt-NHEJ pathway, but it is independent of 53BP1. We compared 53BP1 to BRCA1 function in homologous recombination, which has a major pathway of HDR and the minor SSA pathway. We utilized HeLa-DR cells and HeLa-SA cells to conduct the HDR and SSA assays, respectively. Repair by each pathway is measured by the conversion of cells to GFP-positive. Depletion of 53BP1 had no effect in HDR (Figure 4.1.D) but increased SSA (Figure 4.1.E). The increase in SSA activity in 53BP1-depleted cells probably reflected relief from 53BP1-mediated inhibition of DNA resection needed for the SSA pathway. BRCA1 depletion affected both homologous recombination pathways (Figure 4.1.D, E). Co-depletion of 53BP1 partially rescued the deficit caused by single depletion of BRCA1 in HDR or SSA. RNF8 and RNF168 depletions resulted in a statistically significant decrease in HDR but not in SSA. Co-depletion of both RNF8 and RNF168 had an additive effect in HDR and caused a decrease of repair efficiency. In the SSA assay, RNF8 depletion caused a minor decrease in SSA whereas RNF168 depletion minimally increased SSA, although these

modest changes were not statistically significant. Co-depletion of both RNF8 and RNF168 resulted in suppression of the deficit due to RNF8, reflecting their opposite functions to each other in this minor pathway. BRCA2, which suppresses SSA^{20,64}, was used as a negative control (Figure 4.1.E). Co-depletion of 53BP1 and either RNF8 or RNF168 did not show any additive effect in both assays. Depletions of protein by siRNAs were confirmed by immunoblot (Figure 4.1.F). In summary for Figure 4.1., we investigated the role of 53BP1 in DNA DSB repair pathways and identified that it functions positively in conservative-NHEJ pathway, has no function in either HDR or Alt-NHEJ, and 53BP1 suppresses SSA. 53BP1 functions in the same C-NHEJ pathway as RNF8 and RNF168, which are known to regulate 53BP1 localization to sites of DSBs. Combined with prior studies, these results suggest that 53BP1 positively regulates conservative-NHEJ pathway in a RNF8- and RNF168-dependent manner.

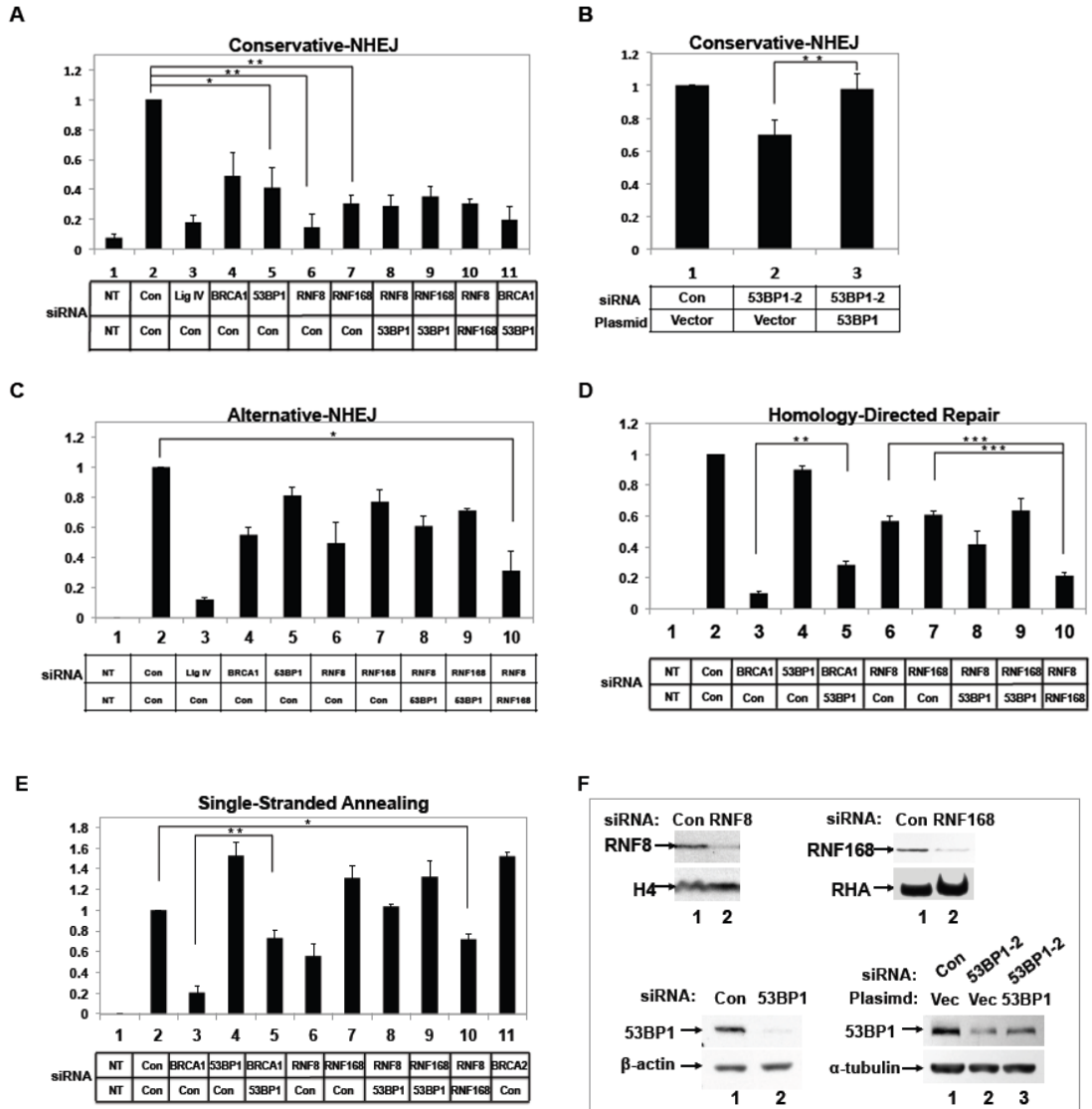


FIGURE 4.1. 53BP1 function in conservative-NEHJ is dependent on RNF8 and RNF168.

A. 293/HW1 cells transfected with indicated siRNAs (bottom grid) followed by transfection of the I-SceI expression plasmid to induced DSB. After 3 days, the repair efficiency was measured by applying quantitative real-time PCR on extracted DNA, represented by the percentage on the Y axis. In each experiment,

the yield of conservatively repaired DNA was normalized relative to the result from the control siRNA transfection. Results (+/- SEM) are from three independent experiments. NT indicates no transfection of the I-SceI expressing plasmid. **B.** same as in panel A except that siRNA targeting the 53BP1 3'UTR was transfected in combination with the wild-type 53BP1 expression plasmid or an empty vector, as indicated. **C-E.** cells were subjected to two rounds of transfections as in A and the percentages of GFP-positive cells were determined by flow cytometry. In each experiment, the percentage of GFP-positive cells from control siRNA transfections was set equal to 1, and the fraction of GFP-positive cells was determined relative to the control siRNA to measure Alt-EJ, HDR, and SSA respectively. **F.** immunoblots show the depletion of indicated protein by RNAi interference, or the expression of 53BP1 protein by plasmid transfection.

4.4.2. 53BP1 is destabilized upon irradiation damage.

53BP1 forms ionizing radiation induced foci (IRIF) in response to DNA damage, and the protein has highest abundance during G1 phase, a stage in the cell cycle associated with NHEJ activity¹²⁴. To investigate 53BP1 protein dynamics in response to irradiation-induced DNA damage, we evaluated changes in 53BP1 protein bulk level 4 hours post-irradiation (10 Gy) by extracting HeLa cell lysates in SDS containing buffer. Surprisingly, the 53BP1 protein level decreased markedly compared to the non-irradiated control (Figure 4.2.A, lanes 1-6). In contrast, the protein abundance of another DSB repair factor involved in

homologous recombination for strand invasion, RAD51, did not change upon irradiation. The DSB damage signal sensor γ -H2AX was used as a positive control and histone H4 served as the loading control. We found that 53BP1 protein levels decreased as early as 15 minutes following ionizing radiation and were restored after 24 hours (Figure 4.2.A, lanes 2-7). Since 53BP1 is a DSB repair protein, we were surprised to observe that its protein abundance in HeLa cells sharply decreased within 15 minutes of DNA damage. To confirm this immunoblot result, we evaluated 53BP1 IRIF 4 h post-IR and then utilized image analysis to measure the total 53BP1 signal in the nuclei following exposure to irradiation. In non-irradiated cells, 53BP1 protein was diffusely abundant in nuclei, whereas upon ionizing radiation, 53BP1 was everywhere diminished except at DNA damage sites (Figure 4.2.B). The 53BP1 signal value of each pixel in each sample of 100 nuclei was normalized according to the size of the nuclei. The nuclear sizes in non-irradiated and irradiated samples did not change (data not shown). By this unbiased method, we found that the 53BP1 protein level in irradiated cells was about 43.7% of that level in non-irradiated cells (Figure 4.2.C, *right*). By plotting the distribution of pixel intensity in the image, we observed that for the irradiated cells, most of the pixels were in the very low intensity group (red trace in Figure 4.2.C, *left*) as would be expected for foci, whereas the 53BP1 stain in the non-irradiated cells was more evenly distributed for pixel intensity and with fewer low intensity pixels but more high intensity pixels (blue trace in Figure 4.2.C, *left*). Even for the foci in the irradiated sample with only a few pixels with very high

intensity (> 200) (bright dots of small area), the diffuse 53BP1 stain in non-irradiated cells was more intense (Figure 4.2.C, *left*, inset). This result clearly suggests that foci appearance is due to degradation in the nucleoplasm, otherwise, if there was recruitment of 53BP1 at sites of damage, pixel intensity at foci would be higher than intensity of pixels throughout the nuclei in non-irradiated cells. We also measured γ -H2AX signal by image analysis in both conditions (No IR versus IR, data not shown) and the protein level increased upon irradiation consistent with observations by immunoblotting (Figure 4.2.A). Though the appearance of 53BP1 at IRIF suggests recruitment from the nucleoplasm to DNA damage sites, these results presented here suggest instead that 53BP1 binding to damage sites renders it resistant to the universal degradation after ionizing radiation. Next we tested if the decrease in 53BP1 protein abundance in response to irradiation in HeLa cells was due to proteasome-dependent degradation. Inclusion in medium of MG132, the proteasome inhibitor, blocked the decrease in 53BP1 protein concentration upon irradiation whereas inclusion of caffeine in the medium, an ATM inhibitor, did not. This result indicated that protein degradation, but not loss of ATM-dependent phosphorylation of 53BP1, caused the decrease of 53BP1 level after irradiation (Figure 4.2.D). 53BP1 immunofluorescence following irradiation in HeLa cells was then analyzed in the presence of MG132, which resulted in the same diffuse 53BP1 pattern as observed in the absence of irradiation (Figure 4.2.E).

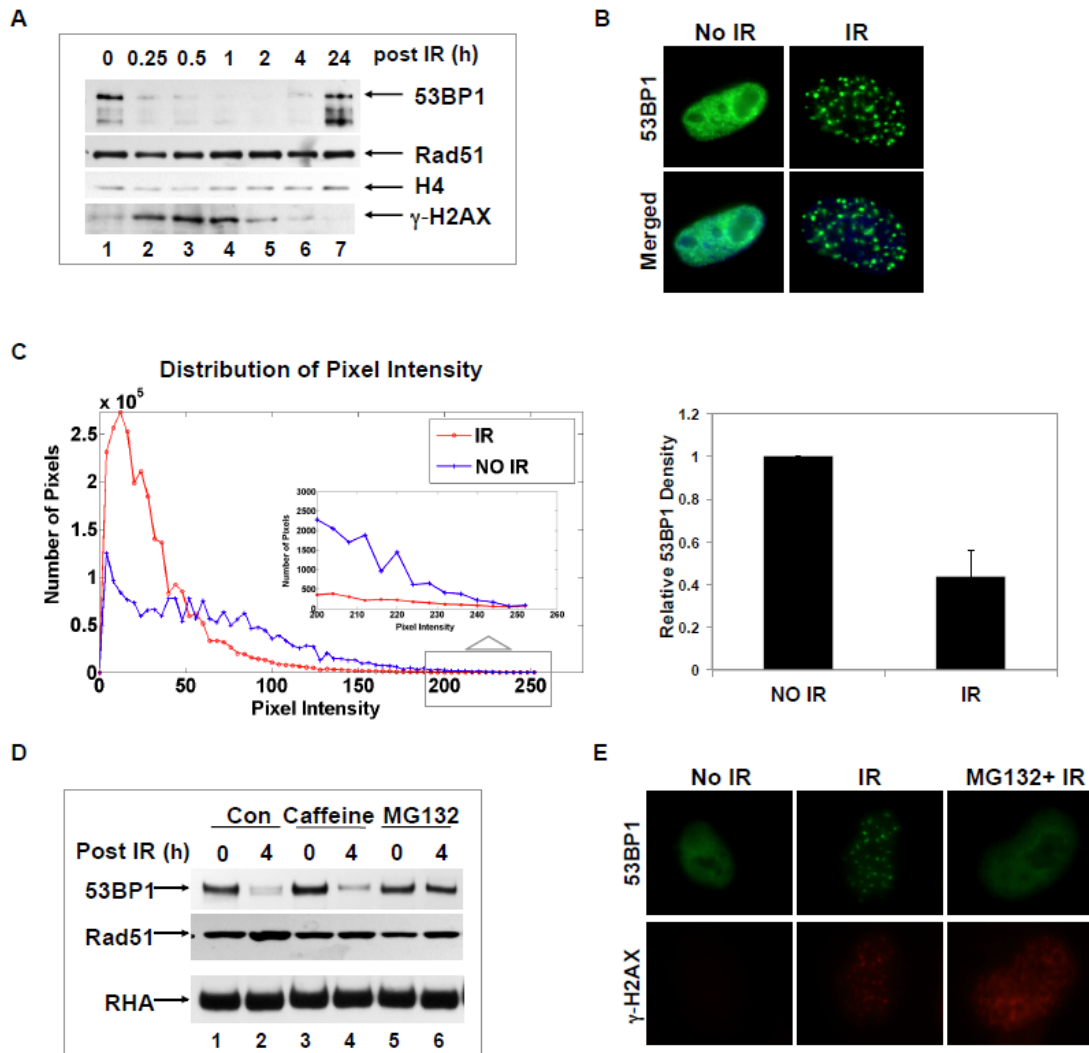


FIGURE 4.2. 53BP1 protein is degraded upon irradiation.

A. HeLa cells were subjected to 10 Gy X-rays and total cell lysates were prepared in SDS containing buffer at the indicated time points. Immunoblots of indicated protein were shown. **B.** HeLa cells were subjected to immunofluorescence microscopy 4 h post-irradiation (10 Gy). Cells were stained for 53BP1 (green; *top*) and merged with DAPI stain of DNA (blue; *bottom*). **C.** the distribution of pixel intensity of 53BP1 nuclear stain in panel B was plotted for No IR (blue) and IR

(red) (*left*). Results shown were typical of all five replicates of 100 cells in each. Pixel intensities within the range of 200- 253 were shown in the enlarged graph. *Right*, relative 53BP1 protein signal intensity (+/- SEM) as shown was obtained by applying five independent experiment repeats. In each experiment of 100 nuclei, 53BP1 intensity was measured by the image analysis in each sample and normalized according to the value from No IR sample. **D.** Before subjection to 10 Gy X-rays, HeLa cells were treated with medium alone (Con; lanes 1, 2), caffeine (10 mM; lane 3, 4) or with MG132 (20 μ M; lane 5, 6). 4 h post irradiation, cell lysates were prepared for immunoblots. **E.** HeLa cells were treated with or without MG132 as in D, and immunofluorescence microscopy was applied after 4 h irradiation (10 Gy) as in panel B.

Image analysis confirmed that the IRIF do not have higher intensity 53BP1 stain than did the unirradiated sample or the IR plus MG132 sample (Figure 4.3.), indicating that 53BP1 protein was degraded upon irradiation.

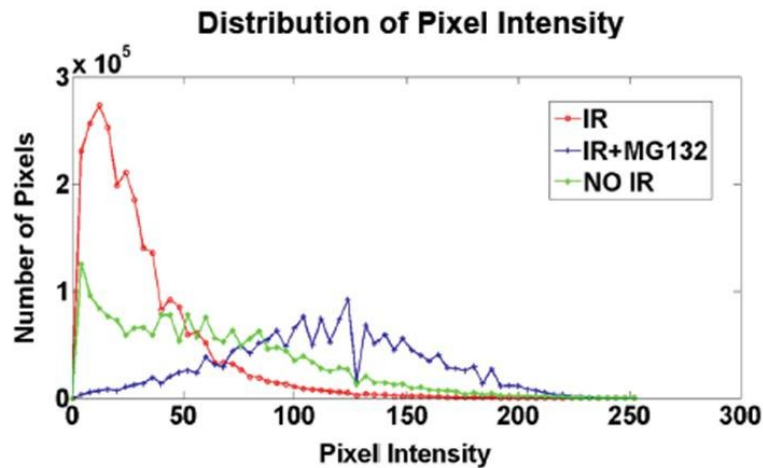


Figure 4.3. 53BP1 protein is degraded upon irradiation.
The distribution of pixel intensity was plotted for each sample in Figure 4.2.E.

4.4.3. Depletion of RNF8 or RNF168 blocks 53BP1 degradation upon irradiation

RNF8 and RNF168 are E3 ubiquitin ligases that mediate the conjugation of ubiquitin multimers on histone H2A via the degradation-independent lysine-63 side-chain of ubiquitin and via this activity recruit other proteins, such as 53BP1 and BRCA1, to the sites of DNA damage^{23,25-28,31}. We tested the possibility that these two enzymes are involved in 53BP1 protein degradation. Indeed, depletion of RNF8 or of RNF168 from HeLa cells and following irradiation-induced DNA damage, 53BP1 degradation was blocked (Figure 4.4.A, lanes 6, 8, 10). Depletion

of BRCA1, another E3 ubiquitin ligase involved in the DNA damage response, did not affect the 53BP1 protein level, indicating RNF8/ RNF168 had a specific role in the control of 53BP1 protein levels upon irradiation (Figure 4.4.A. lane 4). Consistent with the model that 53BP1 is degraded dependent on RNF8 and RNF168, the 53BP1 protein remained diffusely localized in the nucleus (Figure 4.4.B). The distribution of pixel intensity in immunofluorescence images showed that depletion of RNF8 and/or RNF168 had a similar distribution pattern as no irradiation, though with more intense pixels (Figure 4.4.C), suggesting that RNF8 and RNF168 regulate proteasome-dependent degradation and IRIF formation of 53BP1 in response to irradiation-induced DNA damage.

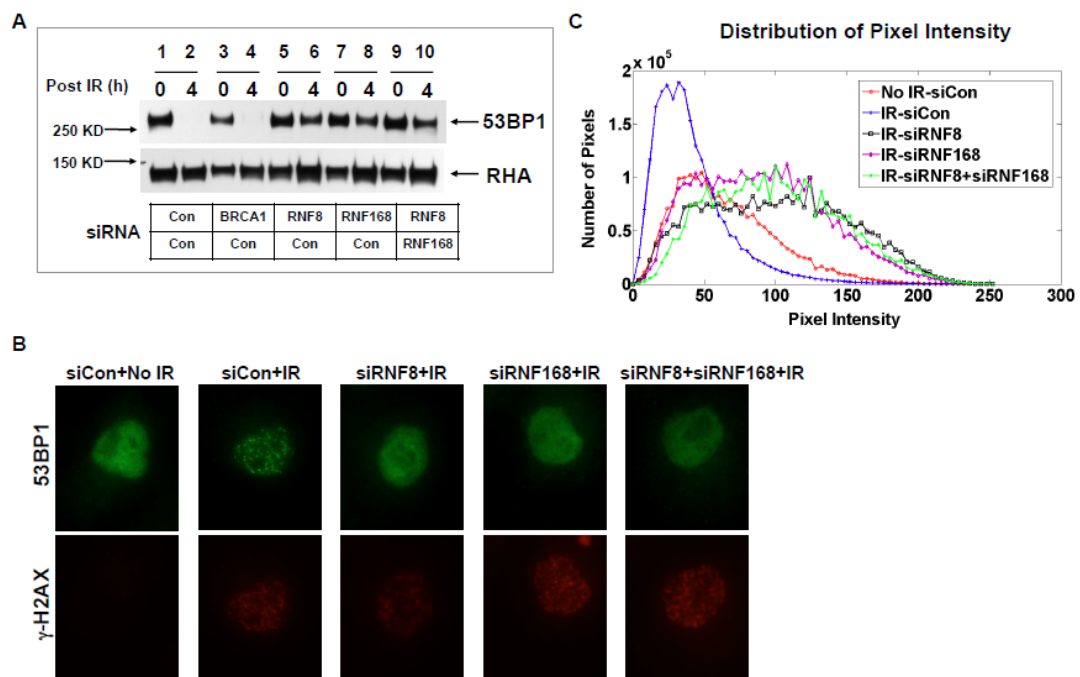


FIGURE 4.4. 53BP1 degradation upon irradiation is regulated by RNF8 and RNF168.

A. HeLa cells transfected with two different siRNAs (indicated in the grid) were

treated with 10 Gy X-rays. 4 hours post-IR cell lysates were prepared for 53BP1 immunoblot analysis. RHA served as a loading control. **B.** Immunofluorescence analysis of cells from panel A which were stained for 53BP1 (green) and γ -H2AX (red). **C.** distribution of pixel intensity was analyzed from microscopic images in panel B.

4.4.4. 53BP1 turnover is accelerated upon irradiation damage

Since 53BP1 protein abundance changed following irradiation, we speculated that irradiation shortened the 53BP1 protein half-life. Using MCF7 (Figure 4.5.) or HeLa cells (Figure 4.6.), we blocked new protein synthesis by the addition of cycloheximide to tissue culture media. In the absence of IR, protein levels were stable (Figure 4.5.A, lanes1-3). By contrast, following IR, 53BP1 turnover was apparent as early as 30 min post-IR, and the protein level decreased to 4% 4 hours post IR (Figure 4.5.A, B). By contrast, RAD51 half-life was not affected by IR (Figure 4.5.A). The results together implicated that ionizing radiation accelerates 53BP1 protein turnover via the proteasome-dependent pathway.

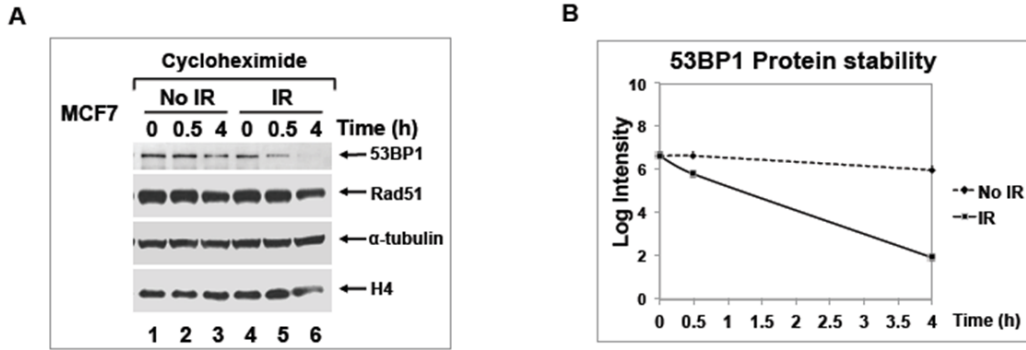


FIGURE 4.5. 53BP1 turnover is accelerated upon irradiation.

A. Cycloheximide (100 µg/mL) was added to MCF7 cells with or without irradiation (10 Gy) and total cell lysates were prepared in SDS containing buffer according to the indicated time course (0, 0.5 h and 4 h) and analyzed by immunoblotting as indicated. **B.** 53BP1 protein signal in immunoblot in A was measured by densitometry in each sample.

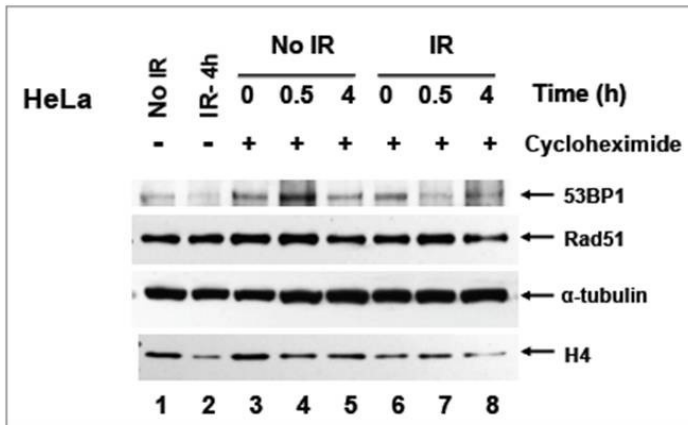


Figure 4.6. In HeLa cells, 53BP1 turnover was accelerated upon irradiation.

Procedure in lane 3-8 was done as in Figure 4.5.A except that HeLa cells were

analyzed and two controls were included: no irradiation and irradiation (post 4h IR) in lane 1 and 2.

4.4.5. 53BP1 does not relocate to damage site but rather is protected from degradation at damage sites

Previous reports had suggested that 53BP1 is recruited to damage sites in an RNF8/ RNF168 dependent manner^{25,28}. We speculated that degradation of 53BP1 upon irradiation, rather than the protein movement to the sites of DSBs, leads to 53BP1 focus formation. To differentiate between movement of the protein versus degradation of bulk 53BP1, we irradiated HeLa cells and after foci formed we blocked proteasome-mediated degradation. If 53BP1 protein relocated within the nucleus, then foci would remain even if the proteasome was blocked. If, on the other hand, the 53BP1 bound to the DNA damage site was stabilized then the foci would be surrounded by diffuse 53BP1 as new protein was synthesized. MG132 was added to the cells 1 hour post-irradiation and a series of time points were analyzed to observe the 53BP1 foci at the damage sites. (Refer to time-line in Figure 4.7.A, *top*.) In the absence of the MG132, 53BP1 containing IRIF were apparent at all post-IR time-points analyzed. By comparison, in the cells in which MG132 was added to the medium one hour post-IR, foci were still apparent, but these IRIF were in the presence of diffuse 53BP1 stain at late time points (Figure 4.7.A, *bottom*). As an indication of the diffuse 53BP1 localization, in the presence of MG132 the nucleoli become apparent as holes in the diffuse pattern. The

results from this experiment were quantified in Figure 4.7.B and show that MG132 treated cells primarily had diffuse nuclear 53BP1 stain or diffuse stain with foci, suggesting that degradation event in the nucleoplasm happened prior to MG132 treatment, and the diffuse stain was due to the appearance of newly synthesized 53BP1. When MG132 was added to the cells prior to IR, no foci were apparent (Figure 4.2.E). These results are most consistent with a model in which 53BP1 is continuously synthesized at a high rate, and post-IR it is rapidly degraded via the ubiquitin-proteasome system (Figure 4.5.A, B). Only at sites of DNA damage is it protected from ubiquitin-dependent degradation. Immunoblot results of 53BP1 protein were consistent with the notion that 53BP1 accumulated to high levels when in the presence of MG132 (Figure 4.7.C). By comparison, the concentration of the downstream effector RIF1 did not change following DNA damage (Figure 4.7.C). Together, these data suggest that following ionizing radiation, 53BP1 is rapidly synthesized and rapidly degraded except when bound to repair sites.

FIGURE 4.7. 53BP1 is degraded except when bound to a damage site.

A. *upper* panel shows the workflow of the experiment. 1 h after 10 Gy X-ray irradiation was applied to the HeLa cells, DMSO or MG132 (20 μ M) was added to the media. At time points 1.5 h, 2 h, 3 h, and 5 h post-IR, cells were either fixed for microscopy (*A, bottom*) or lysed for immunoblot analysis (*C*). **B.** in each sample, the percentage of the cells that have diffuse 53BP1 stain (blue), 53BP1 foci (orange) or diffuse 53BP1 stain with foci (green) was quantified (mean \pm SEM;

N=3). **C.** cell lysates taken from panel A were subjected to immunoblot for 53BP1 and RIF1 stain. Tubulin was a loading control.

A

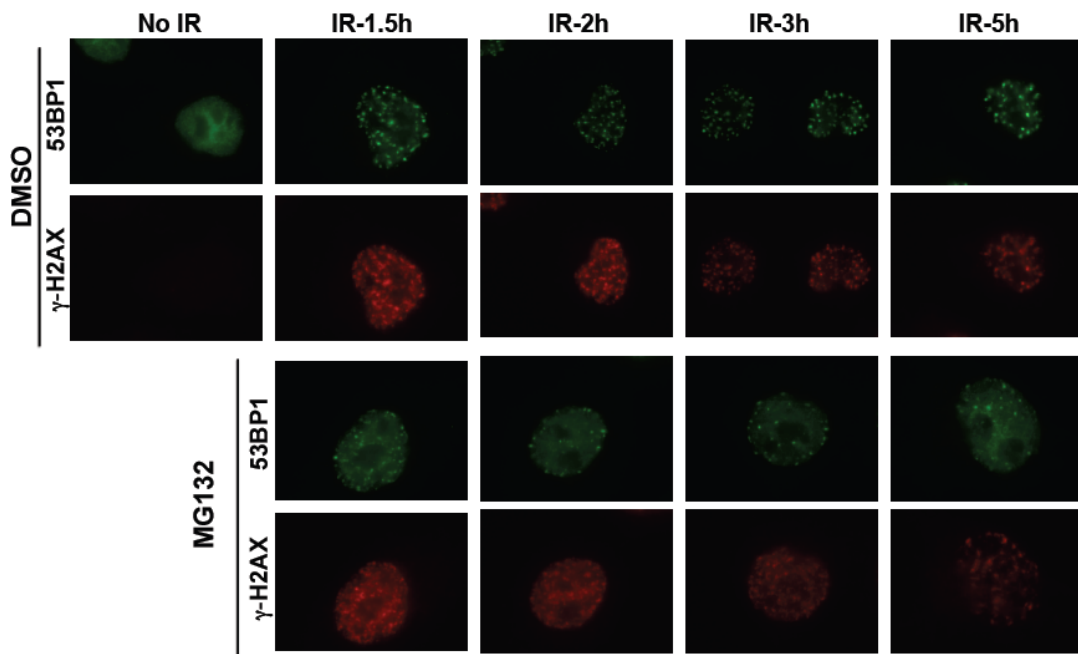
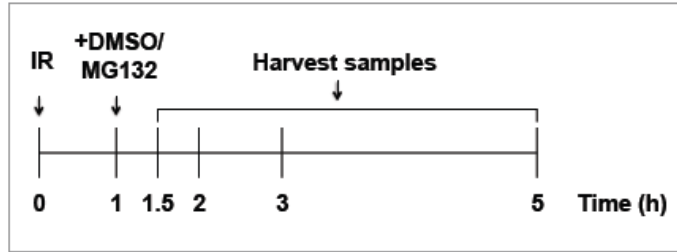
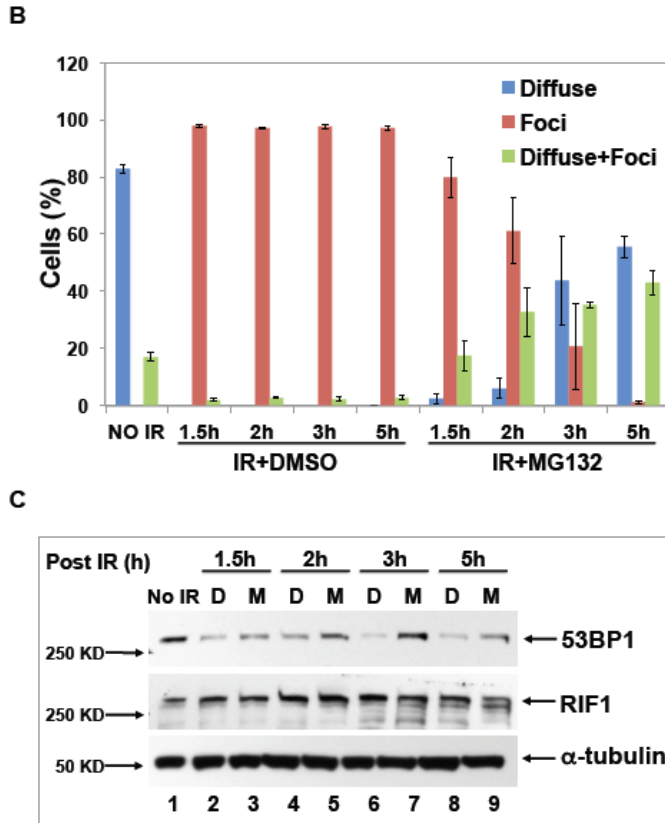


Figure 4.7. continued:



4.4.6. 53BP1 stability is important for RIF1 recruitment

RIF1 is the only known DNA damage repair factor that requires 53BP1 for its recruitment to damage sites⁸⁵, and which indirectly depends on RNF8 and RNF168⁴¹. We tested if inhibition of 53BP1 protein degradation affects RIF1 association with IRIF. In contrast to 53BP1, irradiation did not affect RIF1 protein level in HeLa cells (Figure 4.8.B, lane 2). Inclusion of MG132 abolished RIF1

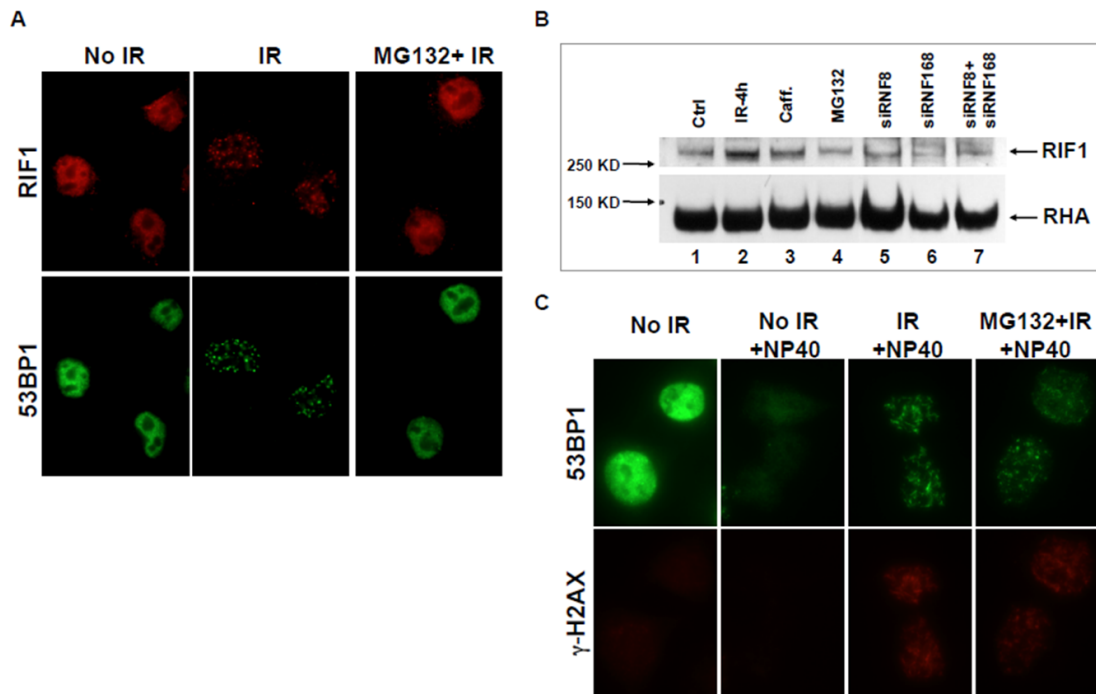
association with IRIF in HeLa cells (Figure 4.8.A) without changing its protein level (Figure 4.8.B, lane 4). Similarly, caffeine or RNF8 and/ or RNF168 depletion did not affect RIF1 abundance (Figure 4.8.B, lane3, 5-7 and⁸⁵). To test whether the inhibition of RIF1 IRIF in the presence of MG132 was directly associated with the failure to degrade the unbound bulk 53BP1, but not due to the impaired ubiquitin-dependent DSB signaling, we modified the immunofluorescence staining protocol to include a detergent extraction step prior to fixation. Following IR in the presence of MG132 in HeLa cells, the unbound bulk 53BP1 protein within the nucleus was removed by using cell lysis buffer containing 0.5% NP40 and 300 mM NaCl, and then cells were fixed and stained as usual. We observed chromatin-bound foci of 53BP1 that co-localized with γ -H2AX stain after irradiation and in the presence of MG132 (Figure 4.8.C). The NP40 in the presence of 300 mM NaCl was sufficient to remove the loosely tethered bulk 53BP1 and reveal 53BP1 bound to the damage sites, consistent with a previous observation¹²⁵. This result indicated that proteasome inhibition 30 minutes prior to DNA damage did not abrogate tight binding of 53BP1 to damage sites in chromatin. While prior studies had suggested that the RNF8-mediated degradation of KDM4A/JMJD2A was required to expose H4K20me2 for the recruitment of 53BP1 to DNA damage sites^{30,86,122}, our result revealed that the block due to KDM4A/JMJD2A binding to the chromatin was not absolute, and 53BP1 could still bind to the damage sites. To conclude from Figure 4.8.C, proteasome inhibition by MG132 does not abolish 53BP1 association with IRIF, but blocks the degradation of the unbound bulk

53BP1, accounting for the failure to recruit RIF1 to the damage sites. Taken together with data of RNF8/ RNF168 regulation on 53BP1 function, stability and IRIF, we conclude that RNF8 and RNF168 ensure proper 53BP1 protein concentration within the nucleus to recruit the downstream response factor RIF1 to damage sites for further efficient repair, consistent with the results that 53BP1 functions in DSB repair (the conservative-NHEJ pathway) dependent on RNF8 and RNF168 (Figure 4.1.A). If 53BP1 degradation fails to occur and it remains in high concentration throughout the nucleus, then the RIF1 fails to bind to the DNA lesion. These results are consistent with there existing in the nucleus of a cell two pools of 53BP1, unbound or bulk 53BP1 and a second small pool of 53BP1 bound to chromatin at the site of DNA damage. Unbound 53BP1 is, in essence, a decoy that inhibits the signal from 53BP1 bound to the damage site.

FIGURE 4.8. Inhibition of 53BP1 degradation causes failure to recruit RIF1 to the DSB sites.

A. HeLa cells were treated with or without MG132 (20 μ M) before exposure to 10 Gy X-irradiation. 4 h post-IR, cells were fixed for immunofluorescence microscopy as indicated. **B.** different treatments were applied to HeLa cells and immunoblot for RIF1 was done. Ctrl, no treatment (lane 1) or 4 h post 10 Gy-IR (lanes 2-7). Additional treatments included caffeine (10 mM; lane 3) MG132 (20 μ M; lane 4), siRNA specific for RNF8 (lane 5) siRNA specific for RNF168 (lane 6), and mixed siRNA specific for both RNF8 and RNF168 (lane 7). **C.** MG132 (20 μ M) was

included in medium 30 minutes prior to exposure to 10 Gy X-ray irradiation. At 4 h post-IR, cells were extracted *in situ* with cell lysis buffer (Materials and Methods) on ice for 15 min (+NP40) or not extracted. Cells were fixed and stained for immunofluorescence microscopy as above.



4.5. Discussion

53BP1 is a DSB repair protein previously identified to influence the NHEJ process^{6,86}, though its specific function had not been clearly defined in this pathway. In this study, we found: 1) 53BP1 positively regulates the conservative-NHEJ pathway; 2) 53BP1 has no effect on the highly mutagenic and deletional alternative-NHEJ pathway or on HDR, but it suppresses SSA; 3) the localization of 53BP1 at sites of DSBs is accompanied by its bulk removal from

the nucleus except at sites of DNA damage; 4) RNF8 and RNF168 are each required for the proteasome-mediated degradation of bulk 53BP1 after DNA damage; and 5) failure to degrade bulk 53BP1 results in the failure for RIF1 to localize appropriately to DNA damage sites.

4.5.1. 53BP1 binding to DSB sites

53BP1 localization to the sites of DNA damage requires the recognition of histone methylation, in particular H4K20me²³⁴ by its tandem Tudor domain^{35,36}. 53BP1 also binds H2A ubiquitinated on Lys-15 (H2AK15ub), a product of RNF168 ubiquitination on chromatin, via its ubiquitin-dependent recruitment (UDR) motif. We suggest that bivalent binding of 53BP1 to epitopes on chromatin may block its ubiquitin targeted degradation, thus distinguishing between bulk 53BP1 in the nucleoplasm and 53BP1 bound at the damage site. Since bulk 53BP1 is not bound to the same epitopes on chromatin, it is susceptible to ubiquitination and degradation. Recruitment thus has a different mechanistic implication: it is not a directed diffusion of 53BP1 from the nucleoplasm to the DSB site, but rather the stabilization signal is in effect a recruitment signal. Previous studies have shown that 53BP1 was transiently immobilized at the sites of damage by Fluorescence Recovery After Photobleaching (FRAP) experiments^{50,51}. Our results indicate that the concept defining that 53BP1 diffuses to high concentration foci upon damage needs to be amended. The immunoblots of total 53BP1 clearly indicate that most of 53BP1 is degraded following ionizing radiation (Figure 4.2.A). Previous studies

using FRAP clearly indicated high mobility of 53BP1^{25,50}, but those measurements began at 30 minutes, after the bulk degradation. Based on our results, we suggest that the high mobility of 53BP1 was in fact the rapid new protein synthesis moving to the sites of damage. Other prior studies have used laser micro-irradiation for generation of localized damage in cellular DNA, and 53BP1 localized to these DNA damage tracts^{28,51}. We suggest that such a result does not contradict our current observations, but rather we posit that the localized damage induced by the laser micro-irradiation may have been local but sent a signal of the damage state throughout the nucleus and causing bulk 53BP1 degradation. The implication for the mechanism of 53BP1 damage site binding is consistent with the observation that inhibition of the proteasome blocks 53BP1 degradation and blocks the appearance of 53BP1-containing IRIF in the nuclei (Figure 4.2.E). When unbound bulk 53BP1 is removed from the nucleus by the detergent, tightly-bound 53BP1 reveals itself as IRIF in the presence of MG132, confirming that upon irradiation, 53BP1 is degraded everywhere with the exception of DNA damage sites where 53BP1 is bound and stabilized (Figure 4.8.C). Excessive 53BP1 has been shown to be repressive to end-joining activity⁸⁶, and we suggest that this repressive activity may be due to the unbound pool of 53BP1 acting as a competitive inhibitor of the damage site bound 53BP1. Bulk 53BP1 prevents RIF1 from binding to the DNA in the undamaged state. After IR, bulk 53BP1 is degraded, and RIF1 is recruited to damage sites by bound 53BP1 to execute inhibition of end resection^{41,71,84}. RIF1 is one of a few proteins identified to date that requires

53BP1 for its recruitment to DSBs and is involved in conservative-NHEJ⁴¹. 53BP1 has been previously implicated as a competitor with BRCA1 in leading to opposite directions in the DSB repair process, NHEJ versus homologous recombination, respectively^{6,41,70,126}. Consistent with this notion, depletion of 53BP1 partially suppressed the effects of depletion of BRCA1 on homologous recombination by partially rescuing defects caused by the loss of BRCA1 (Figure 4.1.D, E). 53BP1 suppressed the single-strand annealing pathway, but had no effect on HDR, however in each case its depletion could partially correct the defect due to depletion of BRCA1. On the other hand, depletion of BRCA1 caused deficits of varying magnitude to all four DSB pathways, suggesting that the opposing function of BRCA1 versus 53BP1 is actually complex.

4.5.2. The roles of RNF8 and RNF168

We found that 53BP1 specific function in the conservative-NHEJ process is RNF8- and RNF168-dependent since these factors regulated the degradation of 53BP1. This result is in agreement with a study that RIF1 accumulation at DSB sites is dependent on the RNF8 and RNF168⁴¹. RNF8 and RNF168 are the E3 ligases that conjugate ubiquitin to histones H2A and H2AX. These provide the binding site for 53BP1 at DSBs³⁵. The simplest interpretation of the results would be that one of RNF8 or RNF168 has the additional activity of ubiquitinating bulk 53BP1 via the Lys-48 side chain, marking it for degradation by the ubiquitin-proteasome system (UPS). Since depletion of either factor stabilizes

53BP1 as effectively as simultaneously depleting both, and since RNF8 is upstream of RNF168, we suggest that RNF168 is the E3 ligase that directly targets 53BP1 for proteasome mediated degradation. Such a condition would imply that RNF168 can change its specificity, targeting ubiquitin Lys-63 when modifying H2A and targeting ubiquitin Lys-48 when modifying bulk 53BP1. It is a possibility that an as yet undetermined E3 ubiquitin ligase is downstream of RNF8 and RNF168 and which mediates the ubiquitination of the bulk 53BP1. Our results indicate that RNF8 and RNF168 function differently in each DSB repair pathway. For the conservative-NHEJ pathway, RNF8, RNF168, and 53BP1 have an epistatic relationship since pairwise depletion in any combination of these three factors equally affected the repair rate as single depletion (Figure 4.1.A). By contrast, simultaneous depletion of RNF8 and RNF168 had a more severe effect on the alternative-NHEJ pathway. We interpret this finding to mean that these two ubiquitin ligases function independently in this NHEJ pathway. Similarly, in the homology directed repair pathway the RNF8 and RNF168 were additive in their effect on this pathway. These results dissect out distinct interrelationships between these two ubiquitin ligases and between these and 53BP1, and these give clues to the mechanisms by which they act in the DSB repair. In summary, this study found that following ionizing radiation, the bulk 53BP1 protein is degraded, and thus preventing competition between bulk 53BP1 and the DSB-bound 53BP1 for the recruitment of RIF1. In addition, it was found that 53BP1 functions positively in the sequence conserving precise NHEJ process, but

53BP1 has no role in the error prone alternative-NHEJ process. Loss of 53BP1 protein would thus be highly mutagenic since the error-prone NHEJ repair would predominate. This study also dissected how the RNF8 and RNF168 protein function in the four DSB repair pathways and indicate that relationships between these factors are pathway-specific.

Chapter 5: SUMO isoforms and conjugation-independent function in DNA double-strand break repair pathways

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Submitted

Author contributions:

- Hu, Y & Parvin JD designed the experiments.
- Hu, Y performed the experiments.
- Parvin JD provided mentorship, advice, and financial support.

5.1. Abstract

SUMO proteins act in DNA double-strand break (DSB) repair but the pathway specificity of the three isoforms has not been defined. In experiments in which we depleted the endogenous SUMO protein by RNAi, we found that SUMO1 functioned in all sub-pathways of either homologous recombination (HR) or non-homologous end-joining (NHEJ), whereas SUMO2/3 was required for conservative-NHEJ but dispensable in other DSB repair pathways. To our surprise, we found that depletion of UBC9, the unique SUMO E2 enzyme, had no effect in HR or alternative-NHEJ. Consistent with this result, non-conjugatable SUMO1 mutant protein rescued repair efficiency in HR and alternative-NHEJ to the similar extent as did wild-type SUMO1. These results detail the functional roles of specific SUMO isoforms in DSB repair in mammalian cells, and reveal that SUMO1 functions in homologous recombination or alternative-NHEJ as a free protein and not a protein conjugate.

5.2. Introduction

DNA double-strand breaks (DSB) present a major problem in genome maintenance since the repair machinery must bridge a gap of indeterminate composition. Two mechanistically distinct pathways are present for DSB repair in mammalian cells: homologous recombination (HR) and non-homologous end-joining (NHEJ), competing for the repair of DSBs^{6,127,128}. There are two major mechanisms present in HR: the error-free Homology-Directed Repair (HDR)

pathway and the error-prone Single-Strand Annealing (SSA). Similarly, eukaryotic cells utilize two pathways of NHEJ, conservative-NHEJ (C-NHEJ), in which DSB ends are ligated without homology and which protects DSB ends with minimal processing^{9,10}, and the alternative-NHEJ (Alt-NHEJ), which depends on resection to generate single strands that anneal via microhomology^{10,12,13}.

In vertebrates, there are three functional forms of SUMO family proteins: SUMO1, SUMO2, and SUMO3. SUMO2 and SUMO3 share about 95% sequence identity but are only 45% identical in sequence to SUMO1, thus forming a distinct subfamily as SUMO2/3⁸⁷. The conjugation of SUMO isoforms onto target protein is designated as SUMOylation, an enzymatic cascade triggered by an E1 SUMO-activating enzyme (SAE1/SAE2), followed by a single specific E2-conjugating enzyme, UBC9, and several E3 SUMO ligases, resulting in a covalent isopeptide bond between the lysine of target protein and di-glycines at carboxyl-terminus of the activated SUMO (reviewed in refs.^{88,89}).

The SUMO system has been shown to have strong ties to double-strand break repair. Abolition of activity of SUMO E3 enzymes in human cells impairs DSB repair^{51,68,90}. Mutation of the single SUMO E2 UBC9 in yeast or human cells results in defects in DNA repair, including recombination abnormality and impaired DSB repair⁹¹⁻⁹³. Furthermore, many HR proteins are modified by SUMO^{51,68,92,94-97}. Nonetheless, cells expressing individual SUMOylation-defective mutant substrate often lack notable phenotypes^{94,101}. SUMO modification of individual NHEJ proteins however, regulates their function in DSB repair^{92,102,103}.

SUMO1 has been discovered as a non-covalent binding partner for human HR proteins RAD51, RAD52 and RPA via SUMO-interacting motifs (SIMs) as well as other DNA repair proteins¹⁰⁴⁻¹⁰⁸. The SIM-dependent non-covalent binding to SUMO is essential for loading of the recombinase RAD51 onto resected DSB ends for HR-mediated repair^{97,109}.

In this study, we identified the roles for SUMO isoforms in all four DSB repair sub-pathways. We found that SUMO1 stimulated all four pathways whereas SUMO2/3 was required only in the C-NHEJ pathway. Surprisingly, the single SUMO E2 enzyme UBC9 was dispensable for HR and Alt-NHEJ, and the conjugation-deficient SUMO1 mutant protein was competent for HR and Alt-NHEJ repair. In contrast, UBC9 was required for C-NHEJ and the SUMO1 mutant was defective in this pathway compared to the wild-type. We conclude that while C-NHEJ is SUMOylation-dependent, the HR and Alt-NHEJ pathways are stimulated by non-covalent SUMO1 interactions.

5.3. Materials and Methods

Homologous recombination and non-homologous end-joining assays.

HDR and SSA assays were performed as previously described^{18,20}. The repair of double-strand break by Alt-NHEJ pathway was based on a vector kindly provided by Jeremy Stark (Beckman Research Institute of the City of Hope)¹⁷ stably integrated into HeLa genome. On day 1, the appropriate cell line was seeded in 15.6-mm-diameter wells in 24-well plates. The next day when cells were 50%

confluent, cells were transfected with 30 pmol of each siRNA in the presence of 3 μ L of Oligofectamine (Life Technologies). On day 3 cells were transferred to 35-mm-diameter wells in six-well plates. At 48 h after transfection, cells were re-transfected with 50 pmol of the same each siRNA in the presence of 5 μ L of Lipofectamine 2000 (Life Technologies), plus 3 μ g of an I-SceI endonuclease expression vector which causes a DSB lesion in the genome. In each transfection, the total siRNA amount was adjusted to be the same in each well by adding siControl. On day 7, cells were trypsinized and those among 10,000 cells from each well were measured by counting the percentage of GFP-positive cells using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) in the Ohio State University Comprehensive Cancer Center Analytical Cytometry shared resource. The C-NHEJ assay utilized quantitative real-time PCR and was carried out as described in²² with the following modification. Double-transfection procedure was done as above. The genomic DNA isolated 3 d after transfection of the I-SceI plasmid was digested with the restriction enzyme XhoI and purified by Qiagen PCR purification kit before real-time PCR was applied. RPS17 probe (Hs00734303_g1, Applied Biosystems) was used as an internal control and quantitative $\Delta\Delta C_T$ method was used to analyze the data.

For plasmid add-back in the rescue assay for all four DSB repair pathways, the transfection procedure was the same except for the amount of reagents used: in the first transfection, 30 pmol of siSUMO1-2 and 0.75 μ g of SUMO1 expression plasmid were added to the cells in the presence of 1.5 μ L of Lipofectamine 2000;

At 48 h after transfection, cells were re-transfected with 50 pmol of siSUMO1-2 and 1.5 µg of SUMO1 plasmid plus 1.5 µg of I-SceI expression vector in 2.5µL of Lipofectamine 2000.

RNA interference and plasmids.

Following siRNAs were used and produced by Sigma: siControl targeting the luciferase gene: 5'-CGUACGCGGAAUACUUCGA-3'¹⁸; siBRCA1:

5'-GCUCCUCUCACUCUUCAGU-3'¹⁸; siLigase IV:

5'-AGGAAGUAUUCUCAGGAAUUA-3'⁵¹; siSUMO1-1:

5'-CUGGGAAUGGAGGAAGAAG-3'¹²⁹; siSUMO2/3:

5'-GUCAAUGAGGCAGAUCAGA-3'¹³⁰; siUBC9:

5'-CAAAAAUCCCGAUGGCAC-3'¹²⁹; siSUMO1-2 starting at nucleotide 850:

5'-GGAAUUGCACAUGGUACA-3'. I-SceI expression plasmid has been

previously described¹⁸ and was a kind gift from Maria Jasin (Memorial

Sloan-Kettering Cancer Institute). Wild-type SUMO1 expression plasmid pFLAG-

CMV-2 was a kind gift from Lirim Shemshedini (University of Toledo, Department

of Biological Sciences). SUMO1-ΔGG expression plasmid was constructed by

PCR amplification from the wild-type SUMO1 plasmid using the following primers:

forward 5'- CGGATCCATGTCTGACCAG-3'; reverse

5'-CCCGGGTCACGTTTGTTCCTG-3'. The PCR amplified fragment was then

annealed into pFLAG-CMV-2 vector by digestion using BamH I and Sma I.

Western Blot Analysis.

Whole-cell lysates were harvested in lysis buffer (50 mM Tris, pH 7.9, 300 mM NaCl, 0.5% Nonidet-40, 1 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1X complete protease inhibitor cocktail from Sigma) after 3 d of the double-transfection procedure in HeLa cells and subjected to immunoblot analysis using following primary antibodies: anti-SUMO1¹²⁹, anti-SUMO2/3 (Abcam), anti-UBC9 (BD Transduction Laboratories), anti- β -actin (Cell Signaling), anti-GAPDH (Advanced ImmunoChemical Inc.) and anti-FLAG M2, Affinity Purified (Sigma).

Statistical Analysis.

Data were compared between different groups for each sample by unpaired and two-tailed Student's t test (*, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

5.4. Results

5.4.1. SUMO1 and SUMO2/3 function differentially in DSB repair pathways.

SUMO proteins have been shown to be involved in DSB repair processes^{51,68,92,97,103}. We tested the specificity of SUMO isoforms in each DSB repair pathway by siRNA-dependent depletion of each isoform in cell lines that specifically probe the homology directed recombination (HDR), single-strand annealing (SSA), alternative-NHEJ (Alt-NHEJ) and conservative-NHEJ (C-NHEJ)

repair pathways^{17,18,20,22}. BRCA1 and Ligase IV, which are known to regulate DSB repair^{18,20,131,132}, served as positive controls in the above functional DSB repair assays: BRCA1 in HDR and SSA (Figure 5.1.A, B) and Ligase IV in Alt-NHEJ and C-NHEJ (Figure 5.1.C, D). Depletion of SUMO1 reduced repair in all four sub-pathways tested to about 62% HDR, 31% SSA, 41% Alt-NHEJ, and 39% C-NHEJ, respectively, relative to the control siRNA (Figure 5.1.A-D), suggesting that the SUMO1 isoform is stimulatory in all mechanisms of DSB repair. On the other hand, depletion of SUMO2/3 had as significant a deficit in the C-NHEJ pathway as did depletion of Ligase IV (Figure 5.1.D). In the assays for the other three DSB pathways, depletion of SUMO2/3 increased the levels of HDR, SSA and Alt-NHEJ, though these increases were not statistically significant (Figure 5.1.A-C). This result reveals that the SUMO2/3 isoforms are required for C-NHEJ and either do not participate in homologous recombination and alternative-NHEJ or these isoforms have a modest inhibitory activity.

Studies have shown that SUMO1 and SUMO2/3 serve distinct functions in mammalian cells, as they are conjugated to different target proteins *in vivo*^{87,133,134}, which could plausibly explain our result that SUMO1 and SUMO2/3 function differentially in the DSB repair pathways.

Co-depletion of SUMO1 and SUMO2/3 in C-NHEJ resulted in similar level of repair as single depletion of SUMO2/3, indicating that the SUMO isoforms were participating in the same pathway (Figure 5.1.D). Depleting SUMO1 and SUMO2/3 reduced HDR to the similar level as SUMO1 depletion alone, and

single depletion of SUMO2/3 suggested that these isoforms do not function in HDR (Figure 5.1.A). By contrast, co-depletion of SUMO2/3 partially rescued the defect caused by SUMO1 depletion in SSA and Alt-NHEJ, consistent with the concept that SUMO2/3 repress these pathways of DSB repair (Figure 5.1.B, C).

Depletions of protein by siRNAs were confirmed by immunoblot (examples shown in Figure 5.1.E). The above results from functional DSB repair assays were summarized in Figure 5.1.F. Depletion of SUMO2/3 minimally impacts homologous recombination or alternative-NHEJ, but these isoforms are required in conservative-NHEJ. By contrast, SUMO1 is stimulatory in all DSB repair sub-pathways.

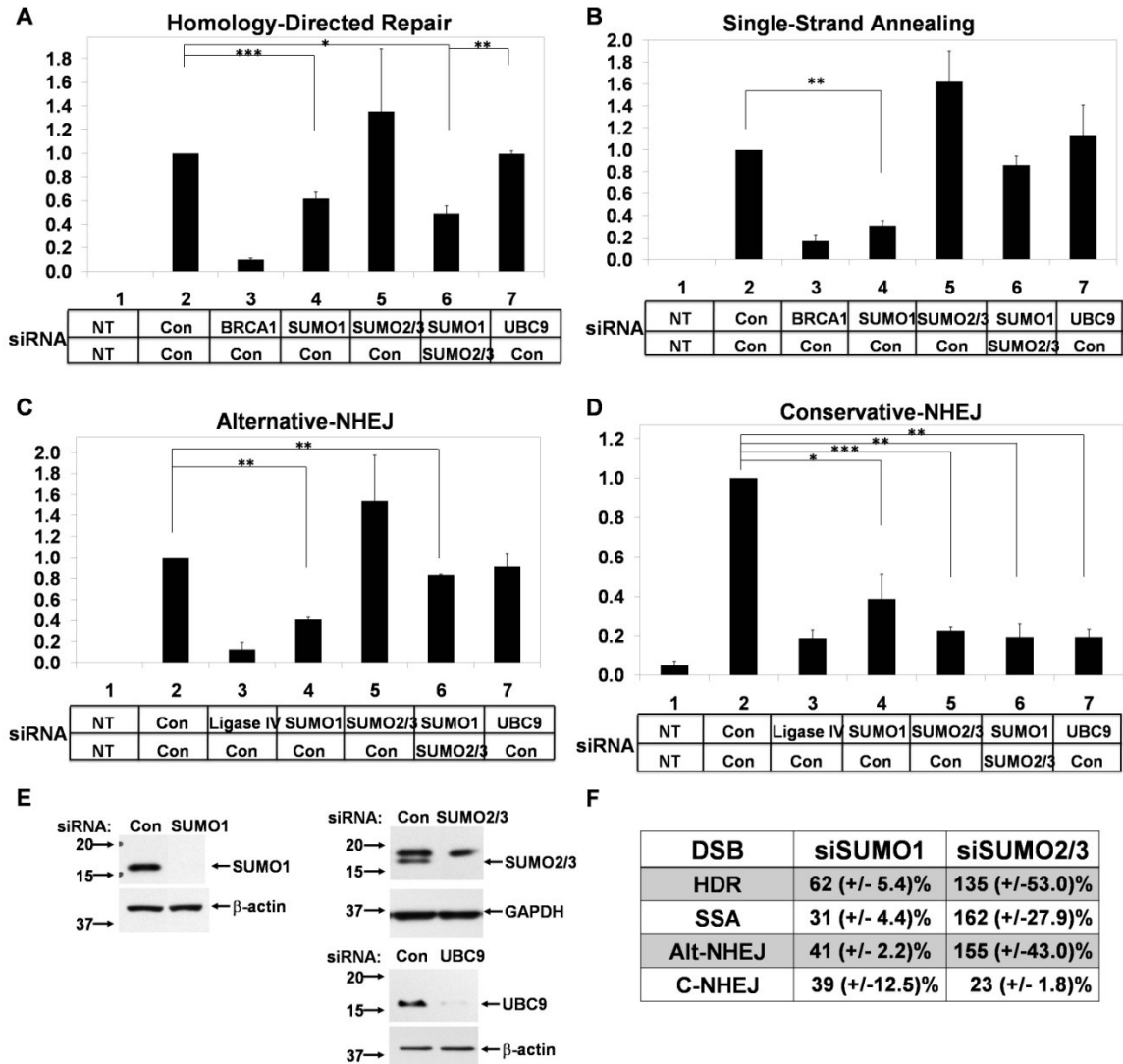


Figure 5.1. SUMO isoforms function differentially in DSB repair pathways.

A-C. cells subjected to two rounds of siRNA transfection indicated in the bottom grid followed by transfection of the I-SceI expression plasmid to induce DSB. After 3 d, the percentages of GFP-positive cells were determined by flow cytometry. In each experiment, the percentage of GFP-positive cells from control siRNA transfections was set equal to 1, and the fraction of GFP-positive cells was determined relative to the control siRNA (Con) to measure HDR, SSA and

Alt-NHEJ, respectively. Results (+/- SEM) are from three independent experimental replicates. NT indicates no transfection of the I-SceI expressing plasmid. **D.** C-NHEJ assay was done by transfecting cells with indicated siRNAs (bottom grid) same as in panel **a**. After 3 d, the repair efficiency was measured by carrying out quantitative real-time PCR on extracted genomic DNA, represented by the percentage on the Y axis. In each experiment, the yield of repaired DNA was normalized relative to the value of result from the control siRNA transfection. **E.** immunoblots show the depletion of indicated protein by RNAi interference. GAPDH and β -actin were used as loading controls. **F.** results (+/- SEM) from each functional DSB repair assay were summarized for the indicated siRNA transfection.

5.4.2. UBC9 is dispensable for HR or Alt-NHEJ.

UBC9, is the only SUMO E2 conjugating enzyme, and it has been implicated in the DNA damage response in animal models and human cells^{51,90,93,102,135,136}. Depletion of UBC9 was included in the experiments in Figure 5.1. to test simultaneously for all three SUMO isoforms, and we were surprised to note that UBC9 depletion did not affect three of the four sub-pathways tested (Figure 5.1. and summarized in Table 1). UBC9 was effectively depleted (Figure 5.1.E), and there was a phenotype due to UBC9 depletion in the C-NHEJ assay, indicating that the observed level of depletion was sufficient to cause a repair defect. UBC9 was required for C-NHEJ since depletion of UBC9 reduced the repair efficiency to

about 19% compared to the control siRNA in conservative-NHEJ, suggesting that UBC9 is important for this pathway, consistent with the result that SUMO2/3 is required for conservative-NHEJ (Figure 5.1.D). We thus hypothesized that SUMO1 function in HDR, SSA, and Alt-NHEJ was independent of conjugation to another protein. There have been other examples in the published literature of SUMO proteins functioning independent of covalent binding to a target protein. As an example, UBC9 has been shown to have no effect on SUMO1-mediated repression of BRCA1-induced transcriptional activity stimulated by DNA damage¹³⁷. Together, our results suggest that the stimulatory effect on homologous recombination or Alt-NHEJ by SUMO1 is not mediated by SUMO conjugation.

DSB	siUBC9
HDR	100 (+/- 2.5)%
SSA	113 (+/-28.2)%
Alt-NHEJ	91 (+/-12.9)%
C-NHEJ	19 (+/- 3.9)%

Table 1 UBC9 is dispensable for HR or Alt-NHEJ.

Results (+/- SEM) from the siUBC9 transfection in each DSB repair assay shown in Figure 5.1. A-C are represented in Table 1.

5.4.3. Free, non-conjugated SUMO1 stimulates HR and Alt-NHEJ.

Conjugation of SUMO onto substrates requires the covalent interaction between the carboxy-terminus of SUMO and lysine acceptors on target proteins via an

isopeptide bond (reviewed in refs^{88,89}). Deletion of the carboxy-terminal di-glycines from the SUMO1 protein renders it incompetent for covalent conjugation to another protein^{105,138,139}. To test whether SUMO1 functions in homologous recombination and Alt-NHEJ without covalent modification, we transfected into cells an siRNA targeting the SUMO1 3'-UTR and a plasmid expressing the SUMOylation-incompetent SUMO1-ΔGG protein, which truncates the critical two carboxy-terminal glycines and which is resistant to the SUMO1-targeting siRNA. Depletion of endogenous SUMO1 and expression of a Flag-tagged wild-type SUMO1 rescued the DSB repair defect in all four DSB repair assays (Figure 5.2. A-D). The exogenous SUMO1 was expressed at slightly lower levels than the endogenous protein (Figure 5.2.E, upper panel, lane 1, 3,4) and in immunoblots probing for the Flag epitope we could detect the major SUMOylation modification of RanGAP1 migrating at a position consistent with a mass of 90 kDa (Figure 5.2.E, middle panel, lane 3). The SUMO1-ΔGG protein was expressed at similar levels as was the wild-type protein and did not result in the SUMO1 conjugation product (Figure 5.2.E, middle panel, lane 4). Using this protocol to replace the endogenous SUMO1 with either wild-type or conjugation-defective SUMO1, we assayed for the specific DSB repair assays as in Figure 5.1. Just as observed in Figure 5.1., transfection of this 3'-UTR specific siRNA (SUMO1-2) depleted endogenous SUMO1 protein (Figure 5.2.E, upper panel, lane 2) and reproducibly yielded inhibition of all four DSB repair pathways as did the siRNA targeting the SUMO1 coding region (Figure 5.2.A-D, lane 3).

When the 3'-UTR specific siRNA was co-transfected with a plasmid expressing wild-type SUMO1 resistant to this siRNA, repair efficiency in HDR was restored back to 87% of activity relative to the control siRNA (Figure 5.2.A, lane 4). Transfection of the non-conjugatable SUMO1- Δ GG plasmid rescued DNA repair in the HDR, SSA and Alt-NHEJ assays to a similar amount as had the wild-type SUMO1 (Figure 5.2.A-C, lane 5). These results unambiguously demonstrate that the stimulatory function of SUMO1 in the HDR, SSA, and Alt-NHEJ assays was independent of conjugation to a target protein. By contrast, in the C-NHEJ assay, transfection of the wild-type SUMO1 expressing plasmid partially rescued repair, but transfection of the SUMO1- Δ GG expressing plasmid did not (Figure 5.2.D). These results together with the data of UBC9 effect on DSB repair pathways (Table 1) further support a SUMOylation-independent mechanism for the action of SUMO1 on homologous recombination and alternative-NHEJ; and a conjugation-dependent role of SUMO protein, SUMO1 and also SUMO2/3, in conservative-NHEJ.

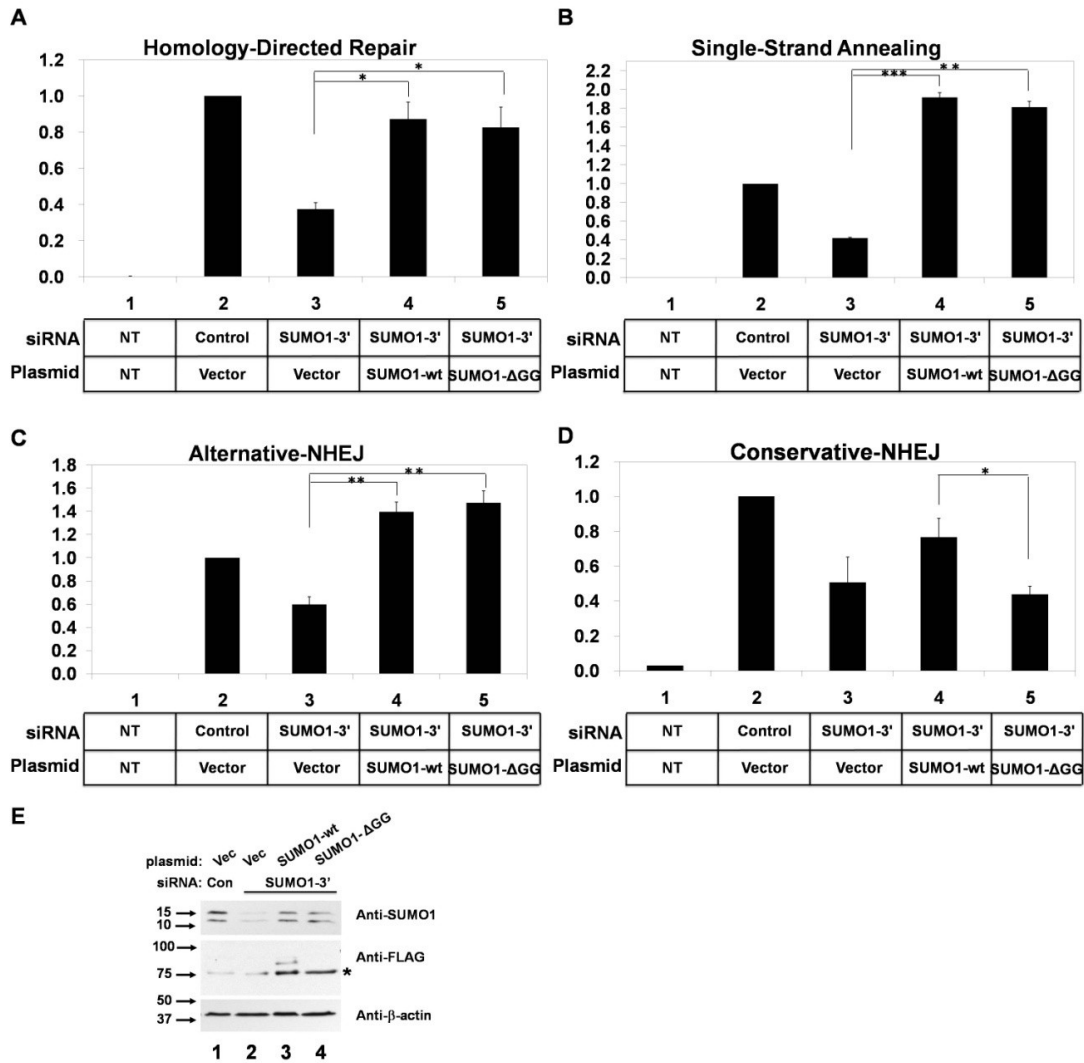


Figure 5.2. Non-conjugated SUMO1 stimulates HR and Alt-NHEJ.

A-D. the appropriate cell line was transfected with siSUMO1-2 targeting the 3'-UTR of the *SUMO1* mRNA plus a SUMO1 wild-type or SUMO1-ΔGG expression plasmid or an empty vector on day 1. On day 3 the cells were re-transfected plus a plasmid expressing the I-SceI. 3 d after transfection, the fraction of GFP-positive cells was determined by flow cytometry to measure repair efficiency of HDR, SSA and Alt-NHEJ respectively (**A-C**), done as in Figure

5.1.A-C, or genomic DNA was extracted from cells for quantitative real-time PCR analysis (**D**) as in Figure 5.1.D. NT represents no transfection of the I-SceI expressing plasmid. **E**. Whole cell lysates were extracted after 3 d of the double-transfection procedure and subjected to immunoblot analysis. SUMO1 specific antibody detected both endogenous SUMO1 and expressed FLAG-SUMO1 protein. FLAG specific antibody detected FLAG-SUMO1 conjugated protein. The asterisk indicates a nonspecific band. The α -actin protein was a loading control.

5.5. Discussion

Together, our findings identify that SUMO isoforms act differentially in double-strand break repair pathways: SUMO1 stimulates all sub-pathways; SUMO2/3 on the other hand, is only required for conservative-NHEJ pathway. Strikingly, our data reveal a novel role of SUMO1 as a free protein, not a protein conjugate in homologous recombination and alternative-NHEJ. Here, we propose a model in which SUMO1 acts via different mechanisms in response to DSB damage in mammalian cells (Figure 5.3.). When cells employ homology-directed repair (HDR) in response to DSB, SUMO1 binds non-covalently to some HR factor via SIM motif of the protein, including the filament-forming recombinase RAD51^{97,104,109,140}, a key HR protein to date known not to be SUMOylated⁹⁷, and this interaction is crucial for the loading of RAD51 onto the resected DNA ends^{97,109} to ensure that subsequent steps could occur (Figure 5.3.A). Similarly,

when single-strand annealing (SSA) or alternative-NHEJ (Alt-NHEJ) pathway takes place to repair DSB, an interaction between SUMO1 and the SIM motif of certain repair factor is essential for the efficient repair which does not depend on SUMOylation of the target (Figure 5.3.B). On the contrary, conservative-NHEJ pathway requires conjugation of SUMO1 and SUMO2/3 to the repair protein substrates for DSB repair (Figure 5.3.C).

Corresponding SUMOylation-defective mutants often barely exhibit deleterious phenotypes^{94,101}. Consistent with these data, our model suggests that non-covalent SUMO1 interaction mediated by SIM motif of the substrate may represent a mechanism that could control both homologous recombination and alternative-NHEJ in DSB repair. Our results of the non-conjugatable SUMO1 mutant effect in the above pathways further support a SUMOylation-independent mechanism for the action of SUMO1 on HR and Alt-NHEJ.

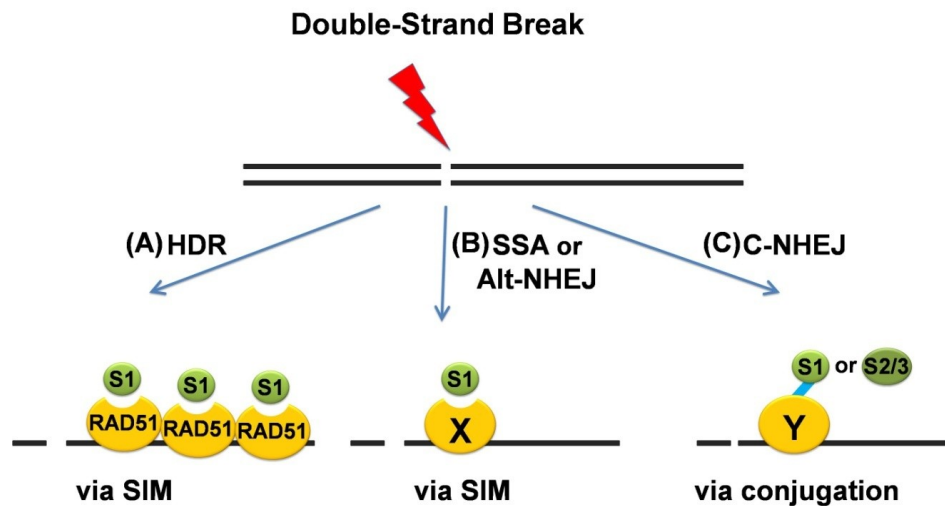


Figure 5.3. Model for SUMO proteins function in DSB repair.

A. in HDR pathway, SUMO1 (S1) stimulates the repair process by a non-covalent

binding to SIM motif of RAD51, thus regulating RAD51 accretion on resected DSB ends. **B.** when SSA or Alt-NHEJ is utilized in response to DSB, SUMO1 interacts non-covalently with a repair factor (X) via SIM motif of the protein at sites of DSB to promote the subsequent repair events. **C.** in C-NHEJ, SUMO1 and SUMO2/3 (S2/3) conjugation to the target repair mediator is essential for efficient repair.

Chapter 6: Concluding remarks and future directions

6.1. Summary of results

In this dissertation study, we investigated the protein functions in double-strand break repair of three important factors, BRCA1, 53BP1 and SUMO isoforms, at levels of biochemistry, protein dynamics and chromosomal DNA repair. Correspondingly, we used methods to test a protein's biochemical activity *in vitro*, as well as tools to measure chromosomal DSB repair frequencies *in vivo*. Here we show novel mechanisms of these proteins function in DSB repair, hence providing new insights and leading to future directions of continuing work.

BRCA1 functions as a tumor suppressor in that it is involved in several cellular processes that ensure genomic stability including centrosome regulation and DSB repair, and one of its roles is that BRCA1 regulates DSB repair as a key repair factor. Therefore mutations in the *BRCA1* gene that contribute to a dysfunctional protein or loss of protein, give rise to increased risk of carcinogenesis. Centrosome regulation is dependent on the ubiquitin ligase function of BRCA1. In DSB repair, the results of involvement of BRCA1 ubiquitination activity has been conflicting in that some studies have shown BRCA1 ubiquitination activity is

responsible for regulation of the recruitment or retention of other repair machineries, while others have argued that this activity is dispensable by using a putative BRCA1(I26A) enzymatic mutant which had no impact on the repair process. The I26A mutation of BRCA1, shown to disrupt its E3 ligase activity without impairing its interaction with its binding partner BARD1, has been thought to be enzymatic inactive as an E3. However, we found that the I26 mutant is still functional in the cellular processes of regulating centrosome number and homologous recombination in DSB repair, thereby raising a question of whether I26A mutation indeed interrupts with BRCA1 ubiquitination activity. We applied an *in vitro* ubiquitination experiment to test the ubiquitin ligase activity of BRCA1/BARD enzyme using purified full length protein heterodimer plus ubiquitination factors purified from bacteria. The reevaluation of the E3 ubiquitin ligase activity of this putative BRCA1(I26A) enzymatic mutant reveals that it is an active E3 ubiquitin ligase when associated with the appropriate E2 factor (Ubc13/Mms2). The BRCA1-dependent control of centrosome duplication in the breast cancer cell line is regulated by monoubiquitination of γ -tubulin, and the BRCA1(I26A)/BARD1 mutant can catalyze the ubiquitination of this substrate. From these results we think that conclusions about the dispensability of the BRCA1-dependent enzymatic activity in various cellular processes should be reconsidered.

Next we studied the unique function of 53BP1, a known NHEJ factor in the specific DSB repair pathways. 53BP1 is known to inhibit DSB end resection, a

critical initial step in HR by the action of its downstream effector RIF1, thus competing with BRCA1-mediated resection-dependent HR mechanism. Nonetheless, studies have not identified directly in which subpathway of NHEJ 53BP1 protein functions, and opposing roles of 53BP1 in HR repair have been implicated. We found that 53BP1 specifically promotes the error-free conservative-NHEJ process, dependent on its upstream recruiters RNF8 and RNF168. 53BP1 has no effect on the highly mutagenic and deletional alternative-NHEJ pathway or on HDR, but it suppresses SSA (Figure 6.1. green labels). Moreover, BRCA1 is positively involved in all four subpathways, including Alt-NHEJ which has not been shown by previous studies (Figure 6.1. red bold arrow). We discovered that the localization of 53BP1 at sites of DSBs is accompanied by its bulk removal from the nucleus except at sites of DNA damage, while prior observations have suggested that 53BP1 is recruited to the sites of damage. The degradation of bulk 53BP1 upon DNA damage is due to each action of RNF8 and RNF168. Further, we showed that failure to degrade bulk 53BP1 results in the failure for RIF1 to localize appropriately to DNA damage sites. These data provide a novel mechanism of 53BP1 responding to DSB damage that after DNA damage is induced in the cells, 53BP1 is divided into two populations, ensuring further functional DSB repair: damage site-bound 53BP1 whose binding signal is known to be generated by RNF8 and RNF168; and unbound bulk 53BP1 whose ensuing degradation is regulated by RNF8 and RNF168.

SUMO has three functional forms which are SUMO1 and a distinct family SUMO2/3. SUMO system including the SUMO E3 enzymes, the single SUMO E2 UBC9 and SUMO isoforms have been implicated to act in DSB repair pathways. However, SUMOylation-defective mutants of SUMO target HR proteins only exhibit mild phenotype, whereas SUMOylation of NHEJ factors regulates their function in DSB repair. SUMO1 has been discovered as a non-covalent binding partner for human HR proteins RAD51, RAD52 and RPA via SUMO-interacting motifs (SIMs) as well as other DNA repair proteins. The SIM-dependent non-covalent binding to SUMO is essential for loading of the recombinase RAD51 onto resected DSB ends for HR-mediated repair. By using chromosomal DSB repair assays to test SUMO proteins, we identify that SUMO isoforms act differentially in DSB repair pathways: SUMO1 stimulates all four subpathways; SUMO2/3 on the other hand, is only required for conservative-NHEJ pathway. Strikingly, the only SUMO E2 UBC9 was required for C-NHEJ but not for HR or Alt-NHEJ. And the conjugation-deficient SUMO1 mutant protein was competent for HR and Alt-NHEJ repair but not for C-NHEJ. Our data together reveal a novel role of SUMO1 as a free protein, not a protein conjugate in homologous recombination and alternative-NHEJ. We propose that this conjugation-independent function of SUMO1 in HR and Alt-NHEJ may be attributed to the non-covalent binding of SUMO1 with a repair protein via SIM motif, while in C-NHEJ which is dependent on SUMOylation, SUMO1 and SUMO2/3 acts as a protein conjugate in regulation of DSB repair (Figure 6.1.).

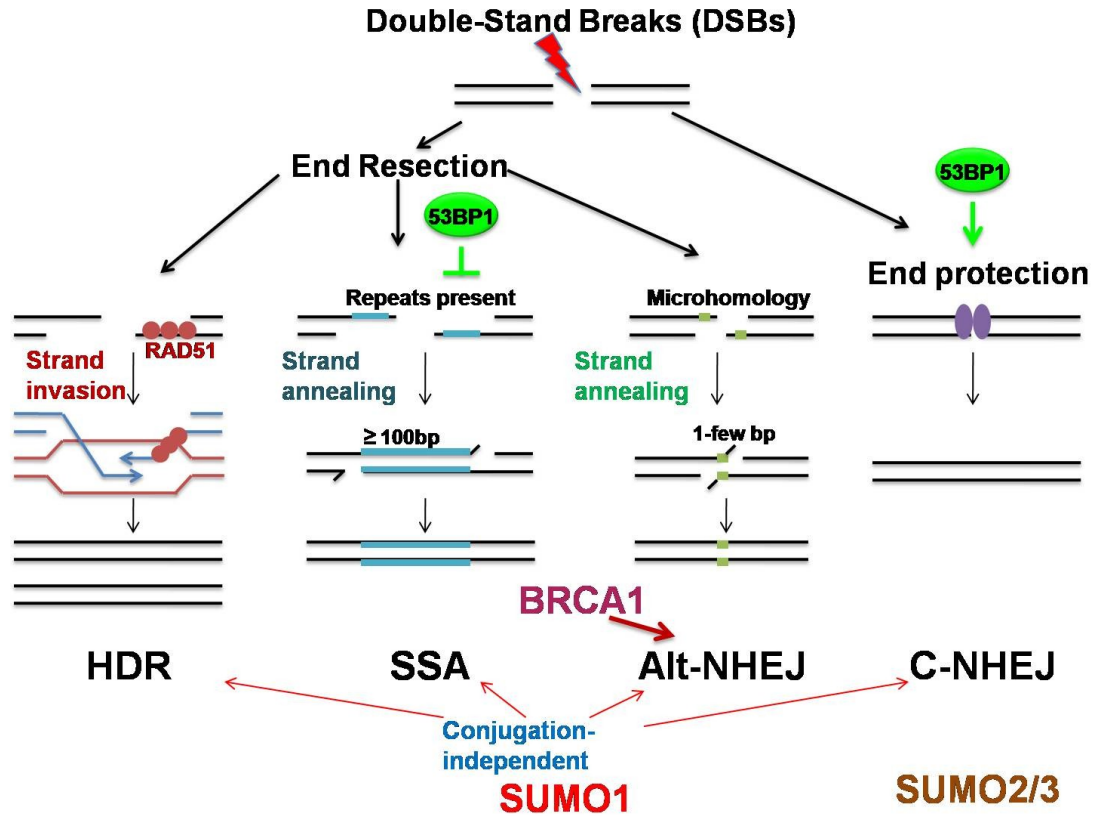


Figure 6.1. Gaps filled: findings of unique functions of BRCA1, 53BP1 and SUMO isoforms in DSB repair subpathways.

The remaining gaps that had not been elucidated for the specific functions of BRCA1, 53BP1 and SUMO proteins in DSB repair pathways were filled by our findings: BRCA1 positively regulates all four subpathways, including Alt-NHEJ (red bold arrow); 53BP1 promotes C-NHEJ and suppresses SSA (green labels); SUMO1 plays a stimulatory role in all four subpathways while SUMO2/3 is only required for C-NHEJ. SUMO1 acts as a free protein in HR and Alt-NHEJ but a protein conjugate in C-NHEJ (red thin arrows).

6.2. Further considerations

6.2.1. BRCA1(I26A) interacting with Ubc13/Mms2: what is the binding surface.

Continuing studies are targeted toward the identification of the Ubc13/Mms2 binding surface on BRCA1(I26A)/BARD1. Since Ubc13/Mms2 interaction with BRCA1(I26A)/BARD1 does not require the isoleucine-26 residue which resides in the E2 binding pocket structure, we speculate that the binding surface for this E2 factor is complex in the context of the full length proteins and may have reduced dependence on I26 residue.

There are other key residues that also reside in or near the E2 binding pocket which is thought to be classical for the common E2 factors such as UbcH5c. UbcH5c binds only to the BRCA1 RING domain but not the BARD1 RING. The binding interface is formed by the first and second structure of the BRCA1 RING domain, a region disrupted by cancer-predisposing mutations. We will use site-directed mutagenesis (or BRCA1 mutant plasmids in hand) to mutate the residues that are known to be important for E2 binding or are in the vicinity of the binding surface, followed by NMR spectroscopy to map the binding site on the BRCA1(I26A)/BARD1 heterodimer for Ubc13/Mms2.

6.2.2. Remaining questions for 53BP1 in DSB repair

Since degradation of bulk 53BP1 in the nucleus except at sites of damage is really the key determinant of 53BP1 function in DSB repair, questions remain to be answered:

A). what is the direct signal that leads to degradation of 53BP1 in response to DNA damage, even though we show that RNF8/RNF168 is responsible for this process? It is a possibility that an as yet undetermined E3 ubiquitin ligase is downstream of RNF8 and RNF168 and which mediates the ubiquitination of the bulk 53BP1. Although no direct evidence of K48-linked ubiquitination of 53BP1 upon damage has been revealed by studies, disruption of 53BP1 protein structure may contribute to the failure of 53BP1 protein degradation. In other words, we will search for domains of 53BP1 that are required for its degradation in a quick response to DNA damage including ionizing radiation. In the context of IR-induced DSB damage, we will test the 53BP1 mutants in which different domains are truncated and check if degradation process still proceeds and downstream effector RIF1 is recruited to DSBs. Figure 6.2. diagrams 53BP1 protein functional domains. Furthermore, deubiquitinase downregulation, in this case, could also contribute to the proteasome-dependent degradation of 53BP1 in combination of RNF8 and RNF168 ubiquitination activity.

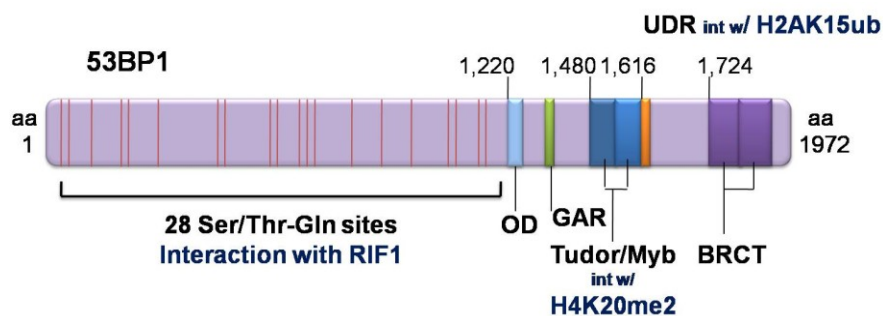


Figure 6.2. 53BP1 domain structure.

53BP1 is large protein of 1,972 amino acids (aa) that has no apparent enzymatic activity but contains interaction surface domains for numerous DSB repair

proteins. 53BP1 contains 28 Ser/Thr-Gln sites in its amino terminus that match the consensus target sequence of ATM kinase. ATM-mediated phosphorylation of the 53BP1 N-terminus is required for the binding of its downstream effector RIF1 to inhibit DSB end resection, thereby important for efficient DSB repair. The 53BP1 C-terminus contains tandem BRCT domains that bind to p53. 53BP1 contains an oligomerization domain (OD), a Gly- and Arg-rich (GAR) motif, a tandem Tudor/Myb motif that binds to H4K20me2 and a ubiquitination-dependent recruitment (UDR) motif that interacts with H2AK15ub, the latter two being critical for 53BP1 localization at DSBs. Amino acid positions are indicated.

B). what is the molecular basis of 53BP unique function in C-NHEJ?

Since 53BP1 is known to inhibit end resection hence favoring towards C-NHEJ mechanism, and it is revealed not being involved in Alt-NHEJ repair by our study, it raises a question that what molecular mechanism is attributed to 53BP1 function in C-NHEJ. Domain studies of 53BP1 also might help find the answer. We will test the 53BP1 truncated mutants directly in chromosomal C-NHEJ repair assay as discussed in Figure 1.5. and identify the domains that are important for 53BP1 function in C-NHEJ. Further, based on the identification of these domains, we will search for potential post-translational modifications within the region that might be responsible for the repair function for C-NHEJ.

C). which step of C-NHEJ does 53BP1 participate in?

Next, after we discover the key domains of 53BP1 for its function in C-NHEJ, it is then critical to understand in which stage/step of C-NHEJ repair process 53BP1 plays the role. We will use immunoprecipitation method to check if 53BP1 interacts with certain NHEJ factors that are active in different stages of C-NHEJ, probably via the important domains found by C-NHEJ repair assay.

6.2.3. *SUMO proteins in C-NHEJ*

Since SUMO1 is stimulatory and SUMO2/3 is required for C-NHEJ, and that this repair process is SUMOylation-dependent, it is then necessary to investigate if SUMO2/3 regulates the C-NHEJ repair as a free protein or a protein conjugate. We will use two different siRNAs to target the 3' UTR sequence of *SUMO2* and *SUMO3* mRNA, respectively, and add back the wild-type plasmid or the non-conjugatable mutant of each isoform in the C-NHEJ repair assay. If the mutant fails to rescue the repair efficiency to the same extent as the wild-type, then SUMO2/3 function in C-NHEJ is via a SUMOylation-dependent mechanism, similar to SUMO1.

Furthermore, since the SIM-dependent non-covalent interaction between SUMO and a protein is common among DSB repair factors, and that HR factor RAD51 binding to SUMO1 via SIM is important for its association with DSBs, it is therefore necessary to identify the SUMO1 non-covalent-binding repair factors that are involved in Alt-NHEJ and SSA, which are conjugation-independent processes (Figure 5.3.). To accomplish this future aim, we will use

immunoprecipitation by anti-SUMO1 antibody that we have in hand to pull down the SUMO1-binding protein pool in the context of DSB damage (i.e., irradiation), then apply mass spectrometry combined with proper data analysis to search for candidate proteins that are known or potentially to be SSA or Alt-NHEJ factors (which can be tested by the chromosomal SSA or Alt-NHEJ assay). Because SIMs are very short sequence motifs, it is a possibility that many proteins would possess multiple SIMs. By using a bioinformatics approach we can predict the SIMs within the properly select candidate repair proteins. By mutating the aa in these motifs, we may be able to identify the impact on the function of the repair factor in the DSB repair signaling pathway.

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