Université de Montréal

The role of ubiquitination and deubiquitination in the regulation of BRCA1 function during genotoxic stress

Par
Helen Pak

Université de Montréal
Faculté des arts et des sciences

Mémoire présenté à la faculté des arts et des sciences
en vue de l'obtention du grade de maîtrise en biochimie
Option génétique et moléculaire

Avril, 2012

Université de Montréal
Faculté des études supérieures et postdoctorales

Ce mémoire intitulé:

The role of ubiquitination and deubiquitination in the regulation of BRCA1 function during genotoxic stress

Présentée par:
Helen Pak

Évalué par un jury composé des personnes suivantes:

Sylvie Mader, présidente-rapporteur
El Bachir Affar, directeur de recherche
Hugo Wurtele, membre du jury

RÉSUMÉ

BRCA1 est un suppresseur de tumeur majeur jouant un rôle dans la transcription, la réparation de l'ADN et le maintien de la stabilité génomique. En effet, des mutations dans le gène BRCA1 augmentent considerablement le risque de cancers du sein et de l'ovaire. BRCA1 a été en majorité caractérisé pour son rôle dans la réparation de l'ADN par la voie de recombinaison homologue (HR) en présence de bris double brins, par example, induits par l'irradiation gamma (IR). Cependant, la fonction de BRCA1 dans d'autres voies de réparation de l'ADN, comme la réparation par excision de nucléotides (NER) ou par excision de base (BER), demeurent toutefois obscures. Il est donc important de comprendre la régulation de BRCA1 en présence d'agents génotoxiques comme le méthyle méthanesulfonate (MMS) ou l'UV, qui promouvoient le BER et le NER respectivement. Nos observations suggèrent que BRCA1 est dégradée par le protéasome après traitement avec le MMS ou les UV, et non avec l'IR. Par ailleurs, cette dégradation semble compromettre le recrutement de Rad51, suggérant que la voie de HR est inhibée. Nos résultats suggèrent que la HR est inhibée afin d'éviter l'activation simultanée de multiples voies de réparation. Nous avons aussi observé que la dégradation BRCA1 est réversible et que la restauration des niveaux de BRCA1 coïncide avec le recrutement de Rad51 aux sites de dommages. Cela suggère que la HR est réactivée tardivement par les bris double brins générés suite à l'effondrement des fourches de réplication. Ayant observé que BRCA1 est hautement régulé par l'ubiquitination et est ciblé par le protéasome pour dégradation, nous avons émis une hypothèse que BRCA1 est régulé par des déubiquitinases. Cela amène à caractériser plus en profondeur par un criblage en déplétant les déubiquitinases individuellement par RNAi et en observant leur effet sur le recrutement de BRCA1 et des protéines reliées à cette voie. Un criblage préliminaire nous a permi d'identifié candidats potentiels tel que BAP1, CXORF53, DUB3, OTUB1 et USP36.

Mots-clés : BRCA1, Dommage à l'ADN, Réparation de l'ADN, Recombinaison Homologue, Ubiquitination, Ubiquitin ligase, Déubiquitinase, Dégradation protéasomale, Méthyle méthanesulfonate, Irradiation gamma.

ABSTRACT

BRCA1 is a tumour suppressor involved in transcription, DNA repair and maintenance of genomic stability. Indeed, BRCA1 mutation carriers have an exceptionally higher risk of breast and ovarian cancers. BRCA1 is mainly known for its role in homologous recombination repair (HR) by recruiting HR proteins to chromatin upon double strand break (DSBs) formation, e.g., following treatment with ionizing irradiation (IR). However, the function of BRCA1 in other DNA repair pathways such as nucleotide excision repair (NER) or base excision repair (BER) is still obscure. It is thus of fundamental and clinical importance to investigate BRCA1 function following exposure to diverse genotoxic agents. Using human cultured cell, we observed that BRCA1 is downregulated by the proteasome upon treatment with MMS or UV, but not with IR. Moreover, this downregulation prevents Rad51 recruitment to chromatin following exposure to MMS. Given that DNA damage induced by UV and MMS trigger NER and BER pathways respectively, this implies that HR could be inhibited in order to prevent competition between independent DNA repair pathways. We also found that BRCA1 downregulation is reversible and the recovery of BRCA1 levels correlates with the reappearance of BRCA1 and Rad51 on chromatin. This implies that the HR has been reactivated at the late stage of DNA damage for the repair of double strand breaks generated by replication fork collapse. Since BRCA1 stability is highly regulated by ubiquitination and is downregulated following MMS treatment, one would expect that a deubiquitinase is responsible for relieving this downregulation to promote the reactivation of the HR pathway. To characterize this aspect further, we conducted DUB RNAi screens in which a particular DUB is depleted and the localization of BRCA1 and other related proteins were observed. According to a preliminary screen, a few DUBs (BAP1, CXORF53, DUB3, OTUB1, and USP36) were identified as potential regulators of the stability and localization of BRCA1 and proteins involved in homologous recombination.

Keywords: BRCA1, DNA damage, DNA repair, Homologous recombination, Ubiquitination, Ubiquitin ligase, Deubiquitinase, Proteasomal degradation, Methyl methanesulfonate, Ionizing radiation

LIST OF ABBREVIATIONS

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and Rad3 Related

BER: Base Excision Repair

BRCA1: Brest Cancer Type1 Susceptibility

BRCA2: Brest Cancer Type 2 Susceptibility

BRCC: BRCA1 BRCA2 Containing Complex

CAK: CDK Activating Kinase

CDK: Cyclin Dependent Kinase

CDKI: Cyclin Dependent Kinase Inhibitor

CSA: Cockayne Syndrome type A

CSB: Cockayne Syndrome type B

DDB1: DNA Damage-Binding Protein 1

DDB2: DNA Damage-Binding Protein 2

DNA: Deoxyribonucleic Acid

DSB: Double Strand Break

DUB: Deubiquitinase

GG-NER: Global Genomic NER

HECT: Homologous to the E6-AP Carboxyl Terminus

HR: Homologous Recombination

IR: Ionizing Irradiation

IRIF: IR Induced Foci

JAMM: JAB1/MPN/Mov34

MDC1: Mediator of DNA damage Checkpoint protein 1

MJD: Machado-Joshephin Domain

MMR: Mismatch Repair

MMS: Methyl Methanesulfonate

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End Joining

PCNA: Proliferating Cell Nuclear Antigen

OTU: Ovarian Tumour Superfamily

RING: Really Interesting New Gene

RFC: Replication Factor C

RNA: Ribonucleic Acid

RNAi: RNA interference

RPA: Replication Protein A

shRNA: Short hairpin RNA

siRNA: Small Interfering RNA

TC-NER: Transcription Coupled-NER

TLS: Translesion Synthesis

UCH: Ubiquitin C-terminal Hydrolase

USP: Ubiquitin-Specific Processing Protease

UV: Ultraviolet

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to express gratitude to my supervisor, Dr. El Bachir Affar, for having me in his laboratory and for his constant presence during my learning experience. He has provided me guidance not only scientifically, but about life in general as well. I would also like to show gratitude to my lab members Ian Hammond-Martel, Helen Yu, Nazar Mashtalir, Salima Daou and Diana Adjaoud for their advices and support with the DUB screen. I especially want to thank Ian Hammond-Martel for his supervision and for introducing me to various techniques. It was all thanks to my supervisor and lab members that I had such a positive learning environment and it was a pleasure working with them. I also want to thank Raphael Rouget, Dr. Andrew A. Horwitz, Dr. Jeffrey Parvin and Dr. Elliot Drobetsky for their expertise in the DNA damage field and Dr. Natsuko Chiba for kindly providing the GFP-BRCA1 deletion constructs. Last but not least, I would like to show appreciation to the members of the jury, Dr. Sylvie Mader and Dr. Hugo Wurtele, for taking their time to correct this work.

A) INTRODUCTION

A) INTRODUCTION

1 Ubiquitination

Ubiquitination is a post-translational modification consisting of the covalent attachment of a small protein of 76 amino acids, ubiquitin, to a lysine residue of a target protein [1]. Ubiquitination is known to regulate various fundamental cellular processes such as the cell cycle, DNA repair and chromatin structure. In fact, deregulation of the ubiquitin pathway is greatly linked to various diseases including cancer [2,3].

1.1 Ubiquitination process

Three enzymes are required for the ubiquitination process. The ubiquitin activating enzyme, E1, activates the ubiquitin in an ATP dependent manner by adenylation, which allows the interaction of ubiquitin with E1 through a thiolester bond between the active cysteine residue of E1 and the C-terminus of ubiquitin [1,4-6]. The E1 then transfers the ubiquitin to the ubiquitin-conjugating enzyme E2 through a thioester bond formation with the active cysteine of E2 [1,4-6]. The ubiquitin ligase E3 catalyses the ubiquitination of the substrate as it promotes the formation of an isopeptide bond between the C-terminus of ubiquitin and a lysine residue of the target protein (Figure 1A) [1,4-6].

1.2 Different types of E3s

While there are only a few E1 and E2 enzymes, there are a myriad of E3 ligases given that they are responsible for specifically recognizing and ubiquitinating target proteins (Figure 1A). There are two major classes of E3 ubiquitin ligases mediating the transfer of ubiquitin to the substrate: the RING finger and the HECT domain.

1.2.1 The RING finger E3s

The RING (Really Interesting New Gene) finger enzymes are the most abundant class of ubiquitin ligases consisting of a domain of 40-60 amino acids with eight conserved cysteines and histidines residues required for coordinating two zinc ions [7,8]. The consensus sequence of the RING finger is: Cys-X(2)-Cys-X(9-39)-*Cys*-X(l-3)-*His*-X(2-3)-Cys-X(2)-Cys-X(4-48)-*Cys*-X(2)-*Cys*, where X represents any amino acids [7-9]. The underlined residues interact with the first zinc ion, whereas the residues in italic interact with the second zinc ion; forming a finger-like structure required for the interaction with ubiquitin conjugating enzymes E2 [7-9]. The RING ubiquitin ligases do not directly interact with ubiquitin, but interact with E2 and promote ubiquitin transfer from the E2 to the substrate (Figure 1 A) [5]. For example, SCF (skip1, cullin1, F-box) is a multi-component E3 ligase containing a RING finger protein RBX1 and a scaffold protein Cullin 1 connecting the RING finger with the adaptor protein SKP1, which interacts with different F-box proteins (i.e. SKP2, FBW7, β-TCR) to confer specificity. Indeed, each of the F-box protein recognizes a specific target to trigger its ubiquitination [6,10]. Another ligase APC (anaphase promoting complex) has some similarities with the SCF as it contains a RING finger protein APC11 and a scaffold protein APC2 connecting the RING finger with several adaptor proteins interacting with the F-box proteins (e.g., cdc20, cdh1) [6,10]. Both SCF and APC are important ligases required for cell cycle regulation.

1.2.2 The HECT domain E3s

The HECT (Homologous to the E6-AP Carboxyl Terminus) is a domain of approximately 350 amino acids and is termed after the ligase E6-AP due to structural similarity [11,12]. The E6-AP ligase was first observed to interact with the E6 protein of human papillomavirus (HPV) and trigger the degradation of the tumour suppressor p53, suggesting a link between viral infection and cancer [11,13-16]. The HECT domain consists of an N-terminal region required for interaction with E2 enzymes and a C-terminal region including a conserved catalytic cysteine residue [12]. The N-terminal and the C-terminal regions of the HECT domain are linked by a flexible hinge that allows conformation change upon E2 interaction, bringing the catalytic cysteines of the E2 and the HECT domain closer to facilitate ubiquitin transfer[12,17,18]. The ubiquitination process by the HECT ligases starts with the interaction with ubiquitin-conjugated E2, allowing the transfer of ubiquitin to the E3 ligase through a thioester bond between ubiquitin and the catalytic cysteine of the HECT ligase [12]. The HECT ligase then transfers the ubiquitin to the substrate [5,12]. An example of a HECT class E3 ligase is HERC2, which was observed to ubiquitinate and degrade BRCA1 through the proteasome [19].

1.3 Ubiquitination and Signaling

The signaling outcome of ubiquitination depends on the type of ubiquitination. The ubiquitin can be attached individually (monoubiquitination) or as a chain (polyubiquitination) on the target protein. Indeed, the ubiquitin possesses 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63) capable of attachment with other ubiquitin molecules, allowing the formation of various ubiquitin chains [5,20]. Depending on the type of chains, ubiquitination can trigger different cellular outcomes and signaling events. For example, monoubiquitination can signal endocytosis, virus budding, gene expression, DNA repair, or nuclear export[5]. In contrast, lysine 48 polyubiquitin chains signal proteasomal degradation. Ubiquitination through lysine 11, 29 or 63 is less understood, but recent studies suggest that these polyubiquitin chains are involved in several cellular processes such as DNA repair, endocytosis, NF-kB activation, or ribosome function (Figure 1 B) [5].

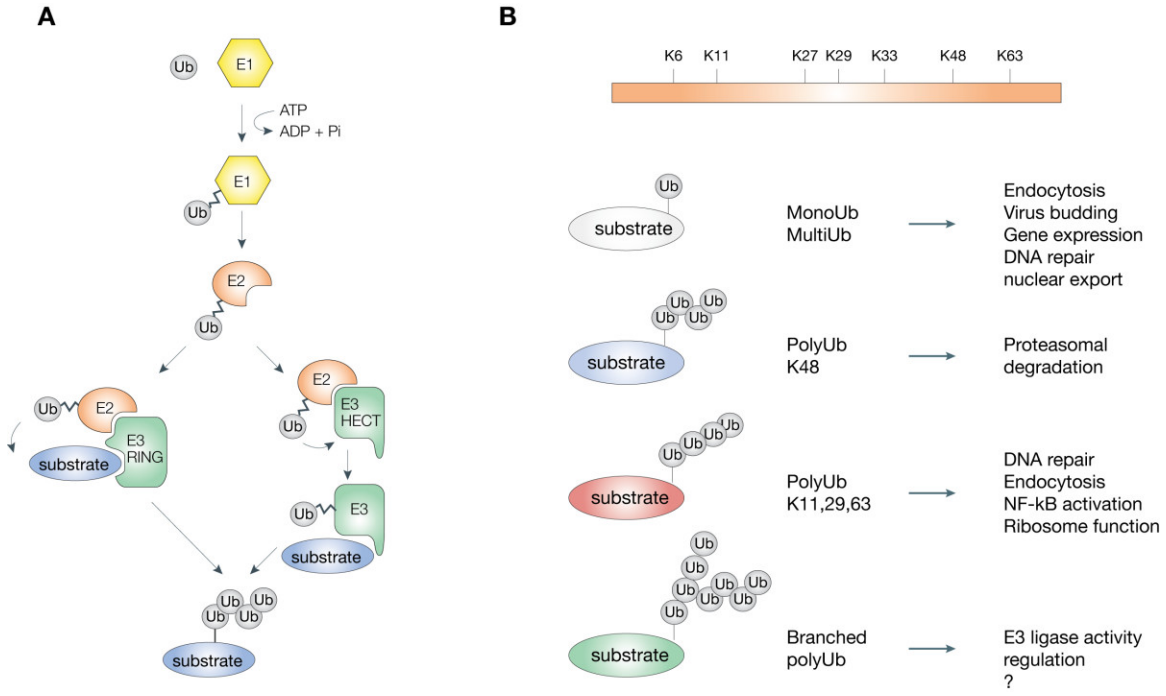


Figure 1 : Description of the ubiquitination process and cellular outcomes of different ubiquitination modifications.

A) Ubiquitination is a sequential process consisting of the transfer of ubiquitin to an E1, E2 and E3 before attachment to the target protein.

B) Ubiquitin possesses 7 different lysine residues (K6, K11, K27, K29, K33, K48, and K63) that could potentially be attachment sites for other ubiquitin molecules, allowing the formation of diverse ubiquitin chains. Ubiquitination plays important roles in cell function and the outcome is dependent on the nature of the ubiquitin chain. There is also evidence for the formation of mixed ubiquitin chains for which the cellular outcome is not well studied. Reference: Woelk et al. (2007) Cell Division 2:11. [5]

2 Deubiquitination

Deubiquitination is the reverse process of ubiquitination[4]. The balance between the activity of the E3 ligases and the deubiquitinases often determines the outcome of cellular processes[4]. Deubiquitinases (DUB) are enzymes that remove ubiquitin from protein substrates through proteolytic activity[4]. These proteases are quite novel and some of their functions are still not well understood. Many DUBs are modified post-translationally through phosphorylation by ATM or ATR upon DNA damage and are regulators of cell cycle checkpoints[4], suggesting roles in tumour development/suppression. DUBs can reverse protein degradation and thus ensure protein stabilization and function[4]. DUBs can also participate in signaling events in a proteasome-independent manner, e.g., by reversing monoubiquitination and K63 chains[4]. Finally, DUBs are also important for recycling free ubiquitin moieties to replenish the cellular pool of this critical signaling molecule[4].

2.1 Different Classes of DUBs

The DUBs can be divided into two major classes: the cysteine proteases (USP, UCH, OTU, and MJD) and metalloproteases (JAMM). Each class of DUB has a specific secondary structures in which their conformation change following interaction with ubiquitin (Figure 2) [21,22].

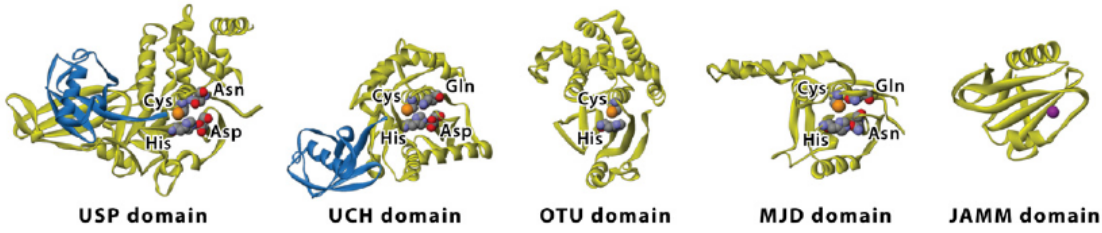


Figure 2: Crystal structures of the catalytic sites of different classes of DUBs.

The catalytic structure of the proteases is in yellow and ubiquitin is in blue. The catalytic centers are shown as spheres (carbon, gray; nitrogen, blue; oxygen, red; sulfur, orange; zinc, purple) Reference: Sebastian M.B. Nijman et al. (2005) Cell 123: 773-786. [22]

2.1.1 Papain-like Cysteine Proteases

The cysteine proteases' catalytic activity involves 2 or 3 critical amino acids forming the catalytic diad or triad: i) a cysteine residue containing a reactive thiol group, ii) a histidine residue that lowers the pka of the catalytic cysteine by deprotonation, and iii) an asparagine or aspartic acid residue which polarizes the histidine, although the latter is not absolutely required as observed for the OTU class (Figure 2)[21,22]. The general mechanism consists of polarization and alignment of the histidine by an asparagine or aspartic acid, the polarized histidine then deprotonates the cysteine to lower the pka of the acidic thiol group to generate a nucleophile group for a nucleophilic attack between the substrate and the ubiquitin [21,22]. This frees the substrate and promotes the formation of an acyl-intermediate between the ubiquitin and the DUB which is stabilized by the oxy-anion hole, which is an environment near the catalytic triad responsible for stabilizing the negatively charged acyl-intermediate though a hydrogen bond donation by either a glutamine, glutamate or asparagine residue [21,22]. Finally, a water molecule cleaves the acyl-intermediate by hydrolysis to free the DUB and Ubiquitin [21,22].

2.1.1.1 Ubiquitin-specific processing protease (USP)

USP is the largest class of DUBs and a typical USP DUB has three subdomains consisting of a finger, a palm, and a thumb [4,21-23]. The catalytic center consists of the palm and thumb and the finger allows the interaction with ubiquitin [4,21-23]. Studies have shown that ubiquitin binding is crucial for activating the catalytic triad. For example, ubiquitin binding on USP7 is required for bringing the catalytic cysteine closer to the histidine [4,21-23]. Although the cysteine and histidine residues of the catalytic triad of USP14 are well aligned, the catalytic center is blocked by the ubiquitin binding surface and is only freed upon interaction with ubiquitin [22,24].

2.1.1.2 Ubiquitin C-terminal Hydrolase (UCH)

The UCH class has a large core catalytic domain of approximately 230aa and is responsible for cleaving small ubiquitin adducts [4,22]. Structural studies on UCHL3 show that the catalytic center is blocked by a large loop and that only small ubiquitin chains can reach it [22,25,26]. However, it is probable that UCH can deubiquitinate proteins from the end, where the chain can reach the catalytic center [22,27].

2.1.1.3 Ovarian tumour (OTU) superfamily

The OTU superfamily is named according to homology with the ovarian tumour gene[4]. Its core catalytic domain consists of five β-strands found between helical domains[4]. Structural studies on yeast OTU1 bound to ubiquitin show that the surface for ubiquitin interaction is disordered in OTUB1 and OTUB2 when not bound to ubiquitin [22,28-31].

2.1.1.4 Machado-Josephin domain (MJD)

The MJD class seems to have a similar structural domain as UCH. Studies on ATXN3 show that another ubiquitin binding site is present in a helical arm further away from the catalytic center. This suggests that 2 ubiquitins might be required to maintain it in an active conformation [22,32-34].

2.1.2 Metalloproteases JAB1/MPN/Mov34 (JAMM)

The JAMM DUBs are metalloproteases containing a zinc ion in the catalytic site which is stabilized by an aspartate and 2 histidine residues [21,35]. The mechanism of JAMM requires a zinc ion to polarize and activate the water molecule[21,35]. The water molecule bound to zinc can interact with the substrate through non-covalent interaction and hydrolyze the isopeptide bond between the substrate and ubiquitin [21,35]. The DUB is then released after a series of proton transfer [21,35]. This class appears to bind specifically to K63-linked ubiquitin [4,22]. An example of a metalloprotease would be BRCC36, which is known to be in the BRCA1-abraxas complex and regulates DNA damage signaling [22].

3 DNA damage and Repair

Every hour, each cell undergoes about 800 DNA lesions induced by endogenous DNA damaging agents (e.g., by-products of cellular metabolism and replication errors) [36]. DNA damage is the foremost cause of tumourigenesis as it induces genomic instability (chromosomal aberrations or translocation) and mutations that impair the function of tumour suppressor genes or activate oncogenes. For instance, mutations in crucial tumour suppressor genes involved in cell cycle checkpoints and DNA repair such as BRCA1 highly promotes tumour formation[37-41]; mutations in the transcription factor c-Myc, which regulates genes involved in cell proliferation, can result in its constitutive activation[42-44]. A hyper-activated c-Myc would thus promote cells to proliferate continuously and eventually transformed into cancer cells[42-44]. DNA damage often impairs the normal structure of the DNA double helix and can provoke stalling of DNA and RNA polymerases, blocking replication and transcription[45]. The replication fork collapse at the site of DNA damage results in the production of double strand breaks, which are considered to be the most genotoxic of all DNA damages as they may result in loss of genomic information and chromosomal rearrangements [46,47]. Exogenous damage can be caused by exposure to UV rays, chemicals, smoke or ionizing radiations. Endogenous damage can be an outcome of a metabolic process such as the generation of reactive oxygen species by the mitochondria during electron transport [48,49], chromosomal rearrangement, or errors during DNA synthesis. Depending on the nature of the DNA damage, specific pathways are activated to repair DNA and to restore genomic stability.

3.1 DNA repair Pathways

DNA damage occurrence is extremely common. DNA repair plays a major role in maintaining the cells free of damage, thus preventing carcinogenesis. DNA repair proteins are crucial for detecting DNA damage and activating specific repair mechanisms. There are several DNA repair pathways that are activated in a tightly regulated manner depending on the nature of the DNA damage in order to ensure a prompt and accurate repair of the lesion. For example, ionizing radiations induce DNA double strand breaks that are repaired by non-homologous end joining or homologous recombination [50]. Reactive oxygen species induce single strand breaks, through oxidization of DNA bases, which are repaired by base excision repair [51,52]. The chemical methyl methanesulfonate (MMS) induces DNA alkylation by methylating guanines or adenines at position N7 and N3 respectively and these DNA lesions are also repaired by base excision repair [53,54]. DNA base mismatch caused by replication errors are repaired by DNA mismatch repair [55]. UV rays or benzo[a]pyrene induce bulky DNA adducts distorting the DNA helix and are repaired by nucleotide excision repair. UV rays are well known to induce cross-linking of adjacent pyrimidines through their cyclobutane rings between position 5 and 6 referred to as cyclobutane pyrimidine dimers (CPD) [56,57]. Benzo [a] pyrene is an environmental carcinogen consisting of a polycyclic aromatic hydrocarbon issued from combustion [58]. Benzo [a] pyrene can intercalate into DNA through a nucleophilic attack at position 2 of a guanine, generating N^2-dG lesions [58,59]. UV and benzo [a] pyrene are highly genotoxic carcinogens present in everyday life as we are constantly exposed to the sun and smoke.

3.1.1 Mismatch Repair (MMR)

The DNA polymerases involved in DNA replication pol δ (lagging strand) and ε (leading strand) are not error proof [60-63]. DNA polymerases can make errors approximately every 10^4-10^5 nucleotides by incorporating the wrong bases, by omitting or inserting extra bases; resulting in 100,000 to 1,000,000 mistakes during every replication [60,63,64]. Fortunately, the 5' to 3' exonuclease proofreading activity of these DNA polymerases and the DNA mismatch repair mechanism work together in order to significantly minimize the errors to approximately every 10^{-10} nucleotides per replication[60,65]. The DNA mismatch repair is the main mechanism involved in repairing mistakes of the polymerases[55]. MMR consists of recognizing the misincorporated base and excising it. The DNA is then re-synthesized using the parental strand as the template and then ligated (

Figure 3 A) [66]. The error is recognized by MutS upon detection of the instability resulting from the kinked DNA structure near the site of mismatch [66,67]. MutS is divided into 2 forms: MutSα (consisting of MSH2 and MSH6) and MutSβ (consisting of MSH2 and MSH3) [60,66]. MutSα is involved in recognizing short mismatch loops of 1-2 nucleotides whereas as MutSβ is involved in recognizing longer mismatch loops up to 10 nucleotides [60,66]. MutL (consisting of MLH1 and PMS2) is then recruited, forming a sliding clamp with MutS [60,66]. It was shown that MLH1 or MSH2 are important components of the MMR pathway as their deficiency leads to lethality upon the inhibition of polymerases involved in base excision repair such as polG and polB [66,68]. The MutS possesses ATPase activity that is enhanced upon the recognition of a mismatched loop and exchanges ATP to ADP in order to trigger the release and sliding of the MutS/MutL clamp along the DNA in the opposite direction of DNA synthesis [66,69]. The MutS/MutL clamp will eventually encounter an unreplicated single strand DNA gap along with replicative sliding clamp and replication factor C (RFC) [66,70]. The MutS/MutL clamp displaces RFC while recruiting the exonuclease EXO1 and guides the latter in order to excise the DNA lesion [66,71]. RPA is recruited to protect the single stranded DNA and once EXO1 has excised DNA beyond the lesion, EXO1 activity is inhibited by MutL [66,71]. The replicative polymerases and PCNA then resume DNA replication to synthesize new strand and ends by ligating the gaps together (

Figure 3B) [66,72]. It is still unclear how the MutS/MutL clamp distinguishes the daughter strand from the parental strand. The distinction between the parent and the daughter strand is a very crucial step, as the repair needs to be triggered on the newly synthesized daughter strand holding the damaged base and not on the undamaged parent strand. It has been well established that in *Escherichia Coli*, DNA methylation plays a major role in differentiating the daughther strand from the parent strand[73,74]. DNA is known to be hemimethylated after replication as the parent strand is methylated while the newly synthesized strand is temporarily unmethylated. The distinction between the parent and daughter strand is made by the endonuclease MutH which is activated and recruited by the ATPase activity of MutS [73,74]. MutH recognizes hemimethylated dGATC sequences and incises the unmethylated strand close to the mismatch in order to initiate MMR[72-74]. However, there is no known human homolog of MutH up to date and thus it is still not well known whether the strand discrimination mechanism is conserved from bacteria to humans. However, it was suggested that MutS/MutL clamp could possibly sense the gaps in between the Okazaki fragments or the single strand gap when it encounters PCNA [66,71,72]. In fact, PCNA was suggested to interact with MutSα and MutSβ in order to assist their recruitment to the nascent daughter stand [75-79].

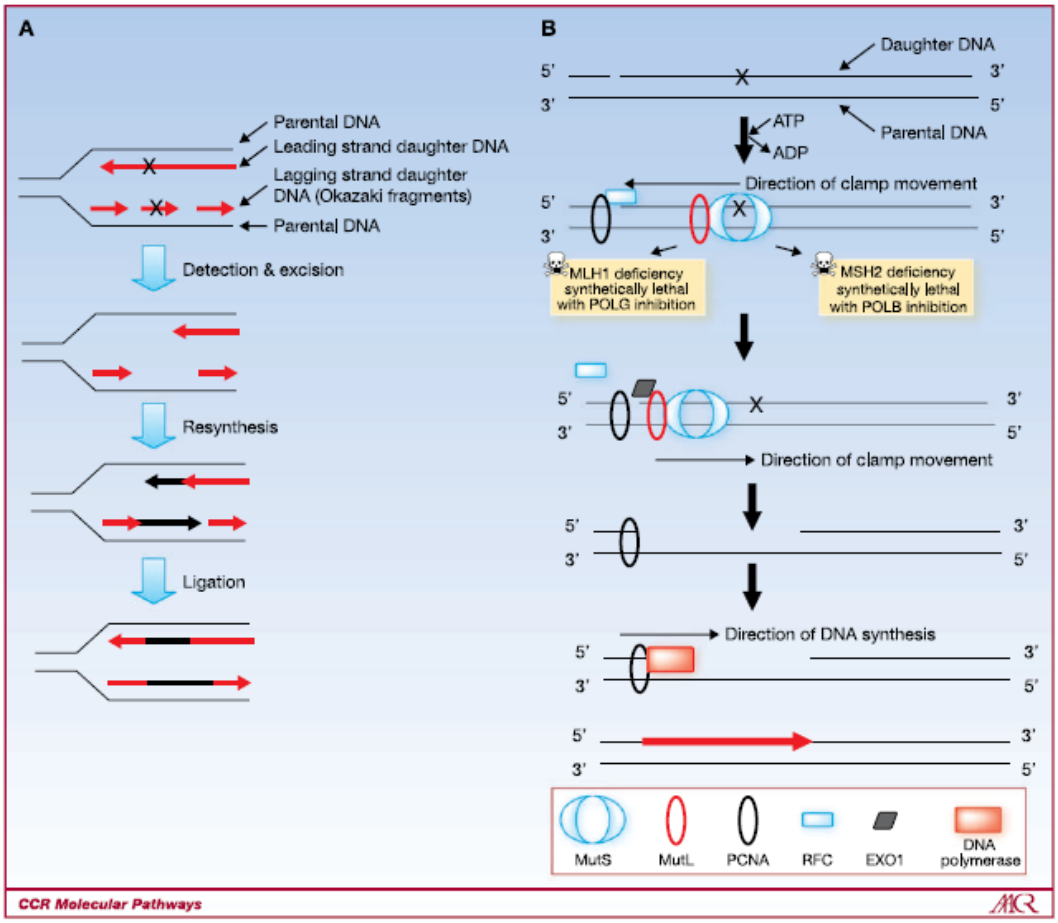


Figure 3 : Overview of the mismatch repair (MMR) pathway

A) Overview of the MMR pathway showing the detection and excision of the DNA lesion followed by resynthesis and ligation.

B) Overview of the MMR pathway including the proteins involved. The MutS recognizes the DNA region and promotes the formation of the MutS/MutL clamp. Upon ATP-ADP exchange by the ATPase activity of MutS, the MutS/MutL clamp slides along the DNA and eventually meets PCNA. The exonuclease EXO1 is then recruited and excises the DNA lesion. The MMR pathway is completed by DNA resynthesis and ligation.

Reference: Martin S.A, Lord C.J, Ashworth A (2010) Clin Cancer Res. 16(21):5107-5113.[66]

3.1.2 Base Excision Repair (BER)

BER is activated throughout the cell cycle upon DNA alkylation, methylation, or oxidation that does not create distortion of the DNA helix backbone. The BER pathway is divided into two sub pathways: the short-patch (SP) pathway, also known as the single nucleotide (SN) pathway and the long-patch pathway (LP) [80]. The short-patch pathway consists of replacing a single nucleotide whereas the long-patch pathway involves the displacement and the replacement of more than 1 nucleotide [80]. The choice between these two pathways remains not so well understood although it was suggested to be dependent on the nature of the damage or the cell cycle [81]. For example, synthetic abasic sites generated by tetrahydrofuran or methoxyamine are repaired by the long patch-pathway given that these reduced AP sites are resistant to the dRP lyase activity of DNA polymerase β [81]. In addition, the DNA polymerases δ and ε involved in the long patch repair are known to be the polymerases implicated in DNA synthesis and thus, the long-patch repair might be favoured during the S phase of the cell cycle [81]. Upon DNA lesion, the damaged base is recognized and cleaved by a DNA glycosylase, which hydrolyzes the glycosidic bond between the base and the sugar phosphate backbone [80]. The removal of the damaged base generates an abasic apurinic or apyrimidinic site known as an AP site [80,81]. The strand with the AP site is recognized and cleaved by AP endonucleases such as APE1 in order to generate a single stranded DNA with a 5' deoxyribosephosphate (dRP) [80-82]. DNA polymerases are then recruited in order to fill in the gaps. In the short-patch pathway, the main polymerase involved is the DNA polymerases β [80-82]. However, DNA polymerase λ can substitute pol β in the absence of the latter [80-82]. In the long-patch pathway, the gap filling is mediated by the DNA polymerases δ or ε, along with their accessory proteins proliferating cell nuclear antigen (PCNA) and the replication promoting factor C (RFC) to coordinate DNA synthesis and increase the processivity of the DNA polymerases[80,81]. Of note, the bases are displaced as new DNA is synthesized, forming a flap cleaved by flap endonucleases such as FEN1[80,81]. However, in the short patch pathway, the dRP is cleaved by the dRP lyase activity of the DNA polymerase β or λ [80,81]. Finally, the ends are ligated by the DNA ligase III in the short-patch pathway and the DNA ligase I in the long patch complex (Figure 4) [80,81].

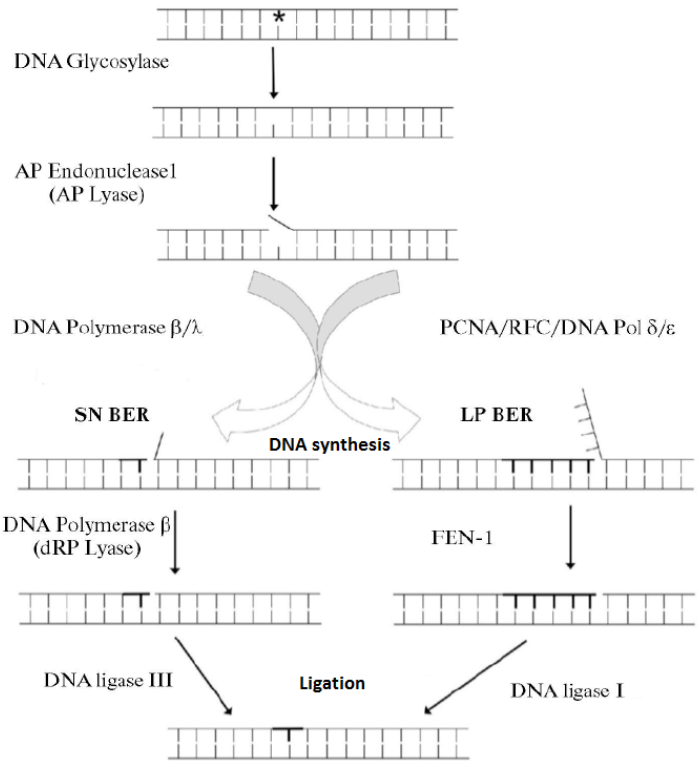


Figure 4: Overview of the base excision repair (BER) pathway.

The left side shows the short-patch repair pathway whereas the right side shows the long-patch repair pathway. The damaged nucleotide is recognized and hydrolyzed by a DNA glycosylase, generating an abasic site. The abasic site is then incised by the AP endonuclease APE1. In the short-patch repair, the DNA polymerases β or λ is recruited to fill in the gap and remove the abasic site. The DNA is then ligated by the DNA ligase III. In the long patch-repair, the DNA polymerases ε or δ, along with PCNA and RFC, are recruited to synthesize new nucleotides while the damaged strand is being displaced, generating a flap. The flap is then cleaved by the flap endonuclease FEN1 and the ends are ligated by DNA ligase I. Reference: Prasad R et al. (2011) Mol Biol (Mosk) 45(4): 586–600. [80]

3.1.3 Nucleotide Excision Repair (NER)

NER is a repair mechanism activated in presence of DNA damage resulting in helix distortion. This type of damage is highly genotoxic as it blocks DNA replication and transcription [83]. There are two different NER pathways: the global-genomic NER (GG-NER) repairs damage in the genome whereas the transcription-coupled NER (TC-NER) repairs damage of transcriptionally active genes [83-85]. These two repair pathways differ in DNA damage recognition but share the same repair pathway called the core pathway. In the GG-NER, the damage is recognized by the XPE (CUL4-DDB-ROC1) complex and the XPC complex, which sense DNA lesions [83-86]. The XPC complex has the role of confirming the presence of damage by detecting DNA destabilization upon loss of Watson-Crick base paring[83]. The XPC complex binds the undamaged strand of the DNA whereas the XPE complex interacts with and inserts its β-hairpin structure into the damaged base in order to extrude and expose it on the surface[83]. The CUL4-DDB-ROC1 complex is an E3 ligase complex inhibited by COP9 [83,86,87]. In presence of damage, COP9 dissociates from the E3 ligase complex so it can be recruited to the lesion site and monoubiquitinates histones [83,86,87]. Histone monoubiquitination results in chromatin relaxation to allow exposure of the damaged site [83,86,87]. The XPC and DDB2 are also ubiquitinated via K63 and K48 chains respectively [83,86-88]. The XPE complex dissociates from DNA upon DDB2 degradation whereas XPC binding to DNA is enhanced, promoting NER activation [83,86-88]. In the TC-NER pathway, upon encounter of DNA lesion, RNA pol II stalls and recruits CSB and the CUL4-CSA-ROC1 ubiquitin ligase complex [83,85]. RNA pol II is then degraded by the E3 ubiquitin ligase Nedd4 [89,90]. This initiates the NER repair pathway. The ubiquitin ligase activity of the CUL4-CSA-ROC1 complex is still poorly understood. It was suggested that CSB is a target of the CUL4-CSA-ROC1 upon dissociation of COP9[91]. CSB ubiquitination and degradation was observed to occur during late TC-NER and was required for elimination of DNA damage signaling and allowing transcription to resume [91]. Upon recognition of the damage and activation of NER, the transcription factor TFIIH is recruited along with the helicases XPB and XPD [83,85,91]. TFIIH is recruited by XPC and CSB in GG-NER and TC-NER respectively [83,85,91-95]. The helicases unwind the DNA at the damaged site. The DNA binding proteins XPA and

RPA bind to the damaged and undamaged strand respectively as a damage verification step and protecting DNA from degradation [83,85,91]. The unwounded DNA facilitates the recruitment and activity of the endonucleases XPF and XPG to excise the damaged DNA site [83,85,91]. PCNA and DNA polymerase are then recruited to resynthesize the DNA and finally the ligase I is recruited in order to ligate the ends together (Figure 5) [83-85].

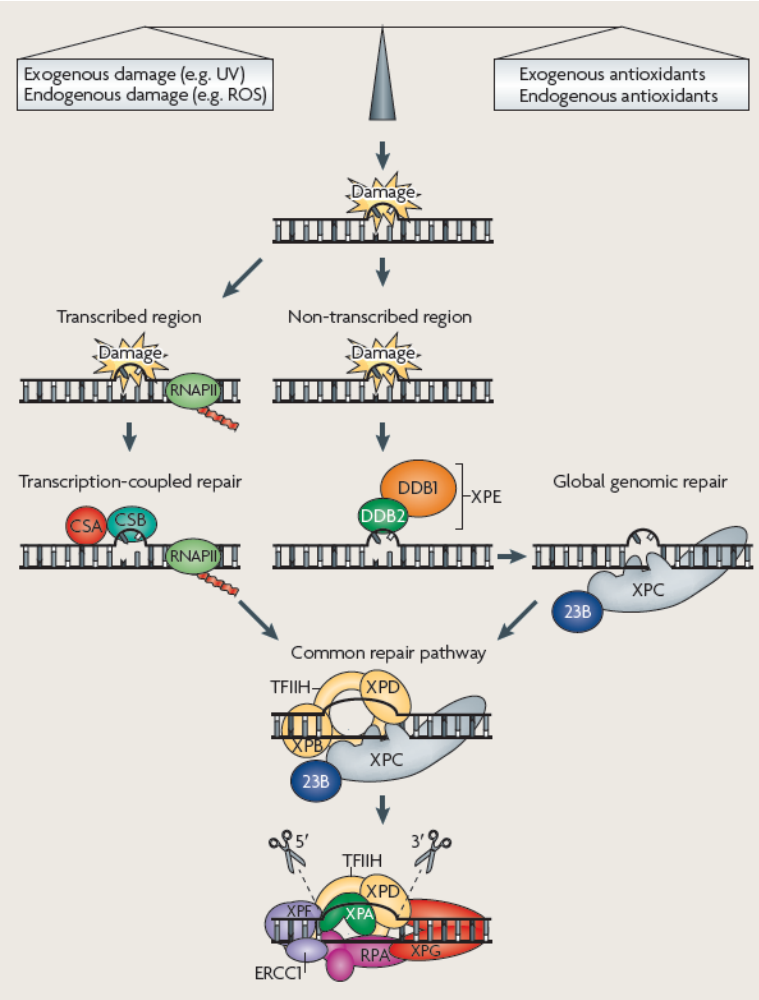


Figure 5: Overview of the nucleotide excision repair (NER) pathway

The DNA damage is first recognized by the CSA and CSB complex (during transcription) or the DDB1 and DDB2 complex (in the genome overall). The transcription factor TFIIH is then recruited along with the helicase XPB and XPD to unwind the damaged DNA to favour the recruitment of XPA and RPA, which bind and stabilize single stranded DNA. The endonucleases XPF and XPG are then recruited to excise the damaged DNA to allow the resynthesis by PCNA and DNA polymerase. The newly synthesized DNA is finally ligated by ligase I. Reference: Cleaver J.E (2009) Nature Reviews Genetics 10, 756-768[85]

3.1.4 Non-Homologous End joining (NHEJ)

NHEJ is a pathway activated upon double strand break functions throughout the cell cycle[96]. Although the NHEJ is error prone, it is the main pathway activated upon DNA double strand break[96]. The double strand break is recognized by the heterodimer Ku70/80 and recruits the DNA-dependent protein kinase subunit DNA-PKcs, a serine/threonine kinase[96]. The Ku heterodimers and DNA-PKcs together form the DNA-PK complex, which is recruited at both ends of the DNA double strand break and is suggested to be important for bridging the DNA ends at a close proximity to allow proper repair [97-100]. Upon interaction with DNA-PKcs, the Ku heterodimer translocates itself and positions DNA-PKcs at the extremities of the DNA[100]. The DNA-PKcs kinase activity is enhanced when two DNA-PKcs are in proximity[100]. Nucleases such as Artemis are recruited in order to process the ends by removing excess overhanging nucleotides that are not compatible for ligation [96-100]. Artemis is a 5' exonuclease; however, upon phosphorylation by DNA-PKcs, Artemis can have exonuclease activity at both the 5' and 3' extremities [96,101]. The gaps are then filled by DNA polymerases and DNA-PKcs autophosphorylates itself and is released allowing ligation of the DNA ends by the XRCC4/DNA ligase 4 complex[100] (Figure 6).

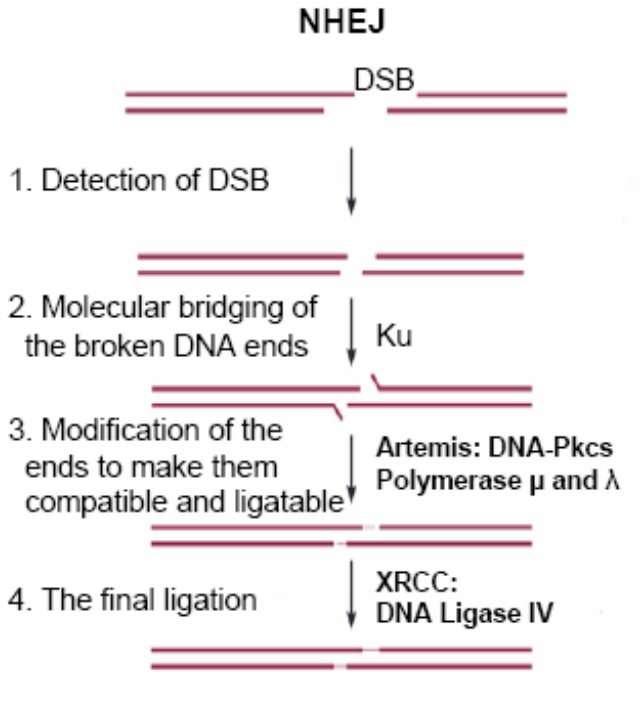


Figure 6: Overview of the non-homologous end joining (NHEJ) repair pathway.

The NHEJ repair pathway specifically repairs double strand breaks. The MRN complex recognizes the double stranded break. The Ku heterodimers and DNA-PK is then recruited to bridge the broken DNA ends. The nuclease Artemis is then recruited to generate ends that are compatible for ligation and after DNA polymerases fill the gaps at the extremities, the XRCC/DNA ligase 4 complex ligates the ends together. Reference: Gu et al. (2008) PathoGenetics 1(1): 4. [102]

3.1.5 Homologous Recombination (HR)

HR is another repair pathway activated upon DNA double strand break. This pathway requires the information of homologous sister chromatids. Since it uses the sister chromatid as a template for repair, this pathway is error-free and is favoured during the S phase and the G2 phase of the cell cycle. Upon double strand break, the MRN complex is recruited and the 3' to 5' exonuclease MRE11 promotes DNA resection [103,104]. DNA resection generates 3' overhangs that are proficient for invasion and DNA synthesis as polymerases synthesize DNA in 5' to 3' direction[103,104]. The Replication Protein A (RPA) is then recruited to prevent degradation of the less stable single stranded DNA ends and formation of secondary structures[104]. Rad51 then displaces RPA, forming nucleoprotein filaments crucial for homology searching and invading the homologous sister chromatids[104]. Another homologous recombination repair protein, Rad52, was reported to interact with Rad51 through its C-terminal domain to enhance Rad51 recombinase activity[105]. The N-terminal domain of Rad52 contains a single strand DNA binding domain that allows annealing to the complementary strand [105]. As the DNA invades the sister chromatid template, a D-loop is formed from the displacement resulting from DNA synthesis[104]. As the 3' ends extends from each strand, the DNA strands will generate two crossing overs referred as Holliday junctions (HJs)[104]. The HJs will be cleaved by resolvases in order to recover the double strand structure of DNA. Depending on the cleavage direction (longitude or latitude), two possible products can be generated (Figure 7) [104].

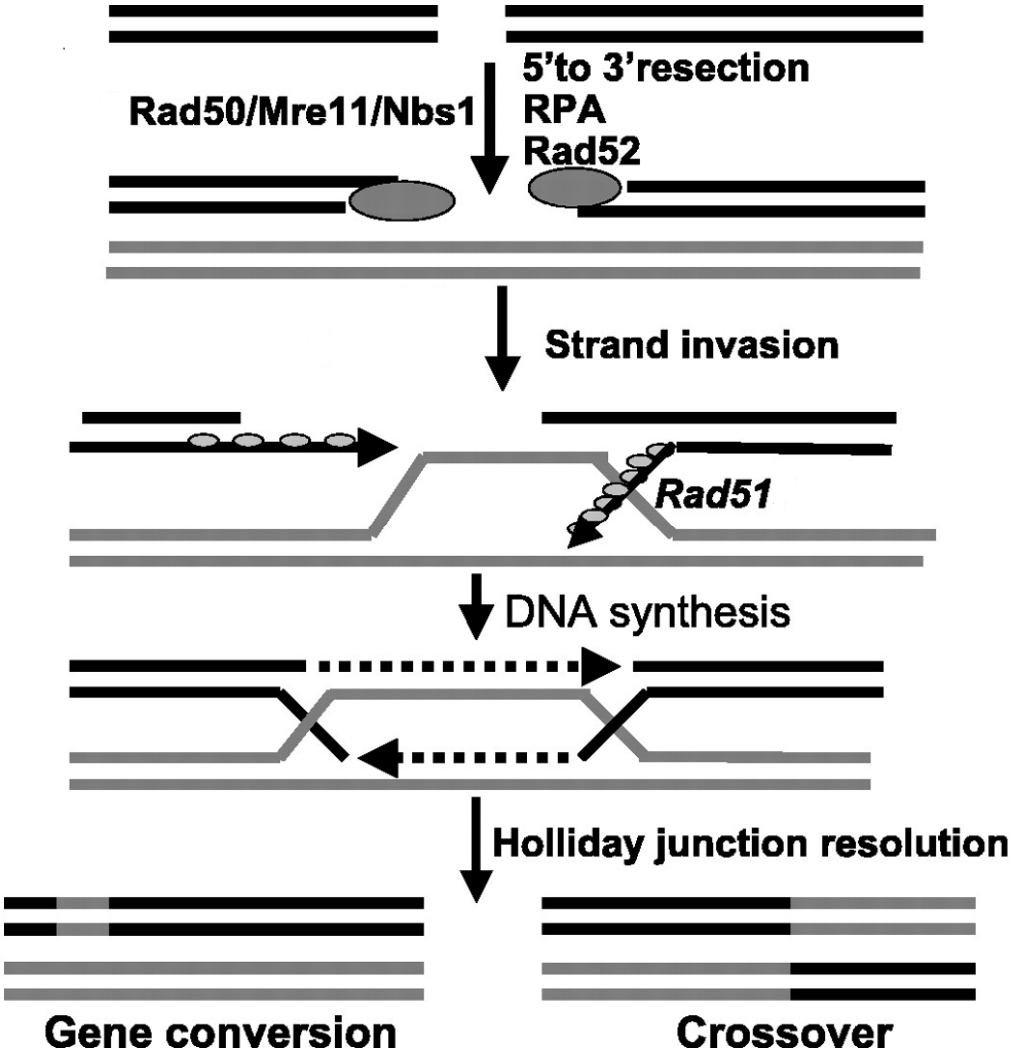


Figure 7: Overview of the homologous recombination (HR) repair pathway.

Upon double strand breaks, the MRN complex (MRE11, RAD50, and NBS1) recognizes these lesions and generates overhangs by end resection to allow DNA synthesis. RPA is then recruited to protect the single strand DNA and is replaced by Rad51 to initiate strand inversion. The Holliday junctions generated during DNA synthesis are resolved in order to form a double strand DNA. Reference: Junran Zhang and Simon N. Powell (2005) Mol Cancer Res: 3 (10) 531-539. [104]

3.1.6 Translesion Synthesis (TLS)

During replication, the DNA polymerase δ or ε encounters countless DNA lesions and results in replication stalling which impairs cell function [57,60-62,64]. Although it is not directly a DNA repair mechanism, translesion synthesis is an approach to bypass DNA lesions and complete replication despite having damage[57,60-62,64]. Unlike DNA polymerases, TLS polymerases are proficient in synthesizing DNA past DNA lesions (Figure 8) [57]. Translesion synthesis is mediated by a different family of polymerases referred to as the Y family. In humans, the Y family polymerases include polη, polι, polκ, polζ, and REV1 [57]. It is still not well understood which TLS polymerase will be recruited to the site of lesion. It is speculated that the nature of damage mediates the choice of TLS polymerases recruited [57]. It is also suggested that they might have redundant roles so that one can compensate for the loss of function of the other [57]. Polη was reported to very accurately bypass cyclobutane pyrimidine dimers (CPD) generated by UV [57]. Polι was found to have high fidelity replicating dA templates whereas it has low fidelity at replicating dT templates [57]. Polκ was shown to have a tendency to incorporate frameshifts whereas it could bypass N^2-dG lesions induced by benzo [a] pyrene accurately [57-59]. Polζ was suggested to form a complex with polι or polκ as an alternative to polη [57,106,107]. REV1 was reported to incorporate dC opposite to dG although it is mostly observed to act as a scaffolding protein to recruit polη, polι, polκ to the DNA lesion [57]. TLS is triggered upon the encounter of a DNA lesion by the replicative polymerase. The DNA replication polymerase stalls and signals PCNA monoubiquitination on lysine 164 by the E3 ligase Rad18 and the E2 conjugating enzyme Rad6 [108-112]. Proliferating cell nuclear antigen (PCNA) is a trimeric clamp that slides along the DNA and allows the anchoring of DNA polymerases, which recognize PCNA through their PCNA interacting motif referred as PIP boxes[57,113]. Upon monoubiquitination of PCNA, the recruitment of TLS polymerases is favoured as they possess an ubiquitin interacting motif and thus, promote a switch of position of DNA polymerases [57]. The TLS then incorporates bases at the sites of DNA lesion and beyond before switching back to the DNA polymerases [57]. It is still not clear how DNA synthesis is resumed but there are speculations that a deubiquitinase could be involved in antagonizing PCNA ubiquitination and/or the TLS polymerase is

polyubiquitinated and degraded by the proteasome [57]. Of note, the TLS mechanism is error prone as the translesion synthesis polymerases are not as accurate as the replicative polymerases and only promote the bypass of the lesion to allow the cell to progress in S phase [57]. TLS polymerases might incorporate the correct base opposite of the DNA lesion or the wrong base which has to be repaired by the appropriate DNA repair pathway prior to the next replicative phase of the cell cycle in order to avoid genomic instability [57]. It is interesting to note that, although still not well characterized, there is also an error free mechanism known as template switching regulated by the ubiquitin ligase complex RAD5-MMS2-UBC13 which triggers K63 polyubiquitination chains on lysine 164 of PCNA[108]. Template swithching is suggested to be dependent on Rad51; as the DNA polymerase encounters a DNA lesion and stalls, the newly synthesized strand temporatily invades and uses the sister chromatid on the opposing duplex as a template [114,115].

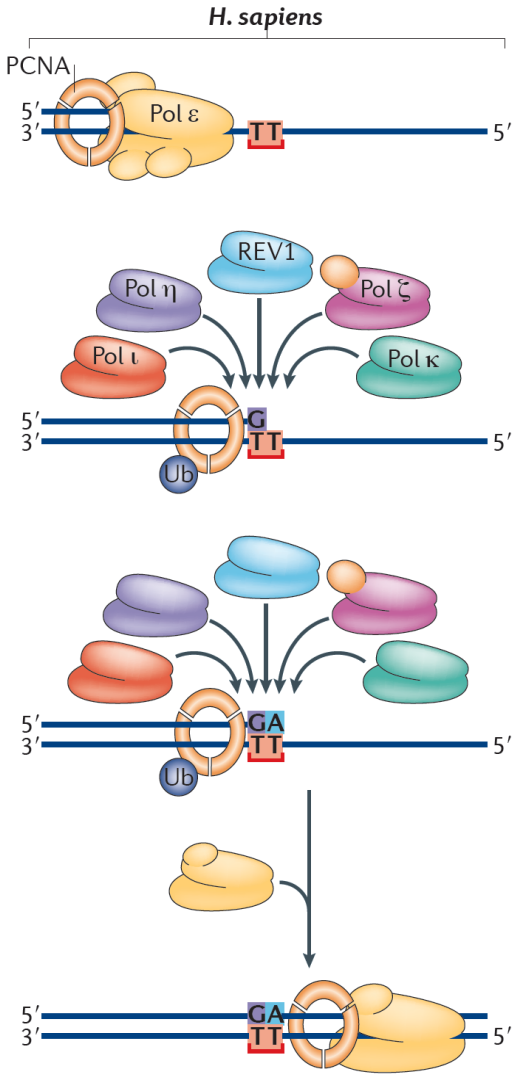


Figure 8: Overview of the translesion synthesis (TLS) mechanism

Upon encounter of a DNA lesion, the replicative polymerase stalls and triggers PCNA ubiquitination and promotes the switch between replicative polymerase and translesion synthesis polymerases. There are 5 translesion synthesis polymerases (polη, polι, polκ, polζ, and REV1) that could possibly be recruited depending on the type of DNA lesion. The translesion synthesis polymerase incorporates a base complementary to the DNA lesion and bypasses the DNA lesion before switching back with the replicative polymerase. Reference: Sale JE et al. (2012) Nat Rev Mol Cell Biol. 13(3): 141-152. [57]

4 The cell cycle regulation and apoptosis

The cell cycle is a process that exists in every living organism that allows cells to divide into identical cells. This important cycle consists of different phases in which each has a specific role and is regulated tightly in a unidirectional way in order to prevent chromosomal aberrations, which lead to tumourigenesis. Cell proliferation is regulated by different mechanisms that arrest cell cycle progression at different stages. These cell cycle arrest mechanisms, called checkpoints, play critical roles in preventing accumulation of mutations and genomic instability. In the presence of chromosomal abnormalities or genotoxic stress, the checkpoints are activated in order to arrest the cell cycle, providing time for DNA repair. Deregulation of checkpoint mechanisms by activation of specific oncogenes or inactivation of certain tumour suppressor genes cause genomic instability, which lead to cancer development.

4.1 The cell cycle regulation

The cell cycle is regulated by different cyclin dependent kinases (CDK) and cyclins. CDK and cyclins assemble as heterodimers and are activated by phosphorylation through CDK activating kinases (CAK) [116]. The CDK/Cyclin dimer phosphorylates and regulates diverse proteins or transcriptional factors required for cell cycle progression. Each CDK/cyclin dimer has its own targets and thus, confers specificity in the cell cycle. Also, the cyclins are synthesized in a sequential manner when necessary and are ubiquitinated and degraded by the proteasome once they have served their purpose to concede their place to other cyclins so that the cell cycle can go on (Figure 9) [116]. The specificity of cyclin degradation is dependent on the E3 ligases, which confer specificity though their F-Box domains recognizing specific targets [6]. The two main E3 ligases regulating the cell cycle are the RING type ligases SCF and APC/C whose F-BOX proteins are expressed in a cell cycle dependent manner [6](Table 1).

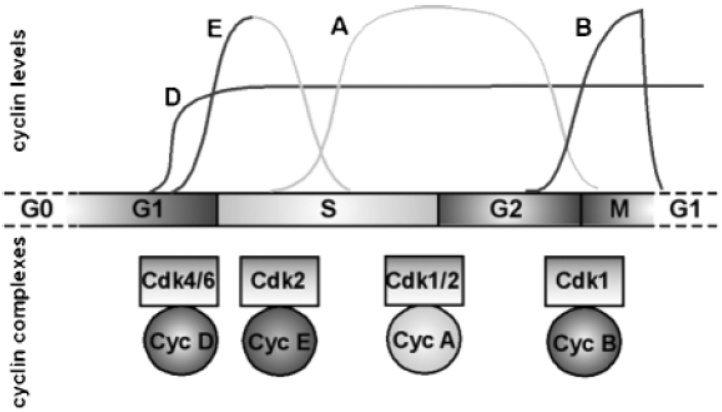


Figure 9: Cyclin levels and complexes during the cell cycle.

Overview of the cyclin levels and the cyclin/cdk heterocomplexes responsible for regulating cell cycle phase transition. Reference: Verschuren E.W. et al. (2004) Journal of General Virology 85, 1347–1361. [116]

The cell cycle can be regulated negatively by the CDK inhibitors or the kinases Wee1/Myt1. CDK inhibitors (CDKI) play an important role in cell cycle checkpoint by interacting with CDKs and inhibiting their activity [117,118]. The CDKI include two families: the INK4 family and the cip/kip family. The INK4 family consists of p15, p16, p18, and p19 mainly known to mediate G1/S checkpoint [117,118]. The cip/kip family consists of p21, p27, and p57 that mediate G1/S checkpoint or G2/M checkpoint [119-121]. The Myt1 and Wee1 kinases are inhibitors of the cell cycle as they inhibit CDK activities by phosphorylation of CDK1 on threonine 14 and tyrosine 15 respectively [122-124]. This inhibition is reversed by the CDC25 family phosphatases (CDC25A, CDC25B, and CDC25C) and thus, plays an important role in promoting cell cycle progression [125,126]. These phosphatases are expressed in a cell cycle dependent manner as CDC25A is mainly expressed from G1 to S; whereas CDC25B is mainly expressed from S to mitosis [125,126]. CDC25C is constitutively expressed throughout the cell cycle [125,126].

4.2 Cell cycle checkpoints

Upon DNA damage, the cell cycle checkpoints are rapidly activated. The G1/S checkpoint is mediated by DNA damage activation of the ATM/ATR kinases which subsequently induce the phosphorylation of CHK2/CHK1 kinases [127]. CHK1 and CHK2 were both observed to phosphorylate CDC25A on serine 126, resulting in CDC25A inhibition [128]. Inhibition of CDC25A inhibits the activity of CDK2 and cell cycle progression[128]. In addition, CHK1/CHK2 phosphorylation activates the tumour suppressor protein p53, which is well known to promote cell cycle arrest as p53 acts as a transcription factor promoting transactivation of the CDK inhibitor p21, known to inhibit the activity of the CDK2 [129-131]. ATM/ATR can as well phosphorylate and inhibit Mdm2, which is the E3 ligase for p53, preventing the degradation of p53 and thus promoting cell cycle checkpoint [132-134].

In the G2/M checkpoint, phosphorylation of CHK1/CHK2 inhibits CDC25B, resulting in CDK1-cyclin B inhibition[128]. Moreover, p21 can also inhibit G2/M transition by inhibiting CDK1 [135]. During the G2/M checkpoint, p53 can induce 14-3-3 and GADD45, inhibiting CDK1 as GADD45 and 14-3-3 sequesters CDK1 and CDC25A respectively in the cytoplasm [41,136,137].

The mitotic checkpoint, also known as the spindle assembly checkpoint (SAC) is important for maintaining genomic integrity. When chromosomes are attached incorrectly on the kinetochores, this checkpoint is triggered to inhibit metaphase to anaphase transition [138]. This checkpoint is regulated by kinetochores. Kinetochores are chromosomal sites containing proteins that serve as attachment sites for the microtubules (the inner kinetochore is attached to the chromosome through the centromeres and the outer kinetochore is attached to the microtubules) [139]. Kinetochores function as a sensor between the chromosomes and the microtubules in order to activate the SAC when chromosomes are not attached properly to the microtubules [138-143]. When the chromosome and the microtubules are attached properly, securin, an inhibitor of separase, is degraded by APC/C^{cdc20} [6,144]. Separase, the enzyme responsible for degrading the cohesins and kinesins maintaining the chromosome together is then released and activated[144]. APC/C^{cdc20} can also activate separase by degrading cyclin B as the latter is required for

maintaining separase inactivated by phosphorylation [6,144]. The active separase degrades the kinesins and cohesins to facilitate chromosomal segregation thus promoting anaphase[144]. In contrast, if the chromosomes are attached incorrectly, a tension is sensed by the kinetochores and the spindle checkpoint is activated by the formation of the mitotic checkpoint complex, Mad1, Mad2, Bub1, BubR1, Bubr3, which inhibits APC/C^{cdc20} to prevent anaphase entry as the latter is the E3 ligase responsible for degrading cyclin B to promote anaphase entry [6,138]. However, if the SAC fails (mitotic slippage), it might promote mitotic catastrophe, in which cell death is induced. Of note, it is possible for the cells to adapt and bypass cell death, leading to aneuploid cells that contain abnormal chromosome numbers, destabilizing genomic integrity and enhancing tumourigenesis.

Phases	Description	Checkpoints	CDK/Cyclins	E3 Ligases (F Box)
G0	Resting phase			
G1 Gap 1	The cells grow in mass	Ensure the size of the cell is appropriate for synthesis	Cyclin D-CDK 4/6 Cyclin E-CDK 2	APC/C (cdh1)
Synthesis	Duplication of DNA content from 2n to 4n		Cyclin A-CDK 2 Cyclin A-CDK 1	SCF (FBW7) SCF (β-TCR)
G2 gap 2	Mitosis preparation	Ensure that no errors occurred during synthesis and that all DNA have been duplicated	Cyclin A-CDK 1 Cyclin B-CDK 1	SCF (SKP2)
Mitosis and Cytokinesis	Division of the cells to generate 2 identical cells	Ensure that chromosome is attached correctly on the spindle to prevent chromosomal aberrations	Cyclin B-CDK 1	APC/C (cdc20)

Table 1: Summary of the cell cycle checkpoints and the proteins involved.

Summary table describing the phases and the checkpoints of the cell cycle along with the cyclin/CDK heterocomplexes and ubiquitin ligases regulating it [6,7].

4.3 Apoptosis

If the DNA damage is too massive to be repaired by DNA repair pathways, apoptosis will be triggered in order to prevent any further proliferation of the cell harbouring damaged DNA. Apoptosis is an irreversible process where cells are programmed to die when the damages are too important to be solved by the cell cycle checkpoints and DNA repair mechanisms. Apoptosis is initiated by particular proteases called caspases, which are found to be inactive under normal conditions. There are two types of caspases: initiator caspases such as Caspase-8 or Caspase-9 and effector caspases such as Caspase-3 [145]. The initiator caspases are first activated, triggering the cleavage and activation of the effector caspases [145]. Apoptosis is negatively regulated by IAPs (inhibitor of apoptosis proteins) as they interact with caspases to constrain their active sites [146-148]. There are two pathways for apoptosis, the intrinsic pathway and the extrinsic pathway, which both ultimately lead to the cleavage and activation of Caspase-3 [145-147]. Caspase-3 then cleaves proteins and DNA in the cell, eventually resulting in cell death [145]. The intrinsic pathway is mediated by the mitochondria. In presence of stress, the mitochondria have increased membrane permeability, promoting the release of cytochrome c and SMAC (small mitochondria-mediated activator of caspases) [145,148]. SMAC interact with IAPs in order to abolish the interaction of the latter with caspases [148]. The cytochrome c released by the mitochondria interacts with the apoptotic protease activating factor 1 (APAF-1) [149]. APAF-1 promotes cleavage and activation of the caspase 9, resulting in Caspase-3 activation [149]. The extrinsic pathway is mediated by membrane receptors such as Fas [150,151]. Upon binding with its ligand FasL, the death-inducing signaling complex (DISC) is formed, allowing the interaction with and the activation of the initiator Caspase-8 [150,151]. Different markers of apoptosis include Caspase-3 and Poly (ADP-ribose) polymerase-1 (PARP-1) cleavage. PARP-1 is a protein required for ADP ribose synthesis for gene transcription and DNA repair[152,153]. In the presence of low DNA damage, PARP-1 activity is enhanced and is recruited to single strand and double strand DNA breaks in order to promote the formation of poly (ADP-ribose) chains [152,153]. The recruitment of PARP-1 to the site of DNA damage promotes chromatin relaxation and

DNA repair [154,155]. During apoptosis, PARP-1 is cleaved and inactivated by Caspase-3 and this cleavage is widely used as a marker for apoptosis [156,157].

5 BRCA1

Breast cancer susceptibility protein type 1 (BRCA1) is a major tumour suppressor. BRCA1 mutation carriers have a higher risk of developing breast cancer (65%) and/or ovarian cancer (39%) by the age of 70 and heterozygous BRCA1 mutation is sufficient for increasing the risk of developing cancer [37,38]. Until now, there are approximately 1600 BRCA1 mutations recorded[158]. However, it is difficult to determine if all mutations are associated with an increased risk of cancer due to the lack of biochemical and functional studies on these mutations[158]. Nevertheless, there is no doubt that BRCA1 is an important tumour suppressor crucial for cell function and maintenance of genomic stability. Consistent with this notion, BRCA1 null mice were reported to be embryonic lethal, likely due to the activation of several checkpoint responses that arrest cell proliferation as a consequence of DNA repair defect [159]. Indeed, a few major roles of BRCA1 include: DNA damage signaling and repair, checkpoint activation, and transcription regulation.

5.1 The Domains of BRCA1

The N-terminal domain of BRCA1 possesses a RING domain and interacts with BRCA1 Associated RING Domain 1 (BARD1) to form a core complex, conferring ubiquitin ligase activity. BARD1 is believed to be crucial for stabilizing the RING domain of BRCA1 for a proper conformation conferring E3 ligase activity (Figure 10) [160]. Although the role of the ubiquitin ligase activity of BRCA1 is still not well understood, it was proposed that it could have a role in amplifying ubiquitination of γH2AX at the site of DNA damage in order to facilitate the recruitment of additional BRCA1 and homologous recombination proteins[161]. Some studies proposed that BRCA1/BARD1 targets RNA polymerase II and has a role in regulating transcription [162-164]. BRCA1/BARD1 ligase complex was also found to auto-ubiquitinate itself in a non-degradative manner in order to signal a yet to be known cellular function [165]. In the middle region, BRCA1 contains a coiled coil domain, which is a hydrophobic region that promotes protein-protein interaction; usually with the coiled coil region of another protein [166-169]. The coiled coil middle region of BRCA1 was observed to interact with the N-terminal coiled-coil region of PALB2 whereas the C-terminal region of PALB2 interacts with the N-terminal region of BRCA2 [166-170]. Both PALB2 and BRCA2 are involved in the repair of double strand DNA breaks[166-170]. At the C terminus, BRCA1 contains 2 phospho-peptide binding domains that are typical of DNA repair proteins, called BRCT [104,171,172] (Figure 10). These BRCT domains recognize a consensus phosphoS-X-X-F motif and are of high importance as they allow the BRCA1/BARD1 core complex to interact with a multitude of proteins in order to form complexes involved in DNA damage repair[171,172]. As a matter of fact, mutations in the BRCT domains of BRCA1 predispose to cancer development [158,173]. The BRCA1/BARD1 core complex interacts with phosphorylated Abraxas, BRIP1 and CtIP through the BRCT domains of BRCA1 to form the complexes A, B and C respectively [104,174]. The complex A is involved in triggering G2-M cell cycle checkpoint and recruiting BRCA1 to DNA damage sites [104,174]. The complex B is involved in the S phase checkpoint of the cell cycle [104,174]. The complex C is involved in DNA resection in order to initiate homologous recombination [104,174]. Finally, the BRCC complex allows the recruitment of Rad51 through the intermediates PALB2 and BRCA2 in order to promote homologous

recombination [104,174] (Table 2). BRCA1 has been reported to be phosphorylated on serine 988 by Chk2 and on serine 1387, 1432 and 1524 by ATM or ATR [104,166] (Figure 10). Phosphorylation of BRCA1 on serine 988 by Chk2 was observed to be crucial for PALB2 interaction with BRCA1 and the recruitment of Rad51 but is not required for cell cycle checkpoint activation [166-169,175]. In contrast, phosphorylation of BRCA1 on serine 1387, 1423 and 1524 were shown to be required for cell cycle checkpoint activation but not for homologous recombination repair [166,176,177].

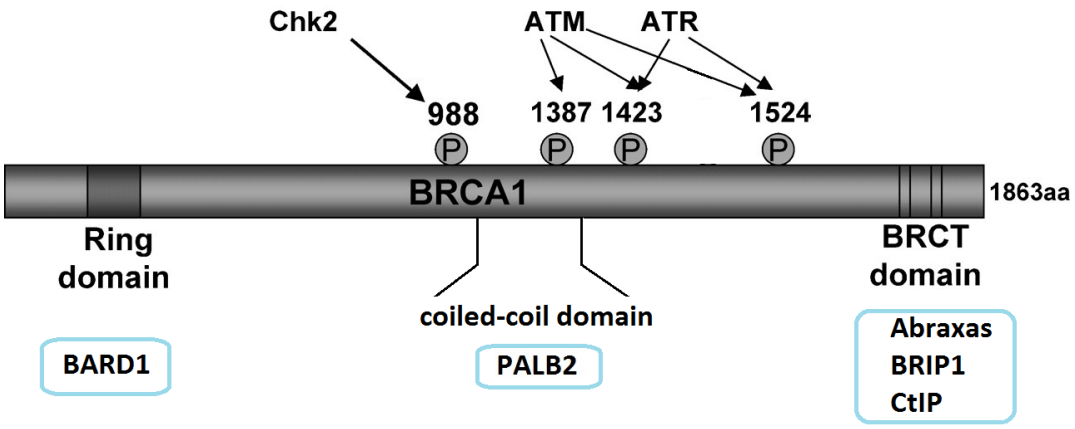


Figure 10: The domains of BRCA1 and its sites of phosphorylation in response of stress.

Schema describing the different domains of BRCA1 along with its interaction sites with BARD1, PALB2, Abraxas, BRIP, and CtIP. The phosphorylation sites by Chk2, ATM, and ATR are also shown. Modified from: Zhang J, Powell S.N (2005) Mol Cancer Res. 3:531-39. [104]

BRCA1 complex	Function	Components
Core complex	Promotes E3 ligase activity	BRCA1 and BARD1 (constitutive heterodimer)
BRCA1 A	Control of G2-M checkpoint and BRCA1 accumulation at damage-induced foci	BRCA1, BARD1, Abraxas, RAP80 and BRCC36
BRCA1 B	DNA replication and S phase progression	BRCA1, BARD1, BRIP and TOPBP1
BRCA1 C	DNA resection and G2-M checkpoint	BRCA1, BARD1, CtIP and MRN complex (MRE11, Rad50, NBS1)
BRCC (BRCA1/2 containing complex)	Homologous recombination-mediated DNA repair	BRCA1, BARD1, BRCA2, PALB2 and Rad51

Table 2: Description of the BRCA1 complexes.

Description of the components and functions of the BRCA1/BARD1 core complex and the complexes A, B, C and BRCC. Modified from: Huen M.S.Y, Sy S.M.H, Chen J. (2010) Nature Review 11(2):138-148. [174]

5.2 The mechanism of action of BRCA1 upon exposure to double strand breaks

Although extensive studies have been conducted on BRCA1, the mechanism of action and the regulation of BRCA1 in presence of stress are still not well established. The following figure (Figure 11) shows a schema of how the BRCA1 complexes are being recruited and how they can recruit homologous recombination proteins. Upon IR, a multitude of DNA damage proteins are recruited to the chromatin, forming IR induced foci (IRIF). MRE11, RAD50, and NBS1 (known as the MRN complex) are recruited to the damaged sites along with the BRCA1 complex C (BRCA1 and CtIP) [178-180]. The MRN complex was shown to be recruited rapidly upon DNA breaks and thus, acts as an important sensor of DNA damage [178-180]. In addition, the MRN complex and CtIP possess exonuclease activity and work together for DNA resection in order to create 3' single stranded DNA overhangs for strand invasion [39,40,174,178,181]. A single strand DNA binding protein, replication protein A (RPA), is then recruited in order to prevent the formation of secondary structures and degradation of the single strand DNA ends[40]. The MRN complex also promotes the recruitment and the activation of the kinase Ataxia Telangiectasia Mutated (ATM) by promoting its autophosphorylation on serine 1981 [182]. ATM is well known for its involvement in signaling DNA damage as it phosphorylates H2A.X on serine 139, an H2A histone variant; generating γH2AX foci at damaged sites on the chromatin [39,183]. The formation of γH2A.X foci is one the of first steps in DNA damage signaling as phosphorylation of H2A.X on serine 139 occurs as early as one minute after DNA double strand breaks induction and thus, γH2AX acts as a marker for DNA damage [184]. Phosphorylation of H2A.X is crucial for the recruitment and maintenance of DNA repair proteins at the sites of DNA damage as it provides binding sites for DNA repair proteins with BRCT domains [185,186]. Subsequently, the DNA damage mediator MDC1 is recruited through its BRCT domain which recognizes phosphorylated H2A.X and stabilizes the MRN complex, creating a positive feed-back loop to amplify the recruitment of γH2A.X and DNA repair proteins [39,187]. The MDC1 and γH2AX interaction also prevents the protein phosphatase 2A (PP2A) from dephosphorylating γH2AX and

thus, prevents γH2AX elimination from the site of DNA damage [187,188]. MDC1 is phosphorylated by ATM on threonine 98, which promotes the recruitment of the ubiquitin conjugating enzyme UBC13 and ubiquitin ligase RNF8 [189]. The RNF8 ligase includes a forkhead associated (FHA) domain that recognizes phosphorylated threonines on proteins [190,191]. RNF8 monoubiquitinates H2A or H2A.X on lysine 119 and monoubiquitination of H2A or H2A.X is recognized by another E3 ligase RNF168 through its ubiquitin-interacting motif [179,191-195]. RNF168 then triggers K63 polyubiquitination chain formation on lysine 119 of H2A and H2A.X, which is crucial for the accumulation of BRCA1 complex A (BRCA1/BARD1/Abraxas/RAP80) at the sites of DNA damage via RAP80, which recognizes ubiquitinated H2A or H2A.X through its ubiquitin interacting motif (UIM) [179,191-196]. It is important to note that lysine 63 polyubiquitin chains are not involved in proteasomal degradation, but involved in DNA damage and other signaling events. It is also interesting to note that H2A.X can also be phosphorylated on tyrosine 142 by the tyrosine kinase WSTF to trigger apoptosis [197,198]. MDC1 only interacts with H2A.X unphosphorylated on tyrosine 142, suggesting that phosphorylation of H2A.X on tyrosine 142 determines the outcome between cell survival by DNA repair or cell death by apoptosis [197,198]. On the other hand, it has been reported that the histone acetyl transferase TIP60 and the E2 conjugating enzyme UBC13 complex can interact with γH2A.X in presence of IR and acetylate histone γH2A.X on lysine 5 and this steps seems to promote histone ubiquitination through UBC13[39,199]. It was also shown that acetylation and ubiquitination induces the eviction of γH2A.X and promotes chromatin reorganization near the site of DSB [39,199]. Although it is still not too well understood, ubiquitination or acetylation of γH2A.X could trigger chromatin modification in order to exposed dimethylated histone H4 to allow the recognition by the DNA damage protein 53BP1[39,191,200,201]. 53BP1, p53 binding protein 1, also interacts with p53 and plays an important role in cell cycle checkpoint [202]. 53BP1 interacts with the MRN complex and was shown to further promote ATM activity to phosphorylate CHK2 for checkpoint signaling [202-205]. Although 53BP1 recruitment to H4K20me2 was shown to be dependent on H2A.X ubiquitination triggered by MDC1, RNF8 and RNF168, little is known on the exact mechanism of action involving 53BP1 [191,200,201] (Figure 11). Finally, following

BRCA1 recruitment, Rad51 is recruited through the scaffolding proteins PALB2 and BRCA2 [40,166]. Rad51 displaces RPA and initiates strand invasion of the sister chromatid for homologous recombination repair [40,166] (Figure 12).

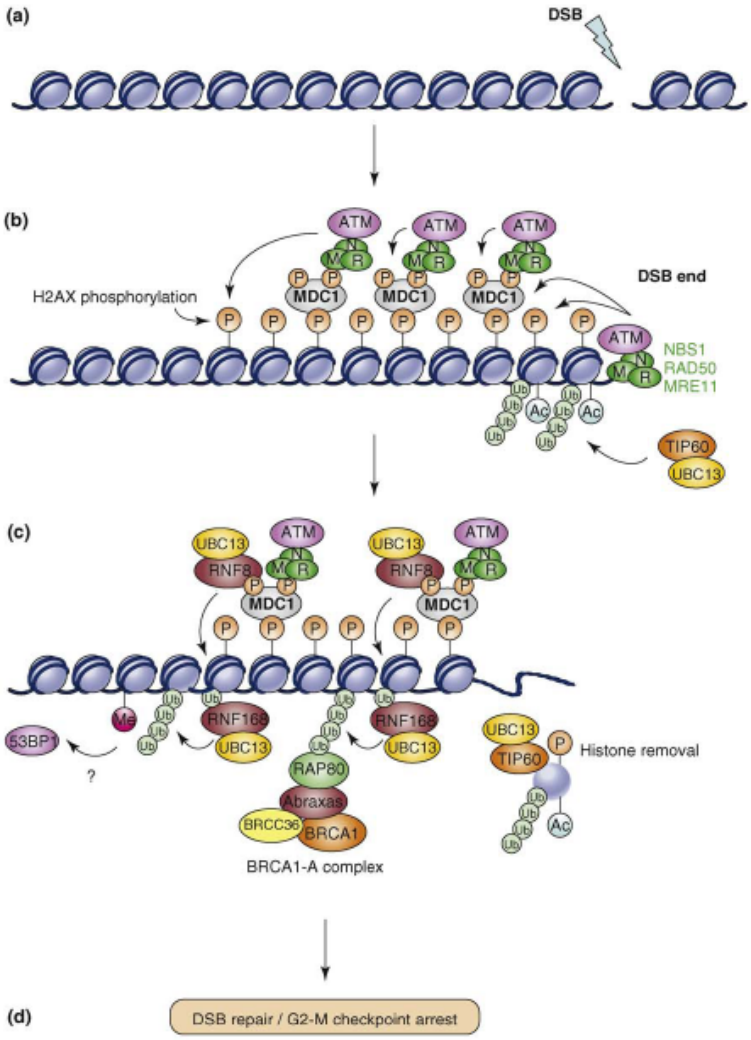


Figure 11: Overview of BRCA1 recruitment to the sites of DNA damage upon double strand breaks.

Upon double strand break, the MRN complex is recruited along with ATM. Phosphorylation of H2A.X by ATM signals the recruitment of the ubiquitin ligases RNF8 and RNF168. Ubiquitination of γH2A.X by RNF8 and RNF168, serve as an interaction site for the BRCA1 complex A consisting of BRCA1, RAP80, Abraxas and BRCC36. BRCA1 recruitment triggers cell cycle checkpoints and DNA damage repair. Reference: Haico van Attikum and Susan M. Gasser (2009) Trends in Cell Biology 19(5): 207-217 [39]

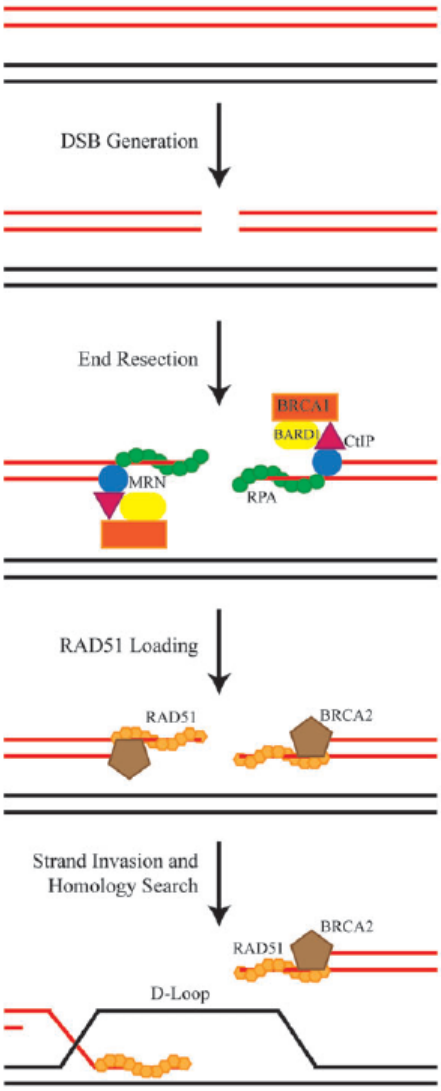


Figure 12: The role of BRCA1 in homologous recombination

BRCA1 has a role is promoting homologous recombination upon double strand break. The BRCA1 complex C consisting of BRCA1, BARD1, CtIP, and the MRN complex promotes ends resection to allow strand invasion. In addition, BRCA1 can recruit RAD 51 through the BRCC complex to promote strand invasion. Reference: P.J.O'Donovan and D.M.Livingston (2010) Carcinogenesis 31(6): 961–967. [40]

5.3 The regulation of DNA double strand break repair pathways: choosing between non-homologous end-joining and homologous recombination

There are two pathways involved in repairing DNA double strand breaks: homologous recombination and non-homologous-end-joining[206]. The homologous recombination repair pathway makes use of the genetic information of the undamaged sister chromatid as a template to repair the damage in an accurate error-free manner[206]. For the reasons mentioned above, this DNA repair pathway is only favoured during late S phase and G2 phase where the sister chromatid template is available[206]. In contrast, the non-homologous repair pathway promotes the ligation of the broken ends together and thus, is less accurate and error-prone[206]. In fact, NHEJ is the main repair pathway for double strand breaks as it can take place throughout the cell cycle and is favoured when sister chromatid templates are not available[206]. It was reported that the ratio between NHEJ and HR occurrence is about 3:1 in mammalian cells [206]. It was also observed that NHEJ is activated and completed more rapidly than HR and proteins associated with NHEJ and HR are recruited simultaneously in an independent matter [50,206,207]. Although there is competition between these two repair pathways, the cell cycle tightly regulates them in order to favour one over the other [207]. BRCA1 is known to be expressed during the S and the G2 phases where sister chromatids are available for homologous recombination [208]. In addition Rad51 and Rad52 are also mainly expressed during the S and G2 phases [207,209,210]. In contrast, DNA-PKcs phosphorylation is decreased during the S phase, resulting in NHEJ inhibition given that DNA-PKcs phosphorylation is crucial for NHEJ [207,211,212]. Also, CDK1, which is mainly expressed from the late G1 phase to mitosis, was observed to phosphorylate CtIP on serine 327 during the S phase and to promote BRCA1-CtIP complex formation and thus favours homologous recombination[207,213,214]. In addition, BRCA2 is being phosphorylated on serine 3291 by CDK1/cyclin B during mitosis and this phosphorylation was shown to block BRCA2 interaction with Rad51, resulting in HR inhibition during mitosis [207,215] (Figure 13). The role of the cell cycle in determining the

choice of DNA double strand break repair between homologous recombination and non-homologous-end-joining.

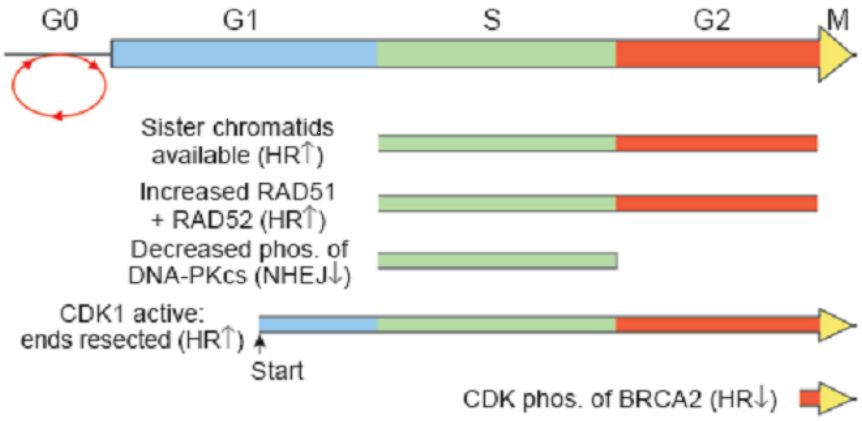


Figure 13: The role of the cell cycle in determining the choice of DNA double strand break repair between homologous recombination and non-homologous-end-joining.

One of the major factors in determining the pathway chosen to repair double strand breaks is the cell cycle. During the S phase, the sister chromatids are available and Rad51, Rad52 expression is increased to favour homologous recombination repair. During the S phase, phosphorylation of DNA-PKcs is decreased and thus, inhibits the non-homologous end-joining repair. Modified from: Shrivastav M et al. (2008) Cell Research 18:134-147.
[207]

5.4 The role of BRCA1 in maintaining genomic integrity

Given the importance of BRCA1 in homologous recombination pathway, BRCA1 mutation impairs this repair process. The NHEJ and TLS become the alternative pathways and could engender genomic instability as they are not error free.

BRCA1 also appears to play an important role in maintaining genomic stability by regulating centrosome duplication [216,217]. The centrosome is an organelle crucial for organizing microtubules as it provides an anchoring site for the microtubules through γ-tubulin, a globular protein found in the centrosome [216,217]. The γ-tubulins in the centrosome are organized into a ring structure referred to as the γ-tubulin ring complex that allows the binding of the microtubules [216,217]. The role of the centrosome is to ensure the bipolarity of the mitotic spindle [216-218]. The centrosome is duplicated once every cell cycle during the late G1 and early S phase [216-218]. During mitosis, the centrosomes migrate to each pole and trigger the mitotic spindle assembly for chromosome segregation and each cell will end up with one centrosome [216-218]. Centrosome duplicated more than once (overamplification) could result in the formation of a multipolar spindle, which is the foremost cause of defects in chromosomal segregation, cytokinesis, thus causing aneuploidy [216,217]. The region encompassing the amino acids 504 to 803 of BRCA1 was observed to interact with centrosomes in the S and G2 phases and monoubiquitinate γ-tubulin upon centrosomes duplication[216-218]. This ubiquitination event might act as a marker to ensure that the centrosome is only duplicated once every cell cycle in order to prevent aneuploidy [216-218] (Figure 14). On the other hand, BRCA1 was shown to regulate the mitotic checkpoint by regulating the transactivation of MAD2, a component of the mitotic checkpoint complex known to inhibit the ligase, APC/C^{cdc20}, responsible for degrading cyclin B1 to promote anaphase entry [128,219].

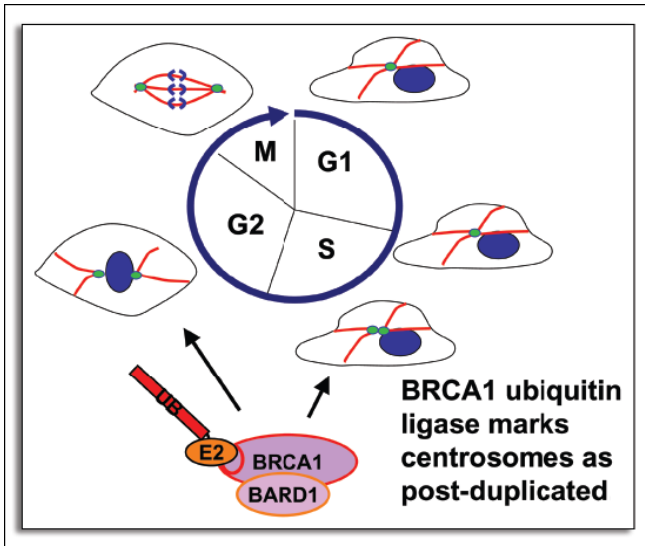


Figure 14: The role of BRCA1 in marking post-duplicated centrosomes.

BRCA1 (in blue) interact with centrosomes (in green) and ubiquitinates duplicated centrosome in order to mark them as post-duplicated to ensure that this event occurs only once during every cell cycle. Reference: Starita L.M., Parvin J.D. (2006) Cancer Biology and Therapy 5(2): 137-141. [218]

Recently, it was reported that BRCA1 plays an important role in maintaining heterochromatin structure by monoubiquitinating histone H2A on lysine 119 in the satellite regions of DNA [220]. BRCA1 appears to play a role in maintaining the transcriptionally silent state of satellite DNA [220]. Satellite DNA are transcriptionally silent and repeated non-coding sequences of different length found near the telomeres and centromeres [60,66,220]. The highly repetitive characteristic of satellite DNA renders them unstable and prone to mutations due to an increase error rate of the polymerases [60,66]. Satellite DNA expression is associated with cancer development as it disrupts the structural integrity of the constitutive heterochromatin; resulting in mitotic defects, overamplification of centrosomes, double strand breaks, defects in cell cycle checkpoints and homologous recombination repair pathway [60,66,220].

5.5 The role of BRCA1 in transcription regulation

Histones are conserved proteins allowing the organization of the large amount of genomic information into a compact structure, termed chromatin, allowing the cell to express, replicate and divide the genomic material between daughter cells. The core histones (H2A, H2B, H3, and H4) form an octamer around which 147 DNA base pairs are wounded, forming the nucleosome which is the basic unit of chromatin[221]. Histones are often subjected to a myriad of post-translational modifications such as phosphorylation, ubiquitination, methylation and acetylation[222]. These modifications have important roles in regulating chromatin structure and function as they can either modify the chromatin into a relaxed state that is transcriptionally potent, or into a condensed state that is transcriptionally silent[222,223]. Transcription, one of the major steps in gene expression, is a crucial process generating mRNA transcripts from DNA. The level of transcripts is tightly regulated by transcriptional factors (bind DNA directly) or cofactors (do not directly bind DNA, but anchor to transcriptional factors and/or chromatin modifications) upon their recruitment near the promoters (region often found upstream of the target genes). Of note, histone modifications can also signal the recruitment of transcriptional activators or repressors, or even signal further histone modifications to regulate chromatin structure and transcription[223]. Chromatin remodelling also has an important role in DNA repair as it can not only expose the damaged site for proper repair, but also initiate signaling cascades that culminate in cell cycle arrest or cell death depending on the extent of DNA damage[223-225].

BRCA1 has a significant role in transcription. The RNA polymerase II was reported to be a target of the BRCA1/BARD1 E3 ligase complex [41,162,218]. Upon DNA damage, RNA polII stalls upon the encounter of a DNA lesion and is phosphorylated on serine 5 and by cdk7, allowing its interaction with BRCA1/BARD1 [163,226]. RNA polII is then ubiquitinated by BRCA1/BARD1 to promote DNA damage signaling and transcription inhibition [162,218]. The outcome of RNA polII ubiquitination is still not so well understood. It was proposed that RNA poll II ubiquitination could either promote its

degradation or inhibit transcription initiation by preventing the assembly of core transcriptional factors such as TFIIE and TFIIH at the promoter[41,162-164,218].

BRCA1 also acts as a transcription co-factor to regulate the expression of many cell cycle checkpoint genes. It can either act as a transcription co-activator or co-repressor. BRCA1 can interact with and stabilize the tumour suppressor and transcription factor p53 to regulate the expression of cell cycle checkpoint genes[41,227,228]. P53 is a transcriptional factor regulating the expression of a myriad of genes involved in cell cycle checkpoints (e.g. p21, GADD45, 14-3-3σ) and apoptosis (e.g. p53-upegulated modulator of apoptosis: PUMA) upon stress in which the cellular outcome is dependent on the transcriptional co-factors it interacts with and the nature of the stress[229]. Although p53 is also known to activate the transcription of genes involved in apoptosis, p53 mediated transcription by BRCA1 was observed to mainly regulate cell cycle checkpoint genes[41,230-232] (Figure 15).

BRCA1 was also observed to repress the expression of growth promoting genes by repressing the activity of the transcriptional factor c-Myc [41,233] (Figure 15). BRCA1 was found to be in a complex with c-Myc and Nmi (N-terminal-Myc-interacting protein) to repress c-myc mediated transcription of the human telomerase reverse transcriptase (hTERT) gene [41,234]. The hTERT gene codes for the catalytic subunit of telomerase, which is involved in resynthesizing telomeres (repetitive sequences that protect chromosome ends) as they shorten during DNA replication[235-238]. The expression of hTERT is often low or inexistent in normal cells, resulting in senescence as telomeres shorten in order to prevent chromosomal fusion or degradation[235-238]. In contrast, hTERT is often expressed in cancer cells and a high expression of hTERT is frequently connected with the immortality characteristic of cancer cells[235-238]. BRCA1 inhibition of c-Myc transcriptional activity might have a role in preventing the immortalization of cancer cells[234].

BRCA1 was reported to regulate the transcription of genes activated by estrogen receptor α[41]. The ERα is a transcription factor found in the cytoplasm that is activated upon binding to an estrogen hormone, which triggers its dimerization and translocation into the nucleus[239]. The activated ERα dimer can then interact with DNA through its DNA

binding domain by recognizing specific response elements and initiate transcription of numerous genes [239,240]. BRCA1 was observed in interact with ERα and inhibit the transcription of the VEGF (vascular endothelial growth factor) gene, coding for a protein involved in angiogenesis (formation of new blood vessels)[241-243] (Figure 15). VEGF was reported to be overexpressed in breast cancer tissues and thus, inhibition of ERα induced transcription by BRCA1 could have a significant role in breast cancer prevention[241,244].

It was also observed that BRCA1 interacts with the SWI/SNF chromatin remodelling complex and histone acetyltransferase CBP/p300 to regulate chromatin structure [245-247]. Thus, it is possible that BRCA1 could promote chromatin relaxation through these chromatin regulators and promote transcription[245-247].

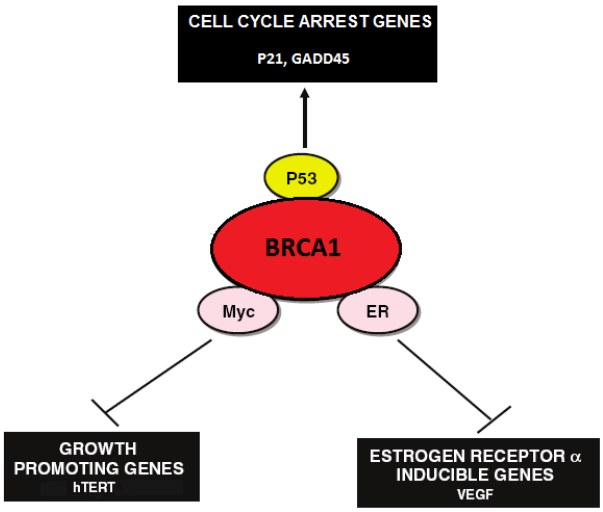


Figure 15: BRCA1 as a transcription co-factor.

BRCA1 can act as a transcriptional co-activator or co-repressor by interacting with transcriptional factors involved in the activation of genes involved in cell cycle arrest or the repression of genes involved in cell growth. Modified from: Mullan P.B., Quinn J.E. Harkin D.P. (2006) Oncogene 25: 5854-5863. [41]

5.6 The role of BRCA1 in cell cycle checkpoints

The recruitment of BRCA1 at the site of DSBs induces checkpoint kinase 1 (CHK1) phosphorylation and a G2-M checkpoint [128]. Also, BRCA1 can activate the transcription of the CDK inhibitor p21 in a p53 dependent or independent manner, resulting in G1-S checkpoint[128]. In addition, BRCA1 phosphorylation on serine 1387 by ATM can trigger cell cycle arrest in the S phase upon IR, likely as a result of p21 activation, which is known to inhibit CDK2 [128,177,248]. BRCA1 can promote G2/M checkpoint in different ways. First, BRCA1 can repress the transcription of cyclin B and thus, inhibit progression to mitosis [128,249]. Second, BRCA1 can transactivate GADD45, known to inhibit CDK1 by sequestering it in the cytoplasm [128,250]. Third, BRCA1 can promote the transcription of the CDK inhibitor Wee1, known to phosphorylate CDK1 on tyrosine 15 to inhibit the CDK1/cyclin B complex [128,251]. BRCA1 can also promote the transcription of 14-3-3σ, known to sequester the phosphatase CDC25C, the antagonist of the kinase Wee1, in the cytoplasm and thus inhibiting CDK1 [128,251]. Another way BRCA1 could inhibit CDC25C is through the transcriptional repression of the kinase PLK, known to activate CDC25C by phosphorylation on serine 191 and 198 (Figure 16) [41,252].

The BRCA1 complex B was reported to regulate S phase checkpoint. In presence of replication fork stalling, ATM phosphorylates BRCA1-interacting protein C-terminal helicase 1 (BRIP1), allowing BRCA1/BARD1 interaction with BRIP and DNA topoisomerase 2-binding protein 1 (TOPBP1) [166,174,253]. This promotes TOPBP1 dissociation from the sites of replication and thus inhibiting the recruitment of the replication initiation factor cdc45, inducing an S phase checkpoint [166,174,253].

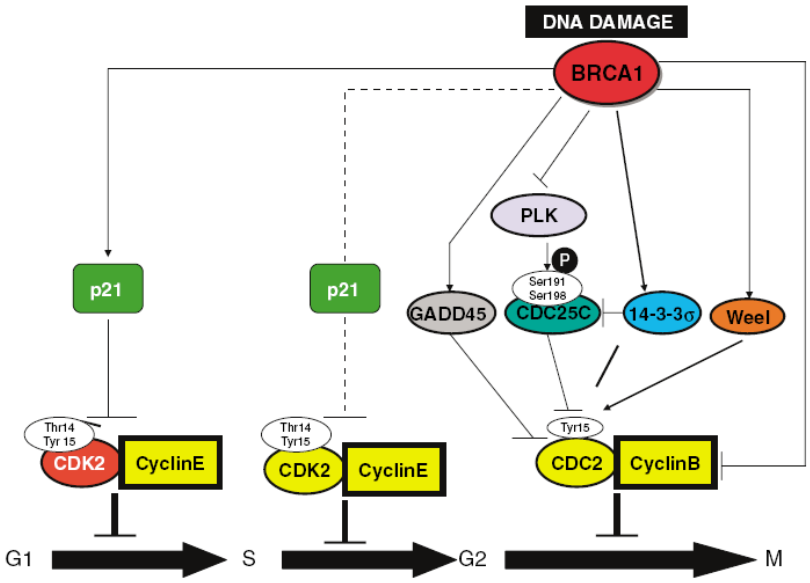


Figure 16: BRCA1 and cell cycle checkpoints

BRCA1 can regulate the transcription of different genes involved in cell cycle upon DNA damage. BRCA 1 can promote p21 transcription, resulting in an inhibition of G1/S or S/G2 transition. BRCA1 can also inhibit mitosis entry by promoting the transcription of GADD45, 14-3-3σ, Weel or repress the transcription of PLK and cyclin B; which results in an inhibition of the cdc2 (CDK1)/cyclin B heterocomplex activity. Reference: Mullan P.B., Quinn J.E. Harkin D.P. (2006) Oncogene 25: 5854-5863. [41]

6 Rationale and Hypothesis

Mutations in the BRCA1 gene confer a high risk of breast and ovarian cancer. BRCA1 is a multifunctional protein that plays a crucial role in maintaining the stability of the genome following genotoxic stress. Indeed, BRCA1 is involved in transcription regulation, DNA damage signaling, and homologous recombination-mediated DNA repair. BRCA1 is phosphorylated by ATM and ATR, kinases that each plays major roles as primary responders to genotoxic stress. Following activation by specific DNA lesions, these kinases initiate signaling cascades that promote DNA repair, as well as cell cycle checkpoints. At the structural level, BRCA1 contains a ring finger domain that confers ubiquitin ligase activity via the formation of a stable complex with another ring finger protein, BARD1. This complex in turn stimulates autoubiquitination of BRCA1. Furthermore, several breast cancer-associated mutations of BRCA1 completely abolish the ubiquitin ligase activity of the protein. In addition to its intrinsic ubiquitin ligase activity, BRCA1 function also involves the actions of other ubiquitin ligases (e.g., RNF8 and RNF168 described earlier). This has been mostly established in the context of DNA double-strand breaks. In addition, BRCC36 is a DUB that interacts with BRCA1 and is required for recruitment of BRCA1 to DSBs. Interestingly BRCC36 expression was also shown to be deregulated in breast cancer. In sum, ubiquitination and deubiquitination play a critical and complex role in regulating BRCA1 function following DSBs formation.

However, the role of ubiquitination in the regulation of BRCA1 in response to other DNA damage/repair pathways that do not directly involve double strand breaks is very poorly studied. Taking into account the major roles of ubiquitination in regulating the DNA damage response, **we hypothesized that BRCA1 function is regulated by ubiquitination in a stress dependent manner**. Thus, our objective was to characterize BRCA1 stability and function under different genotoxic stress conditions involving major DNA damage signaling and repair pathways.

B) MATERIAL AND METHODS

B) MATERIAL AND METHODS

1) Chemicals and plasmids

The thymidine, cycloheximide and MG132 were from Sigma Aldrich. The GFP-BRCA1 vectors were provided by Dr. Chiba [254]. ZL3VS was provided by Dr. B.M. Kessler [255].

2) Cell Culture

HeLa cervical cancer, U2OS osteosarcoma, HEK293 embryonic kidney, HCT116 colon sarcoma, human foreskin fibroblasts (CCD-2056) were from ATCC. The cell lines were cultured in DMEM media containing 10% foetal bovine serum, L-glutamine, penicillin and streptomycin.

The UVC treatment was done by washing the cells with PBS (phosphate buffered saline) and irradiated in PBS at a rate of 5 $J/m^2/s$ using the crosslinker CL-1000 form VWR. After treatment at 30 J/m^2 at 254-nm, the cells were maintained in cell culture media until the time point for harvesting. The IR treatment was done at a rate of 6.3 rad/s with cesium 137 from Gamma Cell Atomic Energy Canada in cell culture media. After treatment at 10Gy, the cells were maintained and collected at the indicated time. The MMS from Sigma Aldrich was added in the cell culture media at the concentration of 200 μM. If the treatment required more than 6H, the MMS was removed after 6H by replacing with fresh media and then collected at the indicated time.

3) Immunoblot

The western blots were done on total cell extracts. The cells were lysed in a buffer containing 50 mM Tris-HCl at pH7.3, 5mM ethylene diaminetetraacetate (EDTA), 50 mM KCl, 0.1% NP-40, 1mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and anti-protease cocktail by Roche Diagnostics[256]. Protein quantification was done by Bradford assay and the western blot was done according to standard procedures using a horseradish peroxidase coupled secondary antibody and revealed using enhanced chemiluminescence (ECL) from Perkin Elmer. The detection of β-actin is used as a loading control. The primary antibodies used and the dilution are described in the annex (Table 6).

4) Immunofluorescence

The cells were fixed in 3% paraformaldehyde diluted in PSB for 20 minutes prior to permeabilization with a phosphate buffered saline (PBS) solution containing 0.5% NP-40. The cells were then blocked for 1H in PBS containing 10% foetal bovine serum and 0.1% NP-40 prior to staining [257]. The primary antibodies used were described in the annex (Table 6) The secondary antibodies used were either goat anti-mouse IgG Alexa Fluor 488 or goat anti-rabbit IgG Alexa Fluor 594 from Invitrogen. The cell nuclei were stained with DAPI.

5) Immunoprecipitation

The cell extracts were prepared as described for immunoblot except that 20 mM of N-EthylMaleimide (NEM) was also added in the buffer [256]. After lysis, the cells were sonicated and the lysate was centrifuged at 14000 rpm for 30 minutes. The supernatant was then incubated with the target antibody or the control IgG antibody for 6 hours at 4°C. The protein sepharose G beads were then added and incubated for an additional 2 hours. After washing with the lysis buffer, the immunocomplexes were eluted with laemmli buffer for immunoblotting.

6) Chromatin Isolation

The cells were first treated with IR, MMS and/or MG132 for the indicated times. The cells were then washed in PBS and centrifuged at 3000 rpm for 2 minutes at 4°C and resuspended in a detergent buffer containing 50mM Tris-HCl pH 7.3; 5mM EDTA; 150mM KCl; 10mM NaF; 1% Triton X-100; 1mM PMSF and 1X protease inhibitor cocktail from Sigma. After resuspension, the pellets were washed 4 times with the same buffer under shaking for 15 minutes at 4°C and then recovered through centrifugation at 6000g for 10 minutes. The chromatin samples and total cell extracts were then used for immunoblotting against the target protein.

7) Cell Synchronization

HeLa cells were synchronized at the G1/S border phase by double thymidine block according to Harper's protocol [258]. The cells were treated with 2 mM thymidine for 12 hours twice[258]. The cells were washed three times with PBS and replaced with media without thymidine for 12 hours in between the treatments[258]. The block was released by washing the cells three times with PBS and replaced with media without thymidine in order to be collected at the indicated time for analysis[258]. Human primary fibroblasts were synchronized in G0/G1 by contact inhibition [259]. The contact inhibition is released by replating the cells at low density. The analysis was done using FACscan Flow Cytometer with the CellQuestPro software from BD Science.

8) siDUB Screen

The cells were transfected with the individual siRNA targeting DUBs (ON-TARGETplus® SMARTpool® siRNA Library - Human Deubiquitinating Enzymes) or the non-target control from Dharmacon. Three days post-transfection, the cells were exposed to DNA damage and collected at the indicated time for immunofluorescence staining for the protein of interest. Approximately 100 cells were counted for each condition and cells with DNA damage foci of the protein of interest were counted as positives.

C) RESULTS

C) RESULTS

I. BRCA1 is downregulated upon genotoxic stresses not directly inducing DNA double strand breaks

In order to determine whether BRCA1 is regulated in a stress dependent manner, HeLa cells were treated with UVC, MMS and IR, which generate DNA helix distortion through pyrimidine dimerization, alkylation and double strand breaks respectively. Surprisingly, BRCA1 is downregulated upon 3 hours of MMS and UVC treatment (Figure 17A, C). The downregulation of BRCA1 is more pronounced after 6 hours of treatment and BRCA1 downregulation coincides with the loss of BRCA1 foci observed by immunofluorescence (Figure 17 A, C). We observed that a low dose of UV ($10J/m^2$) or MMS (50μM) is sufficient for triggering BRCA1 downregulation. Moreover, BRCA1 is downregulated by MMS in a dose dependent manner (Figure 17 D and E). As a reference, $10J/m^2$ of UV generates approximately the same amount of pyrimidine dimers as 1 hour exposure to sunlight [260,261]. The experiment was also repeated with additional BRCA1 antibodies recognizing a different region to exclude the possibility of epitope masking due to posttranslational modification and BRCA1 downregulation was also observed (Figure 17 F and G). In contrast, BRCA1 is stabilized upon IR treatment (Figure 17B). In order to determine whether BRCA1 downregulation is cell-type specific, different cancer cell lines or primary human fibroblasts were treated with MMS and we found that BRCA1 is downregulated independently of cell types. Thus, BRCA1 downregulation is not the consequence of cellular transformation characteristic of cancer cell lines (Figure 17 H). It is interesting to note that a band shift for BRCA1 has been observed following exposure to all genotoxic agents used. This shift in migration suggests that BRCA1 is modified post-translationally; most likely by phosphorylation as it has been reported that BRCA1 is rapidly phosphorylated by ATM or ATR in presence of damage to promote cell cycle checkpoints[104,166]. However, we note that in the case of UV and MMS, BRCA1 is downregulated irrespective of its phosphorylation.

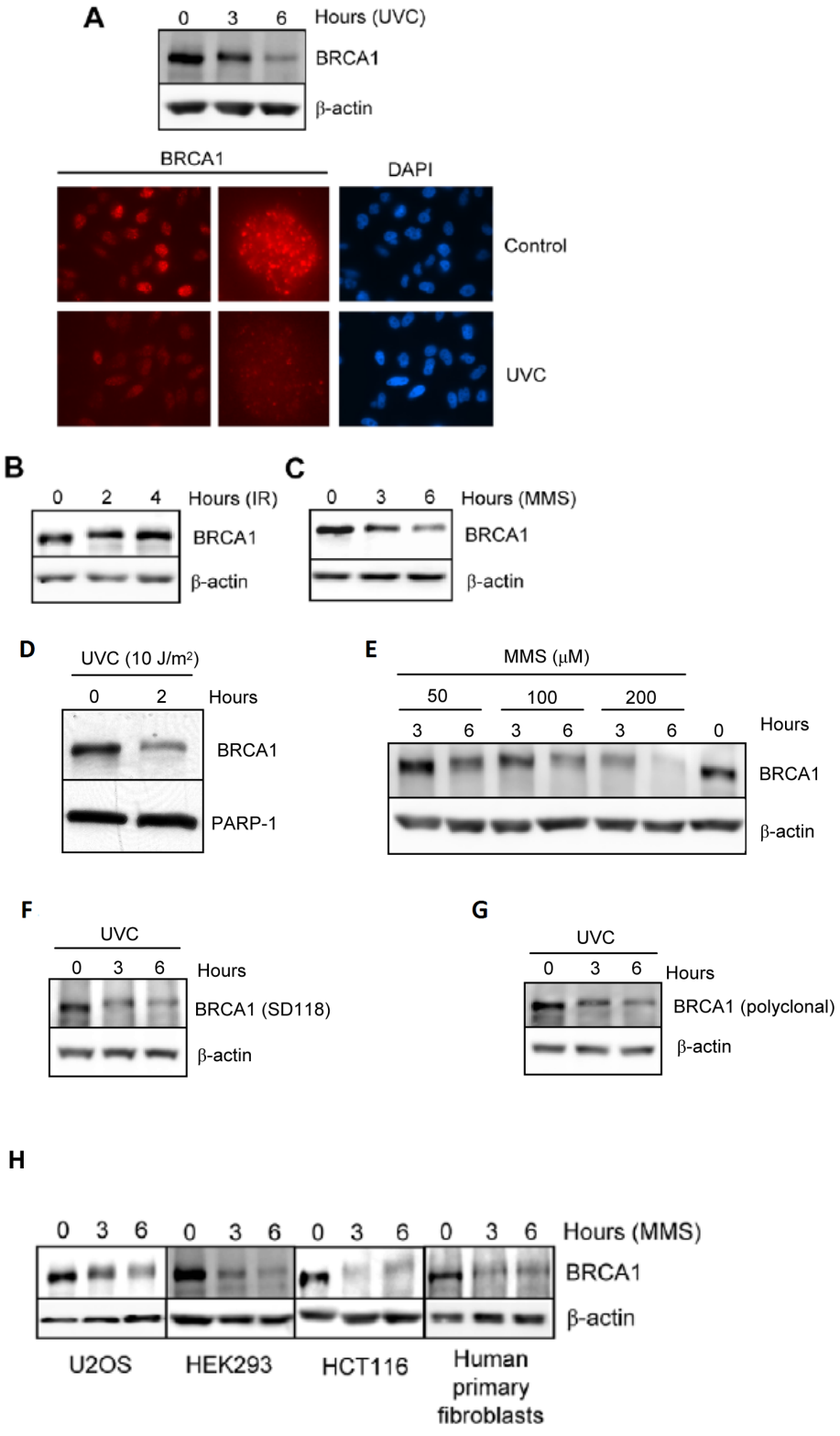

A
0 3 6 Hours (UVC)
BRCA1
β-actin
BRCA1
DAPI
Control
UVC
B
0 2 4 Hours (IR)
BRCA1
β-actin
C
0 3 6 Hours (MMS)
BRCA1
β-actin
D
UVC (10 J/m²)
0 2 Hours
BRCA1
PARP-1
E
MMS (μM)
50 100 200
3 6 3 6 3 6 0 Hours
BRCA1
β-actin
F
UVC
0 3 6 Hours
BRCA1 (SD118)
β-actin
G
UVC
0 3 6 Hours
BRCA1 (polyclonal)
β-actin
H
0 3 6 0 3 6 0 3 6 0 3 6 Hours (MMS)
BRCA1
β-actin
U2OS
HEK293
HCT116
Human primary fibroblasts

Figure 17: BRCA1 is downregulated with MMS and UVC treatment but not IR

A) Top: HeLa cells were treated with UVC at 30J/m^2 and collected at the indicated time for immunoblot against BRCA1 or β-actin as a loading control.

Bottom: HeLa cells treated with UV and fixed after 4 H post-treatment for immunofluorescence against BRCA1. A representative enlarged cell was shown for details. The nuclei were stained with DAPI.

B) Detection of BRCA1 upon IR treatment. HeLa cells were treated with IR at 10 Gy and then collected for immunoblot at the indicated time

C) Detection of BRCA1 upon MMS treatment. HeLa cells were treated with MMS at 200 μM and then collected for immunoblot at the indicated time

D) HeLa cell were treated with a low dose of UVC (10 J/m^2) and harvested 2 H post-treatment for immunodetection with BRCA1. Detection of PARP-1 was used as a loading control.

E) HeLa cells were treated with different doses of MMS and collected at the indicated time for immunodetection with BRCA1. Detection of β-actin was used as a loading control.

F) G) HeLa cell were treated with UVC (30 J/m^2) and harvested at the indicated time for immunodetection with F) anti-BRCA1 (SD118) [262] recognizing the C-terminal region of BRCA1 or G) anti-BRCA1 polyclonal [263] recognizing the middle region of BRCA1. β-actin was used as a loading control.

H) Immunoblot detection of BRCA1 level upon 200 μM MMS treatment in different cells at the indicated time.

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027 [264]

II. BRCA1 downregulation in not caused by transcriptional silencing

Next, we wanted to determine whether BRCA1 downregulation is caused by transcriptional inhibition. Cycloheximide, the protein biosynthesis inhibitor that blocks the elongation step of translation, was used in combination with or without MMS treatment (Figure 18). With cycloheximide alone, BRCA1 seems to have a half-life of approximately 4 hours, confirming the findings of another group [259]. However, when the cells were treated with MMS alone or with MMS and cycloheximide, a strong downregulation of BRCA1 was observed already after 2 hours. This suggests that an active mechanism of degradation is activated and targets BRCA1 for downregulation following MMS treatment. As a positive control for the cycloheximide treatment, the same extracts were blotted for CDC6, a protein with a short half-life, and showed that cdc6 level decreases in a time dependent manner in cycloheximide treated cells whereas it remains essentially constant in MMS only treated cells (Figure 18).

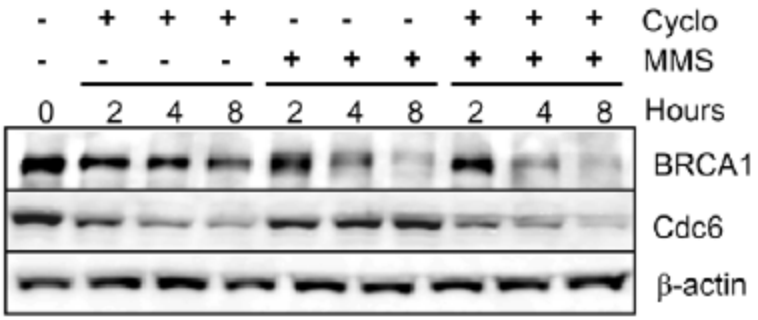


Figure 18: BRCA1 downregulation is not caused by transcription inhibition.

Detection of BRCA1 and CDC6 upon cycloheximide (20 μg/mL) and/or MMS (200 μM) treatment. HeLa cells were treated for the indicated time and then harvested for immunoblotting.

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

III. Downregulation of BRCA1 is reversible and is not a consequence of apoptosis

Having shown that BRCA1 downregulation is independent of cell type or transcription, we hypothesized that the DNA damage treatment might induce cell death by apoptosis, thus resulting in BRCA1 downregulation. In order to investigate this possibility, we treated HeLa cells with MMS and collected them at the indicated time point for either immunofluorescence or immunoblotting. The cells did not show any sign of apoptosis as the nuclear staining by DAPI did not show nuclear condensation (Figure 19A); BRCA1 downregulation by immunostaining coincided with BRCA1 depletion observed by western blot (Figure 19B left). At later time points, BRCA1 downregulation seemed to be reversible as BRCA1 foci reappeared and BRCA1 protein levels increased. Using a band intensity quantification program FUJI multi gauge imager, BRCA1 level was quantified, showing BRCA1 downregulation at early MMS treatment time points and that it is reversible at later MMS time points. BRCA1 degradation reversibility could be explained by the presence of DNA double strand breaks generated by the replication fork collapse induced by MMS. To ensure that BRCA1 downregulation is not a result of apoptosis, the extract was also subjected to a Caspase-3 and PARP-1 immunoblot. These two proteins are known to be cleaved during apoptosis. Given that neither Caspase-3 nor PARP-1 cleavage was observed, the results indicated that BRCA1 downregulation was not a consequence of apoptosis. To ensure that apoptosis can be detected, HeLa cells were treated with a high dose of UVC. Indeed, cleavage of both Caspase-3 and PARP-1 were observed (Figure 19B right)

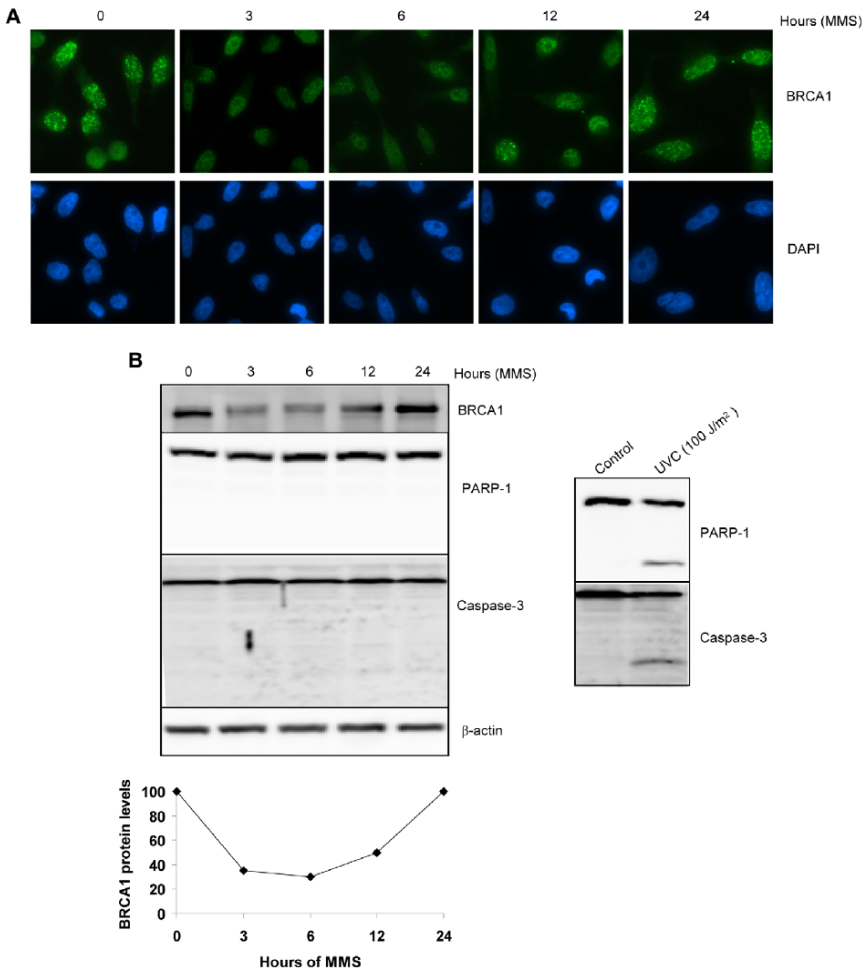


Figure 19: BRCA1 downregulation is reversible

A) Immunofluorescence in HeLa cells for BRCA1 in presence of 200 µM MMS. The cells were fixed at the indicated time following MMS treatment. The nuclei were stained with DAPI.

B) Immunoblot for BRCA1, PARP-1 and Caspase-3 in HeLa cells treated with 200µM MMS. The cells were fixed at the indicated time subsequent to MMS treatment. Quantification of BRCA1 level using FUJI multi gauge imager.

C) Immunoblotting for PARP-1 and Caspase-3 in HeLa cells upon treatment with high dose of UVC (100 J/m^2).

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

IV. BRCA1 downregulation is independent of the cell cycle

BRCA1 regulation is dependent on the cell cycle as it is mainly expressed in the S and G2/M phases[259]. It is thus important to determine if BRCA1 downregulation is specific to a cell cycle phase. HeLa cells were synchronized in G1/S using thymidine double block and human primary fibroblasts were synchronized in G0/G1 by contact inhibition. The cells were released and then treated with either MMS or UVC in order to monitor BRCA1 levels throughout the cell cycle. Cell cycle analysis and immunoblot suggest that BRCA1 downregulation is not dependent on cell cycle as BRCA1 downregulation occurs regardless of cell cycle phases (Figure 20 A and B).

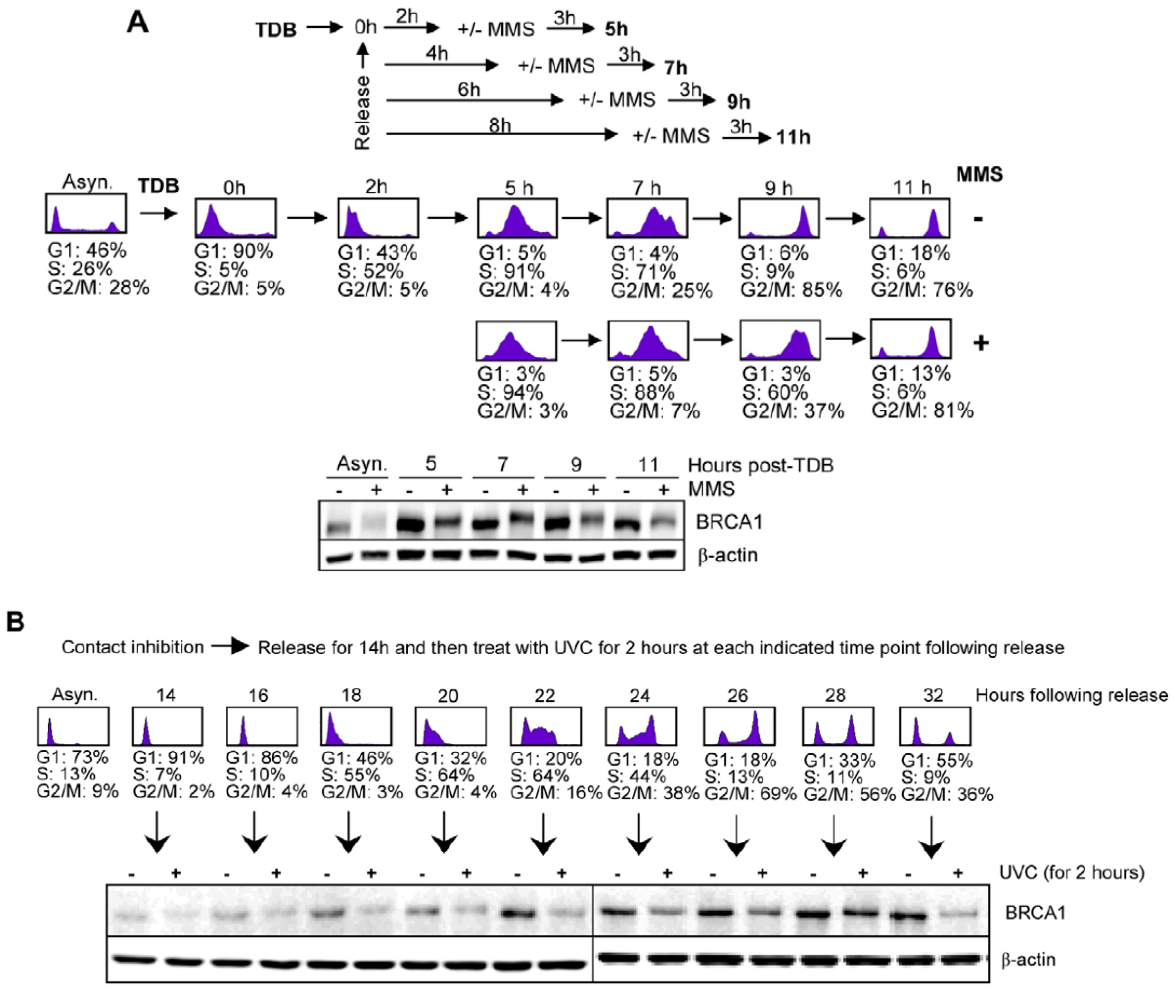


Figure 20: DNA damage-induced BRCA1 downregulation during cell cycle progression.

A) Synchronization of HeLa cells with thymidine double block (TDB). After the release of the block, the cells were treated with 200 μM MMS for 3 hours and harvested for cell cycle analysis or immunoblot.

B) Synchronization of human primary fibroblasts in the G0/G1 phase by contact inhibition. The inhibition is released by replating the cells at low density for 14 hours prior to UV treatment. Cells were then harvested at the indicted times for cell cycle anaylsis or immunoblot.

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

V. BRCA1 downregulation is dependent on proteasomal degradation

Since protein levels are often regulated through proteasomal degradation, we hypothesized that BRCA1 might be degraded by the 26S proteasome upon MMS treatment. In order to test this hypothesis, the proteasome inhibitor MG132 was used. If BRCA1 downregulation is dependent on proteasomal degradation, MG132 should rescue it. Indeed, MG132 treatment prevents BRCA1 downregulation in the presence of MMS treatment. A similar effect is observed with the major partner of BRCA1, BARD1 (Figure 21 A). To ensure that this observation is not a non-specific effect of MG132, the experiment was repeated with another proteasome inhibitor ZL3VS after UV treatment and the same conclusion was drawn (Figure 26A). To confirm that BRCA1 downregulation is dependent on proteasomal degradation, we sought to determine whether BRCA1 is ubiquitinated in the presence of MMS as ubiquitination is the key event for triggering protein degradation by the proteasome. We conducted an immunoprecipitation using an antibody against BRCA1 or a non-related rabbit IgG as control in HEK293T cells treated with MMS or not. The immunoblot against BRCA1 showed that BRCA1 was immunoprecipitated. We next want to know if BRCA1 is ubiquitinated by using an antibody against ubiquitin. As expected, BRCA1 is highly ubiquitinated upon MMS treatment. Band quantification indicates that BRCA1 is approximately 3 times more ubiquitinated compared to non-treated cells (Figure 21B). It is important to note that this experiment was also done in HeLa cells and BRCA1 is also ubiquitinated after MMS treatment (Figure 26 B).

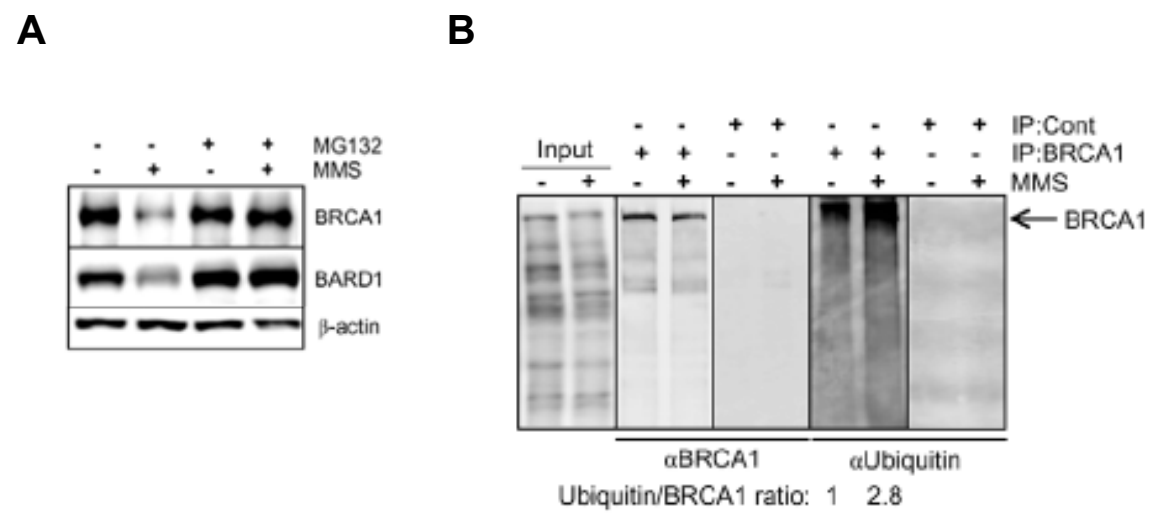


Figure 21: BRCA1 downregulation is mediated by proteasomal degradation

A) HeLa cells were treated with or without 20μM of MG132 for half an hour followed by no treatment or 200 μM MMS treatment for 6 hours prior harvesting for immunoblotting. B) Immunoprecipitation using HEK293T cells with an antibody against BRCA1 or a non-related polyclonal rabbit IgG antibody as a control. Prior to immunoprecipitation, the cells were treated or not with 200 μM of MMS for 3 hours. The immunoprecipitated BRCA1 was then used for immunoblot with anti-BRCA1 or anti-ubiquitin. The ubiquitination of BRCA1 was quantified using the FUJI multi gauge imager.

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

VI. The BRCT domain of BRCA1 is required for its downregulation

Upon the observation of BRCA1 downregulation, we wanted to define the domains of BRCA1 crucial for its downregulation through mapping. To do so, we used the constructs of BRCA1 containing different deletions (Figure 22A) provided by Dr. Chiba[6]. The BRCA1 expression plasmids were transfected in HeLa and were detected with an antibody against GFP as the constructs were fused with GFP cDNA. It was observed that both the Δmid2 (Δ775-1292) and ΔBRCT (Δ1592-1863) mutants are resistant to degradation. Furthermore, these mutants seem to be stabilized upon MMS treatment. The mid2 region encompasses the region for PALB2 interaction and the phosphorylation sites by ATM/ATR whereas the BRCT domain is crucial for forming BRCA1 complexes [104]. This suggested that either Rad51 recruitment through PALB2, posttranslational modifications, or the formation of BRCA1 complexes are necessary for BRCA1 downregulation. In contrast, the RING (Δ1-302) and the mid1 (Δ305-770) deletion mutants are still downregulated following MMS treatment. The RING domain confers ubiquitin ligase activity of BRCA1 and allows its interaction with BARD1[18]. This suggested that neither BRCA1 ubiquitin ligase activity nor its interaction with BARD1 is crucial for BRCA1 downregulation (Figure 22B).

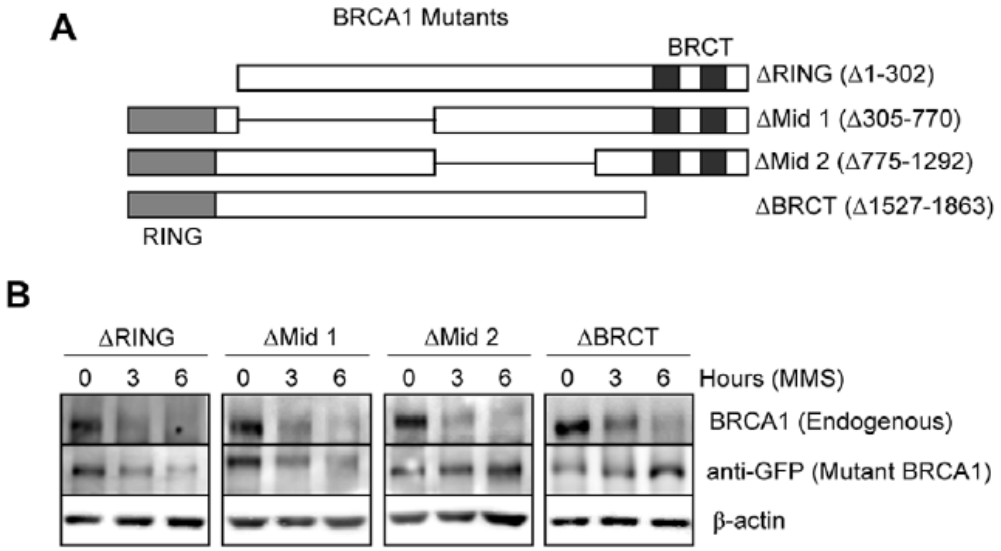


Figure 22: The BRCT domain of BRCA1 is crucial for BRCA1 downregulation

A) Schema showing the GFP-BRCA1 deletion constructs provided by Dr.Chiba [254]

B) Immunoblot of endogenous BRCA1 or GFP-tagged deletion constructs in HeLa cells transfected with the constructs in A. Two days post-transfection, the cells were then treated with 200 μM MMS for the indicated time prior to harvesting.

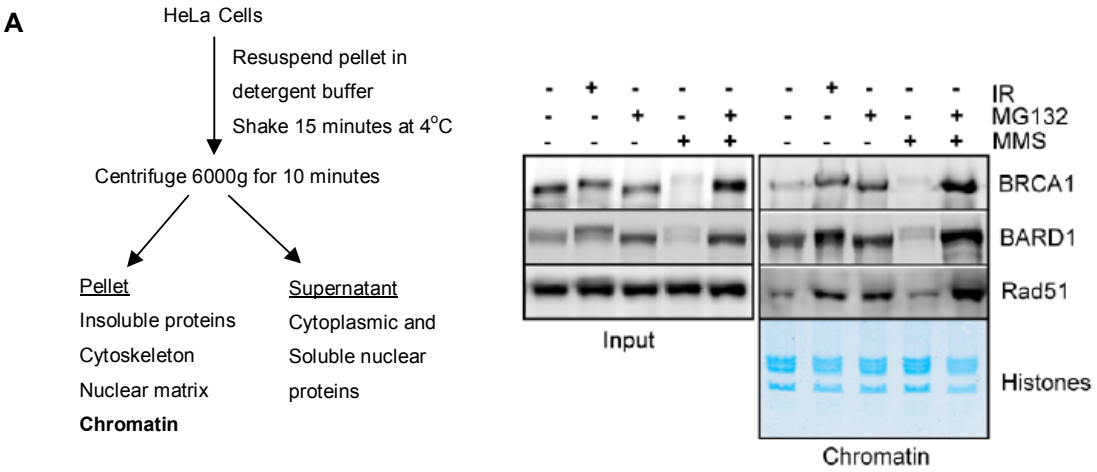
Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

VII. BRCA1 downregulation is an early step to homologous recombination inhibition

We subsequently sought to investigate the significance of BRCA1 downregulation by studying BRCA1, BARD1 and Rad51 recruitment to damaged chromatin following MMS in the condition where downregulation is inhibited by MG132. The IR treatment is used as a control as it is known to trigger the recruitment of BRCA1, BARD1 and Rad51 to chromatin containing double strand breaks. In order to do so, the chromatin fraction of HeLa cells was prepared in various conditions (Figure 23A). In the total extract, we observed as expected a downregulation of BRCA1 and BARD1 in presence of MMS that is blocked by MG132. In the chromatin fraction, we observed the presence of BRCA1, BARD1 and Rad51 indicating that IR indeed induced as expected the recruitment of these proteins to sites of DSBs. MMS induced the downregulation of BRCA1 and BARD1 and these effects are blocked by MG132. Interestingly, in MG132 and MMS treated cells, we observed BRCA1, BARD1 and Rad51 in the chromatin fraction (Figure 23A). These data suggest that BRCA1 and BARD1 might be downregulated in order to prevent an unwanted recruitment of these protein as well as Rad51 to chromatin. It is typical that BRCA1, BARD1 and Rad51 are recruited to the chromatin in presence of double strand breaks induced by IR. However, in presence of MMS that does not directly induce double strand breaks, their recruitment might interfere with DNA repair. In fact, their recruitment to the chromatin could compete with the activation of other DNA repair mechanisms (i.e. base excision repair). The role of BRCA1 and BARD1 downregulation could thus prevent the recruitment Rad51, as they are upstream of this homologous recombination protein.

Altogether, our data suggest that BRCA1 downregulation is an early step that inhibits homologous recombination. To investigate this possibility further, the recruitment of BRCA1 and homologous recombination proteins to foci were analyzed in the presence of MMS at various times post-treatment. Indeed, during the downregulation of BRCA1, no recruitment of the homologous recombination proteins RPA and Rad51 was observed. As BRCA1 returns at later time points, we observed the appearance of RPA foci followed by Rad51 foci. This is expected as RPA recruitment occurs prior to Rad51 recruitment [169]

(Figure 23B). Downregulation of BRCA1 might be needed as MMS or UVC both induce the formation of γH2A.X foci (Figure 23B) and thus, downregulating BRCA1might be a mechanism that prevents the activation of the homologous recombination machinery when not required. The occurrence of RPA and Rad51 foci at later time points strongly suggests that the homologous recombination pathway is reactivated (Figure 23B); likely as a result of the double strand breaks generated by replication fork collapse.



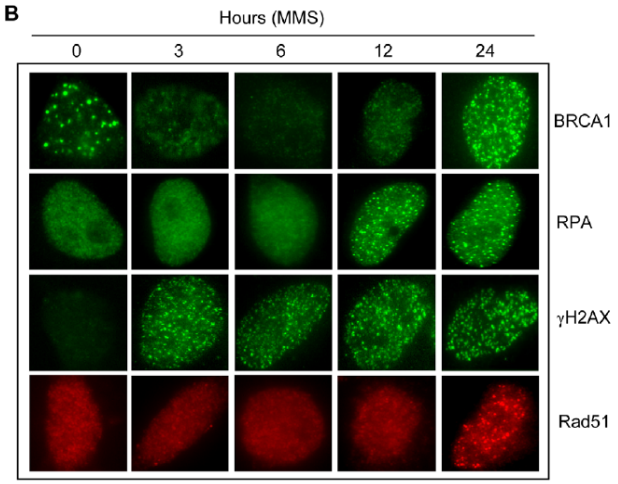


Figure 23: BRCA1 downregulation inhibits the recruitment of homologous repair proteins to the sites of DNA damage.

A) Chromatin from HeLa cells was isolated as in the schema on the left. The histones were stained with coomassie blue as a loading control. Prior to chromatin isolation, the cells were either treated with or without 20 μM of MG132 for 30 minutes prior to IR (10 Gy) or MMS (200 μM) treatment for 6 hours.

B) Immunofluorescence on homologous recombination proteins in HeLa cells treated with 200μM MMS for the indicated time.

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

VIII. BRCA1 regulation in presence of various genotoxic stress inducing agents

In summary, our observations showed that BRCA1 is regulated in a genotoxic stress dependent manner. In presence of DNA double strand break inducers such as IR, BRCA1 and BARD1 are recruited to the chromatin in order to initiate homologous recombination. In presence of UVC or MMS that does not directly induce DNA double strand break, BRCA1 was degraded by the proteasome as an early step to inhibit homologous recombination as the recruitment of RPA and Rad51 to the site of damage was impaired. This downregulation is believed to prevent the activation of conflicting DNA repair pathways as DNA alkylation (induced by MMS) or bulky adducts (induced by UV) trigger base excision repair or nucleotide excision repair respectively. However, longer exposure to these genotoxic agents generates DNA double strand breaks through replication block and thus, homologous recombination proteins are re-recruited to chromatin. Since BRCA1 downregulation is dependent on proteasomal degradation, a deubiquitinase is expected to be required for antagonizing the ligase activity so that BRCA1 dowregulation could be reversible. Taking our data altogether, we propose a model for BRCA1 regulation in presence of different genotoxic stress-inducing agents (Figure 24).

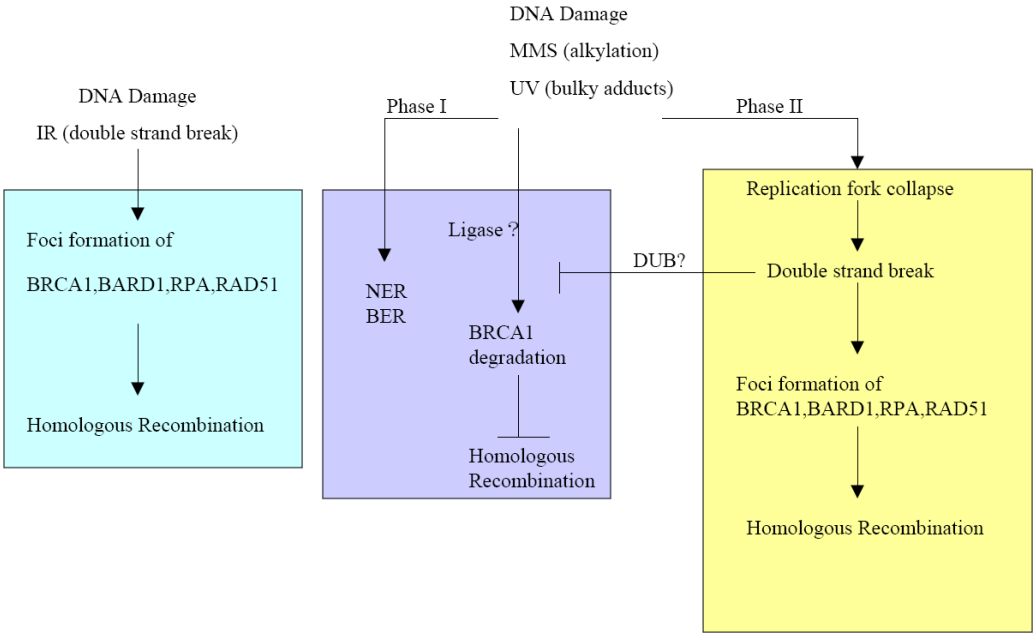


Figure 24: Model describing BRCA1 regulation in the presence of diverse genotoxic stress-inducing agents.

In presence of IR, BRCA1 and BARD1 are recruited to initiate the homologous recombination. In presence of UVC or MMS that does not induce DNA double strand break directly, BRCA1 is degraded by the proteasome by an unknown ubiquitin ligase to inhibit homologous recombination. Longer exposure to these genotoxic agents induce DNA double strand breaks through replication block and thus, homologous recombination proteins are re-recruited. Given that BRCA1 downregulation is dependent on proteasomal degradation, an unknown deubiquitinase is expected to reverse the downregulation.

IX. Identification of the DUBs involved in BRCA1 regulation by a screen using a siRNA library

We demonstrated that BRCA1 is ubiquitinated and targeted for degradation in presence of the genotoxic stress not directly generating DNA double strand breaks such as MMS or UVC; whereas BRCA1 is recruited to the chromatin upon treatment with double strand DNA break inducers. This suggests that there might be different mechanisms for BRCA1 regulation highly dependent on the nature of DNA damage. Taking into account that (i) BRCA1 expression is cell cycle-dependent and (ii) BRCA1 levels are regulated by DNA damage, it was interesting to study the role of ubiquitination in the BRCA1 mechanism under different genotoxic stress conditions. Although we have shown that BRCA1 is degraded by the proteasome upon MMS and UVC treatment, we do not know the identity of the ubiquitin ligase. Unfortunately, the number of existing E3 ubiquitin ligases (more than 600 genes) is too high to be screened without a high throughput screening system. On the other hand, there are approximately 100 DUBs identified up to date and thus, it is more feasible to conduct a screen for DUB enzymes regulating BRCA1 stability and function. Since the recruitment of BRCA1 to the sites of double strand breaks involves ubiquitin ligases and that BRCA1 downregulation after MMS or UVC treatment is reversible, we strongly believed that at least one DUB is responsible for deubiquitinating BRCA1 in order to prevent its degradation by the proteasome or perhaps regulates BRCA1 function in a proteasome-independent manner (Figure 24). RNA interference (RNAi) was used in order to screen deubiquitinases that might be proficient in reversing this downregulation or play a role in the BRCA1 mechanism. RNAi consists of a small double stranded RNA sequence that is complementary to the messenger RNA (mRNA) of a specific gene. The annealing of siRNA with its target gene could either destabilize and degrade the mRNA or prevent its translation by blocking the assembly of the ribosomal subunit 60S [265]. The precursor of these siRNA is synthesized by the RNA polymerase, and forms a double stranded RNA with a hairpin structure which is then processed by Drosha into a short hairpin RNA (shRNA) of approximated 70 nucleotides long[266,267]. The shRNA is then exported to the cytoplasm and processed by Dicer into small fragments of approximately 21 nucleotides long called small interfering RNA

(siRNA) [267]. The double stranded RNA then unwinds into two single stranded RNA: the guide strand and the passenger strand. The guide strand is then recognized by the RNA-induced silencing complex (RISC) and is used as a template to bind the target gene. Argonaut, a nuclease in the RISC complex that is structurally similar to RNase H [267,268], will then degrade the mRNA and thus prevent its translation. The RNAi can be artificially take place by using synthetic siRNA or vectors expressing the shRNA in cell culture. This is a widespread method used to study gene function determining the consequence of knocking down the expression of the target gene. For the screen, we first knocked down the DUBs using a synthetic siRNA library for DUBs from Dharmacon and then treated the cells with MMS for 20H, a time point where BRCA1 downregulation is reversed (Figure 25). The RNAi of each DUB is composed of a pool of 4 siRNA targeting different and non-overlapping regions of the mRNA in order to ensure an efficient knock down. We then investigated the stability and localization of BRCA1 by immunofluorescence. We expected that if the DUB is crucial for reversing BRCA1 downregulation by inhibiting its degradation, knocking it down would inhibit BRCA1 foci formation compared to the non-target control siRNA. We were also interested in determining if some DUBs could actually regulate the mechanism the other way around in which knocking it down would promote BRCA1 foci stability. For example, in the case where ubiquitination is not associated with protein degradation, but in the activation of a particular signaling pathway, deubiquitination might thus have a role in preventing protein recruitment. It was also important to investigate the recruitment of DNA damage sensors or homologous recombination proteins that are part of the BRCA1 complex in presence of either MMS or IR, since the BRCA1 mechanism is still poorly understood (Figure 25).

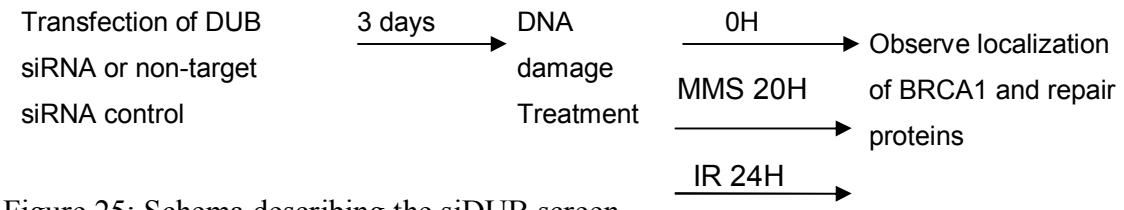


Figure 25: Schema describing the siDUB screen

U2OS cells were transfected with either a DUB siRNA or a non-target siRNA as a control. Three days post-transfection, the cells were treated with the indicated genotoxic stress and harvested at the designated time. BRCA1 and DNA repair proteins localization were then observed by immunofluorescence. Approximately 100 cells were counted and cells with foci of the protein of interest were counted as positives. An example of a hit is shown in annex (Figure 27).

Althought it is still not well understood, it was shown that BRCA1 forms foci in unstressed cells during the S phase [269-272]. These spontaneous foci were suggested to have a role in pericentric heterochromatin replication as they were found to be mainly recruited to centromeres and pericentric heterochromatin[269-272]. We were first interested to investigate whether some DUBs could be responsible for regulating spontaneous BRCA1 foci and thus, a siRNA screen was done in cells without DNA damage treatment. The non-target control siRNA results in 52% of cells with BRCA1 foci, which is within the range of spontaneous foci distribution (40 to 70%) [269-272]. As potential hits, we found BAP1 and UCHL3, which results in fewer cells with BRCA1 foci (Table 3).

BRCA1 spontaneous foci			
RNAi of DUBs with less BRCA1 foci		**RNAi of DUBs with more BRCA1 foci**	
siDUB	**% cells with BRCA1 foci**	**siDUB**	**% cells with BRCA1 foci**
BAP1	24%		
UCHL3	35%		
Non-target control	52%		

Table 3: Potential hits of siDUB screen in untreated U2OS cells for spontaneous BRCA1 foci assembly

The percentage represents the positive cells with foci out of the 100 cells counted. The siDUB that seem to have less or more foci compared to the non-target si control are considered as hits. (See Table 7 in the annex for more details).

Next, we investigated the DUBs that might regulate BRCA1 during DNA damage more specifically to identify potential DUBs responsible for reversing BRCA1 downregulation after MMS treatment. We observed that approximately 70% of the cells have BRCA1 repair foci (as opposed to the spontaneous foci). As potential hits resulting in cells with less BRCA1 foci, we observed BAP1, COPS5, CXORF53, CYLD, DUB3, PSMD14 and USP36. As potential hits resulting in more cells with BRCA1 foci, we found ZA20D1, UCHL1, USP29 and USP3. Of note, a potential hit for the DUB responsible for reversing BRCA1 downregulation could be USP36 found only in the condition of MMS treatment, but not the IR treatment (described below). We also investigated the regulation by DUBs of γH2A.X foci recruitment, as we were interested to know whether the initial DNA damage signal would be regulated by deubiquitination. In addition, γH2A.X staining also served as a positive control for the MMS treatment. In the non-target control siRNA, there was a strong formation of γH2A.X foci in nearly all cells (100%). As potential DUBs whose knock down resulted in less γH2A.X foci, we identified USP2 and USP24 (Table 4)

BRCA1 and γH2A.X staining in response to MMS			
RNAi of DUBs with less BRCA1 foci		**RNAi of DUBs with more BRCA1 foci**	
siDUB	**% cells with BRCA1 foci**	**siDUB**	**% cells with BRCA1 foci**
BAP1	49%	ZA20D1	90%
COPS5	39%	UCHL1	87%
CXORF53	38%	USP29	85%
CYLD	42%	USP3	83%
DUB3	47%	Non-target control	71%
PSMD14	45%		
USP36	46%		
Non-target control	71%		
RNAi of DUBs with less γH2A.X foci		**RNAi of DUBs with more γH2A.X foci**	
siDUB	**% cells with γH2A.X foci**	**siDUB**	**% cells with γH2A.X foci**
USP2	22%		
USP24	71%		
Non-target control	100%		

Table 4: Potential hits of siDUB screen in MMS-treated U2OS cells for BRCA1 and γH2A.X foci assembly

The percentage represents the positive cells with foci out of the 100 cells counted. The siDUB that seem to have less or more foci compared to the non-target si control are considered as hits (see Table 8 and Table 9 in the annex for more details).

Subsequently, we conducted another RNAi screen by treating the cells with IR for 24H. This provided a comparison for the screen in which cells were treated with MMS in order to determine the DUBs specific to DNA damage by MMS and those specific to DNA damage by IR. It is known that BRCA1 and Rad51 are rapidly recruited to the site of damage upon IR. However, how the recruitment of these proteins is reversed after the damage has been repaired is not so well understood. We observed that after 12H of IR treatment, the damage started to be repaired and BRCA1 began to return to basal level in U2OS cells (Figure 28). Here, we conducted a siDUB screen for BRCA1, the homologous repair protein Rad51 and the DNA damage signaling proteins γH2A.X and 53BP1 after 24H of IR treatment in order to determine DUBs that might play a role in reversing the DNA damage signal. In the non-target siRNA control, approximately 63% of the cells still had BRCA1 foci. Potential hits identified for DUBs whose depletion resulted in fewer cell with BRCA1 foci included BAP1, COPS5, CXORF53, DUB3,

PSMD14, SENP2, STAMBPL1, UBL5, UEVLD, USP31 and USP3. A potential hit resulting in more cells with BRCA1 included ZRANB1. The non-target si control for Rad51 foci showed that approximately 51% of cells still had Rad51 foci. Potential hits in which the DUB RNAi resulted in cells with fewer Rad51 foci included BAP1, COPS5, DUB3, OTUB1, OTUD5, OTUD6B, PSMD14, STAMBP, STAMBPL, UBL4 and USP4. Potential candidates resulting in cells with fewer γH2A.X foci included DUB3 and SSBI54; and in cells with fewer 53BP1 foci included DUB3 (Table 5).

BRCA1, γH2A.X, Rad51 and 53BP1 staining in response to IR			
RNAi of DUBs with less BRCA1 foci		**RNAi of DUBs with more BRCA1 foci**	
siDUB	**% cells with BRCA1 foci**	**siDUB**	**% cells with BRCA1 foci**
BAP1	35%	ZRANB1	78%
COPS5	41%	Non-target control	63%
CXORF53	38%		
DUB3	33%		
PSMD14	41%		
SENP2	34%		
STAMBPL1	32%		
UBL5	40%		
UEVLD	42%		
USP31	33%		
USP3	47%		
Non-target control	63%		
RNAi of DUBs with less γH2A.X foci		**RNAi of DUBs with more γH2A.X foci**	
siDUB	**% cells with γH2A.X foci**	**siDUB**	**% cells with γH2A.X foci**
DUB3	39%		
SBBI54	77%		
Non-target control	90%		
RNAi of DUBs with less RAD51 foci		**RNAi of DUBs with more RAD51 foci**	
siDUB	**% cells with RAD51 foci**	**siDUB**	**% cells with RAD51 foci**
BAP1	32%		
COPS5	35%		
DUB3	38%		
OTUB1	33%		
OTUD5	30%		
OTUD6B	38%		
PSMD14	31%		
STAMBP	25%		
STAMBPL	23%		
UBL4	36%		
USP4	33%		
Non-target control	51%		
RNAi of DUBs with less 53BP1 foci		**RNAi of DUBs with more 53BP1 foci**	
siDUB	**% cells with 53BP1 foci**	**siDUB**	**% cells with 53BP1 foci**
DUB3	55%		
Non-target control	81%		

Table 5: Potential hits for siDUB screen in IR-treated U2OS cells for BRCA1, γH2A.X, Rad51 and 53BP1 foci assembly.

The percentage represents the positive cells with foci out of the 100 cells counted. The siDUB that seem to have less or more foci compared to the non-target siRNA control are considered as hits. (See Table 10, Table 11, Table 12 and Table 13 in annex for more details).

D) DISCUSSION

D) DISCUSSION

i. BRCA1 function is regulated by ubiquitination in a stress-dependent manner

BRCA1 and BARD1 are well characterized for their importance in triggering homologous recombination. Following genotoxic stress induced by IR, BRCA1 and BARD1 promote the formation of IR induced foci (IRIF), which are responsible for initiating checkpoint signaling and repair. Here, we showed that although it has been reported that IR induce an early dispersion of the constitutive BRCA1 foci prior to IRIF formation [269], this genotoxic agent does not appear to induce downregulation of BRCA1 as observed for UVC or MMS treatment. BRCA1 downregulation in response to UV and MMS has been observed not only in various cancer cell lines, but also in primary human fibroblasts, thus excluding the possibility of a cell type specific effect. Consistent with previous studies, we observed that BRCA1 is largely expressed in the S and G2/M phases of cell cycle[208]. Following the optimization of cell cycle synchronization, we found that BRCA1 is downregulated by MMS or UV throughout the cell cycle, suggesting that this event is not a result of replication block.

We also showed that BRCA1 downregulation is not a result of apoptosis as the dose of DNA damage used does not induce cell death within the time of analysis and indeed, BRCA1 downregulation was also observed in presence of low doses of UV and MMS. In addition, BRCA1 downregulation is reversible indicating that energy-dependent processes (transcription, translation) are responsible for the recovery of BRCA1 levels, which are only possible for living cells. Previous studies showed that BRCA1 is cleaved by caspase-3 in the presence of a high dose of UV after 3 hours post-treatment[34]. However, in our case, with a low dose of DNA damage, no caspase-3 activation was observed.

We present evidence indicating that BRCA1 is downregulated in a proteasome dependent manner. First, proteasome inhibitors abolish BRCA1 degradation. Second, we observed that BRCA1 is 3 times more ubiquitinated in cells treated with MMS; although

we cannot conclude at this time that the ubiquitination is indeed attached through lysine 48 chains, a signal that target for proteasomal degradation. Thus, it would be interesting to validate our conclusion by conducting immunoprecipitation of BRCA1 and detecting it with an antibody specifically recognizing lysine 48 ubiquitin chains. It would have been interesting to identify the ligase responsible for the degradation of BRCA1 after MMS or UVC treatment. The ligase HERC2 was recently found to be part of the RNF8 and UBC13 complex upon IR treatment. HERC2 was shown to act as a scaffold protein to enhance RNF8 and UBC13 ligase complex formation in order to promote RNF8 ligase activity in ubiquitinating histone H2A [273]. Another study showed that HERC2 can also target unstable BRCA1 that is not interacting with BARD1 for degradation [19]. However, we do not believe that HERC2 is the ligase targeting BRCA1 for degradation in the case of MMS or UVC treatment as HERC2 is recruited in an IR dependent manner [273]. In addition, the study showed that BRCA1 degradation by HERC2 is dependent on the RING domain of BRCA1 as it is the region of interaction between HERC2 and BRCA1[19] whereas we observed that BRCA1 downregulation does not require its RING domain (Figure 22).

It appears unexpected that a tumour suppressor is downregulated in presence of DNA damage. We believe that this downregulation is necessary in order to coordinate and prevent simultaneous activation of independent DNA repair pathways. In fact, we have shown that blocking BRCA1 degradation with MG132 results in the unscheduled recruitment of BRCA1, BARD1 and Rad51 to chromatin. This unwanted recruitment of BRCA1 might interfere with the signaling and repair of DNA damage involving NER or BER. It would be interesting to investigate the outcome of BRCA1 downregulation on the stability and regulation of proteins involved in NER or BER in the presence of UVC or MMS respectively. It is also interesting to determine whether inhibiting BRCA1 downregulation by MG132 could possibly affect the levels of BER and NER proteins. It is important to note that while we have consistent observations that correlate with our model using MG132; it is difficult to make a definitive conclusion as proteasome inhibitors often have pleiotropic effects. Indeed, numerous fundamental cellular processes are regulated by the proteasome and thus their deregulation may possibly lead to indirect effects. It was shown that proteasome inhibitors induce an accumulation of p53 and apoptosis in several several cancer cells[274,275]. For instance, MG132 treatment induces

apoptosis as well as S and G2/M arrests in HeLa cells [276]. Interestingly, it has been reported that proteasome inhibition impairs the recruitment of several proteins involved in DNA damage such as BRCA1, Rad51, and 53BP1 to damaged sites[277]. However, our observations indicate that inhibiting BRCA1 degradation after MMS treatment using MG132 induce the loading of BRCA1 and Rad51 on the chromatin. The identification of the ubiquitin ligase involved in the degradation of BRCA1 will provide a definitive proof on the role of ubiquitination in mediating BRCA1 degradation following DNA damage.

It has been well characterized that ATM-dependent H2A.X phosphorylation signals homologous recombination in presence of IR[179]. Although UV or MMS does not directly induce double strand breaks, the unrepaired damage will eventually result in replication fork collapse and induce double strand breaks, triggering H2A.X phosphorylation in an ATM dependent manner [278-280]. However, studies have also shown that UV and DNA alkylation can induce H2A.X phosphorylation at the sites of damage where no double strand breaks are generated [281] . The role of this phosphorylation event remains unclear and might regulate different DNA damage signaling and repair pathways. Since H2A.X phosphorylation is the initial step in signaling DNA damage upon double strand break, we believe that the significance for BRCA1 downregulation is to inhibit homologous repair by preventing the recruitment of HR proteins at DNA damage sites that are not double strand breaks (e.g. DNA Alkylation or pyrimidine dimers) where γH2A.X is assembled. However, it has also been reported that DNA damage that promote replication blocks such as MMS and UV can be repaired by template swithching during replication[282-284]. In fact, it is possible that H2A.X phosphorylation when no double strand breaks are present is a result of template switching activation in order to recruit Rad51 to stalled replication forks[282,283]. Nonetheless, we believe that base excision repair or nucleotide excision repair are favoured because they can occur throughout the cell cycle whereas template switching is favoured during replication and is triggered if nucleotide excision repair or base excision repair fail to repair the DNA lesion prior entering the S phase.

It would be interesting to confirm our model by conducting different assays to measure the level of DNA repair in the presence of different genotoxic stress conditions According to our model, we would expect that blocking BRCA1 downregulation using specific inhibitors (e.g. E3 ligase) might impair nucleotide excision repair or base excision repair as undegraded BRCA1 would promote HR. For instance, a method to measure nucleotide excision repair is through the detection of cyclobutane pyrimidine dimers (CPD) induced by UVC by flow cytomety [285]. Following UVC treatment, the cells would be collected at different time points and stained with an antibody against CPD and a fluorescent labelled secondary antibody[285]. The amount of CPD remaining proportionally reflects the efficiency of the cells to remove CPD by nucleotide excision repair[285]. Our model predicts that in the absence of BRCA1 downregulation, the NER capacity will be significantly abrogated.

ii. Potential DUBs regulating BRCA1 function

We conducted a DUB RNAi screen in order to define the DUBs that might regulate BRCA1 function and stability. This section consists of a discussion on the potential DUBs identified. We note that for these screens, it would have been useful to co-stain with the respective DUB antibody in order to determine the level of knock down by the RNAi. It is indeed possible that potential hits were missed due to the fact that the knock down was not too successful. We emphasize however that this would have been extremely expensive and moreover, good antibodies are not available for most DUBs. Here, we counted the cells with the assumption that all cells are siRNA-transfected and thus, the effect might have been weakened by the non-transfected cells. Nevertheless, there are a few interesting hits that are worth discussing because they appear to be either recurrent or strong hits in the screens conducted. In contrast, some observations are likely to be false-positive based on the fact that the downstream components in the DNA damage signaling are not affected. For example, if depletion of a DUB affects BRCA1 recruitment without affecting Rad51 recruitment, it might be a false positive as logically, if BRCA1 recruitment is affected; the proteins downstream should also be affected.

In the DUB RNAi screen conducted in untreated cells for spontaneous BRCA1 foci, BAP1 and to a lesser extent UCHL3 appear to be potential hits. There is not much known yet about UCHL3 function in the regulation of the constitutive BRCA1 foci. However, UCHL3 has been observed to also cleave an ubiquitin like protein NEDD8 [286]. Neddylation is known to be an analog process to ubiquitination and also plays important role in regulating different cellular processes including cell cycle by coordinating the activity of cullin-based ligase complexes [287,288]. A possible explanation to less BRCA1 foci could be an indirect effect of UCHL3 depletion on cell cycle progression.

In the DUB RNAi screen for BRCA1 foci in MMS-treated cells, we observed that USP36 seems to be a hit specific for MMS-treated cells. This suggests that USP36 might be required for reversing BRCA1 downregulation by MMS as less BRCA1 foci was observed in USP36 RNAi cells. Moreover, studies have shown that USP36 is overexpressed in ovarian cancers [289] and thus suggest a possible connection between USP36 and BRCA1 regulation. CYLD is another potential hit that seems to be specific to MMS-treated cells. However, it is not likely a DUB involved in reversing BRCA1 degradation as it is a DUB specific for K63 chains [290]. CYLD is a tumour suppressor as CYLD mutation results in cylindromatosi, multiple skin tumours in the scalp [22,290,291]. CYLD is a negative regulator of the NF-κB pathway by deubiquitinating its positive regulators TRAF2 (TNF receptor associated factor 2) and NEMO (a subunit of the IKK complex) which are activated by polyubiquitination on lysine 63 [22,291]. NF-κB is a transcription factor that is responsible for the transcription of more than 150 genes involved in immunological response, cellular survival, and cell cycle progression in presence of the cytokine tumour necrosis factor (TNF) [292-294]. Independently of the NF-κB signaling, CYLD has also been reported to be required for cells to enter mitosis [295]. It has been reported that CYLD can interact with polo-like kinase (PLK) and possibly regulate PLK activity by deubiquitinating PLK or its downstream components [295]. It will be interesting to determine whether CYLD could directly or indirectly regulate BRCA1 through NF-κB signaling or during mitosis regulation. CYLD might act on other proteins in order to impact BRCA1 stability and function. ZA20D1 and USP29 RNAi seem to result in more BRCA1 foci in presence of MMS. Unfortunately, there isn't much known

about these DUBs yet. UCHL1 is another DUB whose depletion results in more BRCA1 foci after MMS treatment. UCHL1 has been mostly known to be expressed in neurons and its gene mutation is associated with neurological disorders such as Parkinson, as it seems to be required for regulating neuronal stability [291,296,297]. However, some observations seem to indicate that UCHL1 could potentially have roles in cancer although it is still not well understood. It was shown that UCHL1 could act as a tumour suppressor as it interacts with p53 and the MDM2 complex to act as a DUB for p53 in order to stabilize the latter by preventing its degradation [298]. It was also reported that silencing of the UCHL1 gene by methylation at its promoter region promote tumourogenesis in nasopharyngeal, colorectal, and ovarian cancers [298,299]. Besides, it was shown that UCHL1 overexpression promotes apoptosis in MCF7 breast cancer cell line [291,300]. We thus cannot exclude the possibility that UCHL1 could regulate BRCA1 function. One potential hit that caught our attention is USP2. It seems that siUSP2 is a very strong hit that diminishes γH2A.X foci after MMS treatment. It is interesting as γH2A.X foci formation is one of the initial steps in DNA damage signaling since it is phosphorylated rapidly upon DNA damage and is not known to be regulated by ubiquitination. This explains the fewer hits of γH2A.X foci obtained following treatment. We did not observe the RNAi of USP2 as a hit in the screen for IR-treated cells and the potential hits observed in the screen for IR-treated cells differ from those obtained in the screen for MMS-treated cells. We thus speculate that γH2A.X is induced differently though an unknown mechanism upon MMS treatment. USP2 has been reported to deubiquitinate MDM2, but not p53 [301]. MDM2 is the negative regulator of p53 as it is the ubiquitin ligase targeting p53 for degradation. If USP2 deubiquitinates MDM2 to prevent its degradation, this would result in p53 downregulation. How p53 accumulation could possibly affect γH2A.X foci formation remains puzzling as p53 activation can have different outcomes. Given the fact that the RNAi of USP2 does not seem to affect BRCA1 recruitment upon MMS treatment, we cannot exclude the possibility that it is a false positive. If γH2A.X foci formation is inhibited, we should expect BRCA1 foci formation also impaired. Another potential hit for γH2A.X is USP24, whose knockdown seems to inhibit γH2A.X foci formation following MMS treatment. Again, this could possibly be a false positive as none of γH2A.X downstream components are affected afterwards. There is not much

known about this DUB in cancer. Population studies have defined USP24 polymorphisms in Parkinson disease [302].

With respect to IR induced DSBs, we obtained some potential hits specific to IR treated cells. We first observed STAMPBPL1 or AMSH-LP whose knockdown impairs BRCA1 and Rad51 recruitment without affecting the upstream components γH2A.X or 53BP1. While there is not much known about STAMBPL1 function, studies have found that its catalytic center is structurally similar to STAMBP or AMSH; suggesting that these DUBs might have overlapping functions [303,304]. Indeed, it seems that STAMBP knock down diminishes Rad51 foci as well after IR treatment. BRCA1 recruitment appears to be somewhat affected as well (63% of cells with BRCA1 foci in the non-target siRNA control against 51% in the STAMBP siRNA knockdown), although the effect on BRCA1 recruitment does not seem to be as obvious and was not included as a potential hit (Table 10). STAMBP is mainly known for its role in endosomal sorting/trafficking and bone formation through the regulation of bone morphology proteins (BMP) [305,306]. Whether STAMBP or STAMBPL1 have a role in cancer remain to be determined. It appears that the RNAi of few members of the OTU class OTUB1, OTUD5 and OTU6B impair the recruitment of Rad51 but not proteins upstream in the pathway. The role of OTUD5 remains to be determined. Studies on lymphocyte B cells suggest that OTU6B expression through cytokine stimulation results in a G1 arrest and OTU6B overexpression results in Cyclin D downregulation. This effect is dependent on OTU6B catalytic activity as the catalytic inactive form had no effect [307]. From this known function of OTUD6B, it is possible that this hit might be an indirect result from cell cycle deregulation. OTUB1 is preferably a K48 DUB and is known to interact with and deubiquitinate estrogen receptor α (ER α) to inhibit transcription mediated by ERα [308]. The catalytic activity of OTUB1 was reported to be necessary for proper interaction with ERα [308]. Ubiquitination and degradation of ERα was shown to be important in ERα turnover as unbound or misfolded ERα is ubiquitinated and degraded [291,308,309]. It is well known that misregulation of ERα is highly linked to breast cancer due to an increase in cell proliferation and that the BRCA1/BARD1 ligase complex was shown to monoubiquitinate ERα to inhibit ERα mediated transcription [310,311]. It is possible that OTUB1 might be linked to BRCA1 and

ERα signaling and deserves more attention. Also, an interesting study showed that OTUB1 interacts with the E2 UBC13 and inhibits RNF168, impairing ubiquitination of H2A [312]. The authors showed that depleting OTUB1 results in an increase in H2A.X ubiquitination [312]. From that observation, we should expect the siRNA of OTUB1 to increase the recruitment of BRCA1 and Rad51 but not the recruitment of γH2A.X as it is upstream in the pathway. Indeed, we observed more BRCA1 foci after MMS treatment, which is consistent with previous studies [312 262]. However, we observed less Rad51 foci and no change in BRCA1 foci formation in IR treated cells. A possible explanation is that the dose of IR used in our study was higher and the cells might have responded differently to this dose. It is possible that the combination of siRNA of OTUB1 and a high dose of IR might have resulted in a major imbalance in H2A.X ubiquitination level and rendered the cells insensitive in activating homologous recombination repair and thus, less Rad51 recruitment was observed. Although that study proposed that there is still more H2A.X ubiquitination in OTUB1 depleted cells at later time points, they mainly focused on shorter time points [312] where the foci are being formed whereas we were observing Rad51 foci at a late time point where the foci are being disassembled, which is believed to be regulated by a different mechanism. Given that OTUB1 was found to be linked with BRCA1 and that we have identified it as a hit, it is worth to study OTUB1 more in depth in order to solve these conflicting observations. The RNAi of UBL4 was another hit resulting in less Rad51 foci. Unfortunately, there isn't much known about its function yet. The RNAi of USP4 was also observed as a hit with less Rad51 foci in presence of IR. Studies showed that USP4 is a DUB for TRAF2 and TRAF6, which are ubiquitinated in order to promote NF-κB transcriptional activity [313]. Another role found for USP4 is that it can interact with ARF-BP1 and deubiquitinate it in order to prevent its downregulation[314]. ARF-BP1 is a ligase targeting p53 for degradation and results in a diminution of p53 levels; suggesting that USP4 could potentially be an oncogene[314]. This finding is hard to grasp as it is difficult to determine the result of NF-κB and p53 accumulation on Rad51 recruitment. BRCA1 is known to interact with p53 in order to regulate its transcriptional activity and could possibly affect homologous recombination by promoting cell cycle arrest, senescence or apoptosis.

A few hits were observed to affect BRCA1 pathway upon IR treatment; however, they could possibly be false positive as the downstream components of the DSB repair pathway are not affected. First, we observed the RNAi of SBBI54, which showed less γH2A.X. SBBI54's function is not well known. UEVLD, UBL5, SENP2 and USP31 knock down seem to diminish BRCA1 recruitment. The function of UEVLD and UBL5 are still unknown. USP31 was observed to be a DUB for TRAF2 and is a positive regulator of NF-κB signaling like CYLD [315]. The RNAi of SENP2 was also observed to affect BRCA1 recruitment. SENP2 was observed to be a SUMO-specific protease that cleaves sumoylated proteins[316]. SUMO is a small protein similar to ubiquitin and is also a key signaling molecule involved in the regulation of many cellular processes [316]. SENP2 was observed to interact with and desumoylate MDM2 and diminish p53 dependent transcriptional activity [316]. ZRANB1 knock down was observed to increase BRCA1 foci. ZRANB1 is a K63 DUB also known as Trabid and is a positive regulator of the WNT signaling [317]. The WNT signaling is known to regulate embryogenesis and cell differentiation; and its deregulation is sometimes associated with cancer[317].

Interestingly, we observed a few recurrent hits that are common to MMS and IR treated cells. First, we observed CXORF53, which is also known has BRCC36. BRCC36 is known to be present in the BRCA1-Abbraxas complex, but its exact role remains not so well understood. Studies suggest that it might have a role in reversing ubiquitination by RNF8[318]. Indeed RNF8 and BRCC36 are recruited and colocalize together probably to establish the appropriate level of H2A ubiquitination required for DNA damage response and repair [318]. Here we observed that knocking down this protein affects the recruitment of BRCA1 upon MMS and IR treatment. Indeed, studies have shown that BRCC36 is required for proper BRCA1 foci formation upon IR treatment as inhibition of BRCC36 expression impairs BRCA1 recruitment without affecting the cell cycle [319,320]. In addition, it was reported that BRCC36 overexpression might promote breast cancer [319,320]. It was intriguing that BRCC36 knockdown affected BRCA1 foci but not Rad51 or 53BP1 foci after 24 H IR. However, what we were observing after 24H of IR was the beginning of foci disassembly, which is likely to be a different mechanism of foci regulation. The recruitment of 53BP1 is still not so well understood, but it is believed to be recruited prior

to Abraxas/BRCA1 complex and possibly explaining why 53BP1 recruitment is not affected. BAP1 appeared to be a hit that is recurrent. BAP1 (BRCA1-associated-protein 1) is a nuclear UCH DUB in which mutations are associated with lung and breast cancers [321]. BAP1 was first found to be interacting with the RING domain of BRCA1 and was proposed to enhance BRCA1 function in cell growth suppression [321,322]. BAP1 was also reported to have cell growth suppression function as it was shown that BAP1 re-expression in BAP1-null lung cancer cell line NCI-H226 suppresses cell growth and induces cell death [322-324]. Given that BRCA1 was shown to also interact with BARD1 through its RING domain, it is believed that BAP1 disrupts BRCA1 and BARD1 interaction [40,322,325,326]. This suggests that BRCA1/BAP1 have a role that is independent of the BRCA1/BARD1 core complex. Although BAP1 was reported to interact with BRCA1, it was shown that BRCA1 does not appear to be a substrate of BAP1 as the latter does not seem to have deubiquitinating activity towards BRCA1[327]. The exact role of BAP1 and BRCA1 interaction remains to be elucidated. It is interesting that in drosophila, Calypso, the ortholog of BAP1, interacts with the polycomb group protein Asx and was observed to have a role in histone H2A deubiquitination resulting in HOX gene repression [323]. Recently, BRCA1 was found to be a histone H2A ligase and play a role in heterochromatin silencing [220]. There might thus be a link between BAP1 and BRCA1 in coordinating H2A ubiquitination and deubiquitination. On the other hand, BAP1 is also known to interact with the transcriptional factor host cell factor 1 (HCF-1) to regulate the transcription of E2F family target genes known to be required G1/S transition in the cell cycle [325]. In fact studies suggest that knocking down BAP1 by RNAi affects the cells cycle by impairing the transition from G1 to S [326,328,329]. BAP1 is thus suggested to be required for proper cell cycle progression and G1-S transition [324]. Thus, an inhibition of G1/S transition would prevent the recruitment of BRCA1 and Rad51 since they are mainly expressed in the S and G2 phase of the cell cycle. We thus cannot exclude that the observed hit from the RNAi of BAP1 is a cell cycle dependent observation. The RNAi of DUB3 was also observed to be a recurrent hit. DUB3 is known to deubiquitinate CDC25A and inhibit its proteasomal degradation [330]. Cyclin dependent kinases are inhibited through phosphorylation by wee1/myt1. CDC25A is the phosphatase responsible for relieving this repression and thus, promotes cell cycle progression. If DUB3 prevents CDC25A degradation, one would

expect that DUB3 RNAi would arrest the cell cycle. Again this hit might be an indirect result of cell cycle arrest. However, the RNAi of DUB3 appeared to have an effect only on DNA damage treated cells and did not affect the constitutive BRCA1 foci. This suggests that the hit obtained with DUB3 might be direct rather than a consequence of cell cycle deregulation. Interestingly, the RNAi of DUB3 seems to have an effect on IR induced γH2A.X foci but not on MMS induced γH2A.X foci. This suggests that γH2A.X foci recruitment mechanism might differ depending on the type of stress. This requires more in depth studies. USP3 RNAi seems to affect BRCA1 recruitment in presence of MMS and IR. However, it is ambiguous since it seems to have opposite effects between the two treatments. USP3 has been observed to be a H2A, H2B and γH2A.X deubiquitinase [331]. This suggests its potential role in reversing BRCA1 recruitment and thus, USP3 knock down would logically result in an increase of BRCA1 foci formation. This was observed in the screen in which the cells were treated with MMS. In contrast, the RNAi of USP3 results in less BRCA1 foci after IR treatment. A possible explanation is that the knock down of USP3 was also shown to induce DNA damage and induce cell cycle checkpoints through ATM-ATR, thus delaying S-phase progression [331]. It is possible that the IR treatment along with USP3 RNAi induces a high amount of DNA damage, leading to a major G1/S checkpoint, explaining the low amount of BRCA1 foci. In the above-cited study, a low dose of IR (1Gy) was used [331] compared to our study and this might explain the discrepancies. In any case, USP3 seems to be a very interesting hit that deserves more investigation in order to address its mechanism of action. COPS5 and PSMD14 seem to be recurrent hits. COPS5 is one of the subunit of the COP9 signalosome, which is known to interact with SCF and cullin ligases and promote their activity [332,333], while PSMD14 is a component of the 26S proteasome [334]. Given that ubiquitination regulates a myriad of cellular processes, we believe that knockdown of these DUBs might induce general effects on the ubiquitin system that in turn impact DNA damage and repair pathways

To summarize, even though many potential DUBs that might regulate BRCA1 were identified, we believe that BAP1, CXORF53, OTUB1, USP36 and DUB3 are the most likely regulators of BRCA1 function. BAP1 was found to interact with BRCA1 and BARD1 although the functional link between these proteins is still not known[321,322] [40,322,325,326]. The data of the screen suggest that BAP1 affects the formation of BRCA1 and Rad51 foci. It was previously found that BAP1 forms a major transcriptional complex and thus BAP1 may perhaps have a role regulating the transcription of BRCA1 or Rad51 genes[335]. It would be of importance to elucidate the functional link between BAP1 and BRCA1. CXORF53 and OTUB1 were both reported to be part of the BRCA1 pathway althought their exact functions are still not well characterized. CXORF53 was suggested to antagonize RNF8 [318] and OTUB1 was shown to be an inhibitor of RNF168 [312]. It would be interesting to investigate on their precise role in the BRCA1 mechanism.
The RNAi of USP36 appears to only impair BRCA1 foci formation in the presence of MMS treatment. USP36 might be the DUB that is responsible for reversing the downregulation of BRCA1 that we initially sought to indentify throught the RNAi screen. It would of interest to confirm this observation by conducting additional experiments. For example, determining whether BRCA1 interacts with USP36 and whether BRCA1 ubiquitination level is affected by the RNAi or overexpression of USP36. Finally, DUB3 has been a very recurrent hit that affects the formation of BRCA1 foci after IR and MMS treatment. In addition, the RNAi of DUB3 does not seem to diminish the formation of constituve BRCA1 foci, suggesting that these effects are not a consequence of cell cycle changes (spontaneous foci of BRCA1 are present in S phase only). In addition, the recruitment of BRCA1 downstream component, Rad51, is also observed to be decreased after IR treatment; again suggesting that DUB3 is a strong candidate for further investigations.

E) CONCLUSION

E) CONCLUSION

To conclude, it appears that BRCA1 is regulated by different signaling mechanisms depending on the genotoxic stress involved. The regulation of BRCA1 stability promotes its function or its degradation to prevent the simultaneous activation of conflicting DNA repair pathways. Thus BRCA1 is highly regulated by ubiquitin signaling and it would be of great importance to investigate this aspect more in depth; as understanding the regulation of BRCA1 under different stress conditions could be critical for breast cancer diagnostic, prognostic and therapy.

Although the DUB siRNA screen provided potential candidates, studies are needed to confirm and validate these screens. Moreover overexpression of DUBs can be conducted to determine whether the opposite effect can be observed. In addition, we cannot exclude the possibility that the observations might be the result of non-direct effects as DUB depletion can affect other cellular functions (cell cycle, different signaling pathways, gene transcription, cell death and survival etc.). Indeed very little or nothing is known about the majority of DUBs. It would be important to determine the cell cycle profile following depletion of the potential DUB candidates to ensure that cell cycle progression is not affected. If the effect of the DUB is confirmed as direct, then it becomes appealing to characterize further its function in the BRCA1 pathway. For example, determining whether its catalytic activity is required by generating and expressing a catalytic inactive mutant. In addition, we can also generate variants of the DUBs with deletions of different domains and characterize the role of each domain in the BRCA1 pathway. Preliminary data suggest that USP36 might be the DUB responsible for reversing BRCA1 downregulation upon MMS. It would be interesting to observe that USP36 is capable of deubiquitinating K48 chains, responsible for proteasomal degradation. If USP36 is indeed a DUB for BRCA1, one could investigate the functional significance of this interaction and determine whether defect in USP36 interaction with BRCA1 can contribute to breast and ovarian cancer development.

Another future perspective would be to conduct a screen to identify the ubiquitin ligases important for regulating the BRCA1 pathway in a proteasomal dependent and independent manner. However, in order to do this, a high throughput screen would be needed due to the large amount of ubiquitin ligases encoded by the human genome.

F) REFERENCES

F) REFERENCES

1. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu Rev Biochem* **67**, 425-79 (1998).
2. Rossi, S. & Loda, M. The role of the ubiquitination-proteasome pathway in breast cancer: use of mouse models for analyzing ubiquitination processes. *Breast Cancer Res* **5**, 16-22 (2003).
3. Lipkowitz, S. The role of the ubiquitination-proteasome pathway in breast cancer: ubiquitin mediated degradation of growth factor receptors in the pathogenesis and treatment of cancer. *Breast Cancer Res* **5**, 8-15 (2003).
4. Reyes-Turcu, F.E., Ventii, K.H. & Wilkinson, K.D. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu Rev Biochem* **78**, 363-97 (2009).
5. Woelk, T., Sigismund, S., Penengo, L. & Polo, S. The ubiquitination code: a signalling problem. *Cell Div* **2**, 11 (2007).
6. Nakayama, K.I. & Nakayama, K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* **6**, 369-81 (2006).
7. Lipkowitz, S. & Weissman, A.M. RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. *Nat Rev Cancer* **11**, 629-43 (2011).
8. Borden, K.L. & Freemont, P.S. The RING finger domain: a recent example of a sequence-structure family. *Curr Opin Struct Biol* **6**, 395-401 (1996).
9. Freemont, P.S. The RING finger. A novel protein sequence motif related to the zinc finger. *Ann N Y Acad Sci* **684**, 174-92 (1993).
10. Fasanaro, P., Capogrossi, M.C. & Martelli, F. Regulation of the endothelial cell cycle by the ubiquitin-proteasome system. *Cardiovasc Res* **85**, 272-80 (2010).
11. Huibregtse, J.M., Scheffner, M., Beaudenon, S. & Howley, P.M. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A* **92**, 2563-7 (1995).
12. Bernassola, F., Karin, M., Ciechanover, A. & Melino, G. The HECT family of E3 ubiquitin ligases: multiple players in cancer development. *Cancer Cell* **14**, 10-21 (2008).
13. Werness, B.A., Levine, A.J. & Howley, P.M. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**, 76-9 (1990).
14. Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J. & Howley, P.M. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**, 1129-36 (1990).
15. Howley, P.M., Scheffner, M., Huibregtse, J. & Munger, K. Oncoproteins encoded by the cancer-associated human papillomaviruses target the products of the retinoblastoma and p53 tumor suppressor genes. *Cold Spring Harb Symp Quant Biol* **56**, 149-55 (1991).
16. Scheffner, M. *et al.* Functions of human papillomavirus proteins. *Curr Top Microbiol Immunol* **186**, 83-99 (1994).

17. Verdecia, M.A. *et al.* Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. *Mol Cell* **11**, 249-59 (2003).
18. Huang, L. *et al.* Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* **286**, 1321-6 (1999).
19. Wu, W. *et al.* HERC2 is an E3 ligase that targets BRCA1 for degradation. *Cancer Res* **70**, 6384-92 (2010).
20. Ikeda, F. & Dikic, I. Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep* **9**, 536-42 (2008).
21. Nijman, S.M. *et al.* A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123**, 773-86 (2005).
22. Komander, D., Clague, M.J. & Urbe, S. Breaking the chains: structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol* **10**, 550-63 (2009).
23. Reyes-Turcu, F.E., Shanks, J.R., Komander, D. & Wilkinson, K.D. Recognition of polyubiquitin isoforms by the multiple ubiquitin binding modules of isopeptidase T. *J Biol Chem* **283**, 19581-92 (2008).
24. Hu, M. *et al.* Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. *EMBO J* **24**, 3747-56 (2005).
25. Johnston, S.C., Larsen, C.N., Cook, W.J., Wilkinson, K.D. & Hill, C.P. Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 A resolution. *EMBO J* **16**, 3787-96 (1997).
26. Komander, D. *et al.* Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO Rep* **10**, 466-73 (2009).
27. Johnston, S.C., Riddle, S.M., Cohen, R.E. & Hill, C.P. Structural basis for the specificity of ubiquitin C-terminal hydrolases. *EMBO J* **18**, 3877-87 (1999).
28. Komander, D. & Barford, D. Structure of the A20 OTU domain and mechanistic insights into deubiquitination. *Biochem J* **409**, 77-85 (2008).
29. Edelmann, M.J. *et al.* Structural basis and specificity of human otubain 1-mediated deubiquitination. *Biochem J* **418**, 379-90 (2009).
30. Lin, S.C. *et al.* Molecular basis for the unique deubiquitinating activity of the NF-kappaB inhibitor A20. *J Mol Biol* **376**, 526-40 (2008).
31. Nanao, M.H. *et al.* Crystal structure of human otubain 2. *EMBO Rep* **5**, 783-8 (2004).
32. Nicastro, G. *et al.* The solution structure of the Josephin domain of ataxin-3: structural determinants for molecular recognition. *Proc Natl Acad Sci U S A* **102**, 10493-8 (2005).
33. Mao, Y. *et al.* Deubiquitinating function of ataxin-3: insights from the solution structure of the Josephin domain. *Proc Natl Acad Sci U S A* **102**, 12700-5 (2005).
34. Nicastro, G. *et al.* Understanding the role of the Josephin domain in the PolyUb binding and cleavage properties of ataxin-3. *PLoS One* **5**, e12430 (2010).
35. Ambroggio, X.I., Rees, D.C. & Deshaies, R.J. JAMM: a metalloprotease-like zinc site in the proteasome and signalosome. *PLoS Biol* **2**, E2 (2004).
36. Vilenchik, M.M. & Knudson, A.G., Jr. Inverse radiation dose-rate effects on somatic and germ-line mutations and DNA damage rates. *Proc Natl Acad Sci U S A* **97**, 5381-6 (2000).

37. Antoniou, A. *et al.* Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *Am J Hum Genet* **72**, 1117-30 (2003).
38. Jeng, Y.M. *et al.* Brca1 heterozygous mice have shortened life span and are prone to ovarian tumorigenesis with haploinsufficiency upon ionizing irradiation. *Oncogene* **26**, 6160-6 (2007).
39. van Attikum, H. & Gasser, S.M. Crosstalk between histone modifications during the DNA damage response. *Trends Cell Biol* **19**, 207-17 (2009).
40. O'Donovan, P.J. & Livingston, D.M. BRCA1 and BRCA2: breast/ovarian cancer susceptibility gene products and participants in DNA double-strand break repair. *Carcinogenesis* **31**, 961-7 (2010).
41. Mullan, P.B., Quinn, J.E. & Harkin, D.P. The role of BRCA1 in transcriptional regulation and cell cycle control. *Oncogene* **25**, 5854-63 (2006).
42. Amati, B., Alevizopoulos, K. & Vlach, J. Myc and the cell cycle. *Front Biosci* **3**, d250-68 (1998).
43. Cole, M.D. Activation of the c-myc oncogene. *Basic Life Sci* **38**, 399-406 (1986).
44. Dang, C.V. *et al.* Function of the c-Myc oncogenic transcription factor. *Exp Cell Res* **253**, 63-77 (1999).
45. Mirkin, E.V. & Mirkin, S.M. Replication fork stalling at natural impediments. *Microbiol Mol Biol Rev* **71**, 13-35 (2007).
46. Saleh-Gohari, N. *et al.* Spontaneous homologous recombination is induced by collapsed replication forks that are caused by endogenous DNA single-strand breaks. *Mol Cell Biol* **25**, 7158-69 (2005).
47. Helleday, T., Lo, J., van Gent, D.C. & Engelward, B.P. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst)* **6**, 923-35 (2007).
48. Muller, F.L., Liu, Y. & Van Remmen, H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem* **279**, 49064-73 (2004).
49. Han, D., Williams, E. & Cadenas, E. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. *Biochem J* **353**, 411-6 (2001).
50. Kim, J.S. *et al.* Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells. *J Cell Biol* **170**, 341-7 (2005).
51. Kanvah, S. *et al.* Oxidation of DNA: damage to nucleobases. *Acc Chem Res* **43**, 280-7 (2010).
52. Maynard, S., Schurman, S.H., Harboe, C., de Souza-Pinto, N.C. & Bohr, V.A. Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis* **30**, 2-10 (2009).
53. Beranek, D.T. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat Res* **231**, 11-30 (1990).
54. Lundin, C. *et al.* Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. *Nucleic Acids Res* **33**, 3799-811 (2005).
55. Aquilina, G. & Bignami, M. Mismatch repair in correction of replication errors and processing of DNA damage. *J Cell Physiol* **187**, 145-54 (2001).

56. Nakajima, S. *et al.* UV light-induced DNA damage and tolerance for the survival of nucleotide excision repair-deficient human cells. *J Biol Chem* **279**, 46674-7 (2004).
57. Sale, J.E., Lehmann, A.R. & Woodgate, R. Y-family DNA polymerases and their role in tolerance of cellular DNA damage. *Nat Rev Mol Cell Biol* **13**, 141-52 (2012).
58. Chandani, S. & Loechler, E.L. Molecular modeling benzo[a]pyrene N2-dG adducts in the two overlapping active sites of the Y-family DNA polymerase Dpo4. *J Mol Graph Model* **25**, 658-70 (2007).
59. Volk, D.E. *et al.* Solution structure of a cis-opened (10R)-N6-deoxyadenosine adduct of (9S,10R)-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene in a DNA duplex. *Biochemistry* **42**, 1410-20 (2003).
60. Preston, B.D., Albertson, T.M. & Herr, A.J. DNA replication fidelity and cancer. *Semin Cancer Biol* **20**, 281-93 (2010).
61. Pursell, Z.F., Isoz, I., Lundstrom, E.B., Johansson, E. & Kunkel, T.A. Yeast DNA polymerase epsilon participates in leading-strand DNA replication. *Science* **317**, 127-30 (2007).
62. Nick McElhinny, S.A., Gordenin, D.A., Stith, C.M., Burgers, P.M. & Kunkel, T.A. Division of labor at the eukaryotic replication fork. *Mol Cell* **30**, 137-44 (2008).
63. McCulloch, S.D. & Kunkel, T.A. The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell Res* **18**, 148-61 (2008).
64. Kunkel, T.A. Evolving views of DNA replication (in)fidelity. *Cold Spring Harb Symp Quant Biol* **74**, 91-101 (2009).
65. Drake, J.W., Charlesworth, B., Charlesworth, D. & Crow, J.F. Rates of spontaneous mutation. *Genetics* **148**, 1667-86 (1998).
66. Martin, S.A., Lord, C.J. & Ashworth, A. Therapeutic targeting of the DNA mismatch repair pathway. *Clin Cancer Res* **16**, 5107-13 (2010).
67. Drotschmann, K., Yang, W., Brownewell, F.E., Kool, E.T. & Kunkel, T.A. Asymmetric recognition of DNA local distortion. Structure-based functional studies of eukaryotic Msh2-Msh6. *J Biol Chem* **276**, 46225-9 (2001).
68. Martin, S.A. *et al.* Methotrexate induces oxidative DNA damage and is selectively lethal to tumour cells with defects in the DNA mismatch repair gene MSH2. *EMBO Mol Med* **1**, 323-37 (2009).
69. Gradia, S. *et al.* hMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on mismatched DNA. *Mol Cell* **3**, 255-61 (1999).
70. Iyer, R.R., Pluciennik, A., Burdett, V. & Modrich, P.L. DNA mismatch repair: functions and mechanisms. *Chem Rev* **106**, 302-23 (2006).
71. Umar, A. *et al.* Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. *Cell* **87**, 65-73 (1996).
72. Modrich, P. & Lahue, R. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu Rev Biochem* **65**, 101-33 (1996).
73. Ban, C. & Yang, W. Structural basis for MutH activation in E.coli mismatch repair and relationship of MutH to restriction endonucleases. *EMBO J* **17**, 1526-34 (1998).
74. Lee, J.Y. *et al.* MutH complexed with hemi- and unmethylated DNAs: coupling base recognition and DNA cleavage. *Mol Cell* **20**, 155-66 (2005).

75. Bowers, J., Tran, P.T., Joshi, A., Liskay, R.M. & Alani, E. MSH-MLH complexes formed at a DNA mismatch are disrupted by the PCNA sliding clamp. *J Mol Biol* **306**, 957-68 (2001).
76. Clark, A.B., Valle, F., Drotschmann, K., Gary, R.K. & Kunkel, T.A. Functional interaction of proliferating cell nuclear antigen with MSH2-MSH6 and MSH2-MSH3 complexes. *J Biol Chem* **275**, 36498-501 (2000).
77. Flores-Rozas, H., Clark, D. & Kolodner, R.D. Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. *Nat Genet* **26**, 375-8 (2000).
78. Kleczkowska, H.E., Marra, G., Lettieri, T. & Jiricny, J. hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. *Genes Dev* **15**, 724-36 (2001).
79. Warbrick, E. The puzzle of PCNA's many partners. *Bioessays* **22**, 997-1006 (2000).
80. Prasad, R. *et al.* A review of recent experiments on step-to-step "hand-off" of the DNA intermediates in mammalian base excision repair pathways. *Mol Biol (Mosk)* **45**, 586-600 (2011).
81. Fortini, P. & Dogliotti, E. Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways. *DNA Repair (Amst)* **6**, 398-409 (2007).
82. Braithwaite, E.K. *et al.* DNA polymerase lambda mediates a back-up base excision repair activity in extracts of mouse embryonic fibroblasts. *J Biol Chem* **280**, 18469-75 (2005).
83. Sugasawa, K. Regulation of damage recognition in mammalian global genomic nucleotide excision repair. *Mutat Res* **685**, 29-37 (2010).
84. Fuss, J.O. & Cooper, P.K. DNA repair: dynamic defenders against cancer and aging. *PLoS Biol* **4**, e203 (2006).
85. Cleaver, J.E., Lam, E.T. & Revet, I. Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity. *Nat Rev Genet* **10**, 756-68 (2009).
86. Groisman, R. *et al.* The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* **113**, 357-67 (2003).
87. Wang, H. *et al.* Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. *Mol Cell* **22**, 383-94 (2006).
88. Sugasawa, K. *et al.* UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. *Cell* **121**, 387-400 (2005).
89. Harreman, M. *et al.* Distinct ubiquitin ligases act sequentially for RNA polymerase II polyubiquitylation. *Proc Natl Acad Sci U S A* **106**, 20705-10 (2009).
90. Anindya, R., Aygun, O. & Svejstrup, J.Q. Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. *Mol Cell* **28**, 386-97 (2007).
91. Groisman, R. *et al.* CSA-dependent degradation of CSB by the ubiquitin-proteasome pathway establishes a link between complementation factors of the Cockayne syndrome. *Genes Dev* **20**, 1429-34 (2006).

92. Araujo, S.J., Nigg, E.A. & Wood, R.D. Strong functional interactions of TFIIH with XPC and XPG in human DNA nucleotide excision repair, without a preassembled repairosome. *Mol Cell Biol* **21**, 2281-91 (2001).
93. Li, R.Y., Calsou, P., Jones, C.J. & Salles, B. Interactions of the transcription/DNA repair factor TFIIH and XP repair proteins with DNA lesions in a cell-free repair assay. *J Mol Biol* **281**, 211-8 (1998).
94. Yokoi, M. *et al.* The xeroderma pigmentosum group C protein complex XPC-HR23B plays an important role in the recruitment of transcription factor IIH to damaged DNA. *J Biol Chem* **275**, 9870-5 (2000).
95. Tantin, D. RNA polymerase II elongation complexes containing the Cockayne syndrome group B protein interact with a molecular complex containing the transcription factor IIH components xeroderma pigmentosum B and p62. *J Biol Chem* **273**, 27794-9 (1998).
96. Lieber, M.R., Ma, Y., Pannicke, U. & Schwarz, K. Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol* **4**, 712-20 (2003).
97. Yaneva, M., Kowalewski, T. & Lieber, M.R. Interaction of DNA-dependent protein kinase with DNA and with Ku: biochemical and atomic-force microscopy studies. *EMBO J* **16**, 5098-112 (1997).
98. Cary, R.B. *et al.* DNA looping by Ku and the DNA-dependent protein kinase. *Proc Natl Acad Sci U S A* **94**, 4267-72 (1997).
99. Pang, D., Yoo, S., Dynan, W.S., Jung, M. & Dritschilo, A. Ku proteins join DNA fragments as shown by atomic force microscopy. *Cancer Res* **57**, 1412-5 (1997).
100. Dobbs, T.A., Tainer, J.A. & Lees-Miller, S.P. A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation. *DNA Repair (Amst)* **9**, 1307-14 (2010).
101. Ma, Y., Pannicke, U., Schwarz, K. & Lieber, M.R. Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell* **108**, 781-94 (2002).
102. Gu, W., Zhang, F. & Lupski, J.R. Mechanisms for human genomic rearrangements. *Pathogenetics* **1**, 4 (2008).
103. Paull, T.T. & Gellert, M. The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol Cell* **1**, 969-79 (1998).
104. Zhang, J. & Powell, S.N. The role of the BRCA1 tumor suppressor in DNA double-strand break repair. *Mol Cancer Res* **3**, 531-9 (2005).
105. Singleton, M.R., Wentzell, L.M., Liu, Y., West, S.C. & Wigley, D.B. Structure of the single-strand annealing domain of human RAD52 protein. *Proc Natl Acad Sci U S A* **99**, 13492-7 (2002).
106. Wang, Y. *et al.* Evidence that in xeroderma pigmentosum variant cells, which lack DNA polymerase eta, DNA polymerase iota causes the very high frequency and unique spectrum of UV-induced mutations. *Cancer Res* **67**, 3018-26 (2007).
107. Ziv, O., Geacintov, N., Nakajima, S., Yasui, A. & Livneh, Z. DNA polymerase zeta cooperates with polymerases kappa and iota in translesion DNA synthesis across pyrimidine photodimers in cells from XPV patients. *Proc Natl Acad Sci U S A* **106**, 11552-7 (2009).
108. Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G. & Jentsch, S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135-41 (2002).

109. Stelter, P. & Ulrich, H.D. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature* **425**, 188-91 (2003).
110. Haracska, L., Torres-Ramos, C.A., Johnson, R.E., Prakash, S. & Prakash, L. Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in Saccharomyces cerevisiae. *Mol Cell Biol* **24**, 4267-74 (2004).
111. Kannouche, P.L., Wing, J. & Lehmann, A.R. Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol Cell* **14**, 491-500 (2004).
112. Kannouche, P.L. & Lehmann, A.R. Ubiquitination of PCNA and the polymerase switch in human cells. *Cell Cycle* **3**, 1011-3 (2004).
113. Naryzhny, S.N. Proliferating cell nuclear antigen: a proteomics view. *Cell Mol Life Sci* **65**, 3789-808 (2008).
114. Minca, E.C. & Kowalski, D. Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks. *Mol Cell* **38**, 649-61 (2010).
115. Goldfless, S.J., Morag, A.S., Belisle, K.A., Sutera, V.A., Jr. & Lovett, S.T. DNA repeat rearrangements mediated by DnaK-dependent replication fork repair. *Mol Cell* **21**, 595-604 (2006).
116. Verschuren, E.W., Jones, N. & Evan, G.I. The cell cycle and how it is steered by Kaposi's sarcoma-associated herpesvirus cyclin. *J Gen Virol* **85**, 1347-61 (2004).
117. Roussel, M.F. The INK4 family of cell cycle inhibitors in cancer. *Oncogene* **18**, 5311-7 (1999).
118. Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A. & Sherr, C.J. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol* **15**, 2672-81 (1995).
119. Ullah, Z., Lee, C.Y. & Depamphilis, M.L. Cip/Kip cyclin-dependent protein kinase inhibitors and the road to polyploidy. *Cell Div* **4**, 10 (2009).
120. Denicourt, C. & Dowdy, S.F. Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev* **18**, 851-5 (2004).
121. Nakayama, K. & Nakayama, K. Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. *Bioessays* **20**, 1020-9 (1998).
122. Fattaey, A. & Booher, R.N. Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14. *Prog Cell Cycle Res* **3**, 233-40 (1997).
123. Wang, Y., Decker, S.J. & Sebolt-Leopold, J. Knockdown of Chk1, Wee1 and Myt1 by RNA interference abrogates G2 checkpoint and induces apoptosis. *Cancer Biol Ther* **3**, 305-13 (2004).
124. Enserink, J.M. & Kolodner, R.D. An overview of Cdk1-controlled targets and processes. *Cell Div* **5**, 11 (2010).
125. Nilsson, I. & Hoffmann, I. Cell cycle regulation by the Cdc25 phosphatase family. *Prog Cell Cycle Res* **4**, 107-14 (2000).
126. Aressy, B. & Ducommun, B. Cell cycle control by the CDC25 phosphatases. *Anticancer Agents Med Chem* **8**, 818-24 (2008).
127. Smith, J., Tho, L.M., Xu, N. & Gillespie, D.A. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res* **108**, 73-112 (2010).

128. Deng, C.X. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Res* **34**, 1416-26 (2006).
129. Shieh, S.Y., Ahn, J., Tamai, K., Taya, Y. & Prives, C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev* **14**, 289-300 (2000).
130. Laptenko, O., Beckerman, R., Freulich, E. & Prives, C. p53 binding to nucleosomes within the p21 promoter in vivo leads to nucleosome loss and transcriptional activation. *Proc Natl Acad Sci U S A* **108**, 10385-90 (2011).
131. Kim, T.K. In vitro transcriptional activation of p21 promoter by p53. *Biochem Biophys Res Commun* **234**, 300-2 (1997).
132. Maya, R. *et al.* ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev* **15**, 1067-77 (2001).
133. Khosravi, R. *et al.* Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. *Proc Natl Acad Sci U S A* **96**, 14973-7 (1999).
134. Shinozaki, T., Nota, A., Taya, Y. & Okamoto, K. Functional role of Mdm2 phosphorylation by ATR in attenuation of p53 nuclear export. *Oncogene* **22**, 8870-80 (2003).
135. Satyanarayana, A., Hilton, M.B. & Kaldis, P. p21 Inhibits Cdk1 in the absence of Cdk2 to maintain the G1/S phase DNA damage checkpoint. *Mol Biol Cell* **19**, 65-77 (2008).
136. Zhan, Q., Chen, I.T., Antinore, M.J. & Fornace, A.J., Jr. Tumor suppressor p53 can participate in transcriptional induction of the GADD45 promoter in the absence of direct DNA binding. *Mol Cell Biol* **18**, 2768-78 (1998).
137. Hermeking, H. *et al.* 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* **1**, 3-11 (1997).
138. Suijkerbuijk, S.J. & Kops, G.J. Preventing aneuploidy: the contribution of mitotic checkpoint proteins. *Biochim Biophys Acta* **1786**, 24-31 (2008).
139. Cheeseman, I.M. & Desai, A. Molecular architecture of the kinetochore-microtubule interface. *Nat Rev Mol Cell Biol* **9**, 33-46 (2008).
140. Przewloka, M.R. & Glover, D.M. The kinetochore and the centromere: a working long distance relationship. *Annu Rev Genet* **43**, 439-65 (2009).
141. Santaguida, S. & Musacchio, A. The life and miracles of kinetochores. *EMBO J* **28**, 2511-31 (2009).
142. Ruchaud, S., Carmena, M. & Earnshaw, W.C. Chromosomal passengers: conducting cell division. *Nat Rev Mol Cell Biol* **8**, 798-812 (2007).
143. Kelly, A.E. & Funabiki, H. Correcting aberrant kinetochore microtubule attachments: an Aurora B-centric view. *Curr Opin Cell Biol* **21**, 51-8 (2009).
144. Peters, J.M. The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol Cell* **9**, 931-43 (2002).
145. Cotter, T.G. Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer* **9**, 501-7 (2009).
146. Fesik, S.W. & Shi, Y. Structural biology. Controlling the caspases. *Science* **294**, 1477-8 (2001).
147. Deveraux, Q.L. & Reed, J.C. IAP family proteins--suppressors of apoptosis. *Genes Dev* **13**, 239-52 (1999).

148. Du, C., Fang, M., Li, Y., Li, L. & Wang, X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**, 33-42 (2000).
149. Hu, Y., Benedict, M.A., Ding, L. & Nunez, G. Role of cytochrome c and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. *EMBO J* **18**, 3586-95 (1999).
150. Wajant, H. The Fas signaling pathway: more than a paradigm. *Science* **296**, 1635-6 (2002).
151. Pennarun, B. *et al.* Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. *Biochim Biophys Acta* **1805**, 123-40 (2010).
152. Ahel, D. *et al.* Poly(ADP-ribose)-dependent regulation of DNA repair by the chromatin remodeling enzyme ALC1. *Science* **325**, 1240-3 (2009).
153. Berger, N.A., Sims, J.L., Catino, D.M. & Berger, S.J. Poly(ADP-ribose) polymerase mediates the suicide response to massive DNA damage: studies in normal and DNA-repair defective cells. *Princess Takamatsu Symp* **13**, 219-26 (1983).
154. Krishnakumar, R. & Kraus, W.L. PARP-1 regulates chromatin structure and transcription through a KDM5B-dependent pathway. *Mol Cell* **39**, 736-49 (2010).
155. Berger, N.A. Poly(ADP-ribose) in the cellular response to DNA damage. *Radiat Res* **101**, 4-15 (1985).
156. Nicholson, D.W. *et al.* Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37-43 (1995).
157. Tewari, M. *et al.* Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**, 801-9 (1995).
158. Coquelle, N., Green, R. & Glover, J.N. Impact of BRCA1 BRCT domain missense substitutions on phosphopeptide recognition. *Biochemistry* **50**, 4579-89 (2011).
159. Ludwig, T., Chapman, D.L., Papaioannou, V.E. & Efstratiadis, A. Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. *Genes Dev* **11**, 1226-41 (1997).
160. Brzovic, P.S. *et al.* Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc Natl Acad Sci U S A* **100**, 5646-51 (2003).
161. Wu, W., Koike, A., Takeshita, T. & Ohta, T. The ubiquitin E3 ligase activity of BRCA1 and its biological functions. *Cell Div* **3**, 1 (2008).
162. Kleiman, F.E. *et al.* BRCA1/BARD1 inhibition of mRNA 3' processing involves targeted degradation of RNA polymerase II. *Genes Dev* **19**, 1227-37 (2005).
163. Starita, L.M. *et al.* BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. *J Biol Chem* **280**, 24498-505 (2005).
164. Horwitz, A.A., Affar el, B., Heine, G.F., Shi, Y. & Parvin, J.D. A mechanism for transcriptional repression dependent on the BRCA1 E3 ubiquitin ligase. *Proc Natl Acad Sci U S A* **104**, 6614-9 (2007).
165. Chen, A., Kleiman, F.E., Manley, J.L., Ouchi, T. & Pan, Z.Q. Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase. *J Biol Chem* **277**, 22085-92 (2002).
166. Roy, R., Chun, J. & Powell, S.N. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer* **12**, 68-78 (2012).

167. Sy, S.M., Huen, M.S. & Chen, J. PALB2 is an integral component of the BRCA complex required for homologous recombination repair. *Proc Natl Acad Sci U S A* **106**, 7155-60 (2009).
168. Zhang, F., Fan, Q., Ren, K. & Andreassen, P.R. PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2. *Mol Cancer Res* **7**, 1110-8 (2009).
169. Zhang, F. *et al.* PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr Biol* **19**, 524-9 (2009).
170. Oliver, A.W., Swift, S., Lord, C.J., Ashworth, A. & Pearl, L.H. Structural basis for recruitment of BRCA2 by PALB2. *EMBO Rep* **10**, 990-6 (2009).
171. Manke, I.A., Lowery, D.M., Nguyen, A. & Yaffe, M.B. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science* **302**, 636-9 (2003).
172. Yu, X., Chini, C.C., He, M., Mer, G. & Chen, J. The BRCT domain is a phospho-protein binding domain. *Science* **302**, 639-42 (2003).
173. Shakya, R. *et al.* BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. *Science* **334**, 525-8 (2011).
174. Huen, M.S., Sy, S.M. & Chen, J. BRCA1 and its toolbox for the maintenance of genome integrity. *Nat Rev Mol Cell Biol* **11**, 138-48 (2010).
175. Zhang, J. *et al.* Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair. *Mol Cell Biol* **24**, 708-18 (2004).
176. Cortez, D., Wang, Y., Qin, J. & Elledge, S.J. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* **286**, 1162-6 (1999).
177. Xu, B., O'Donnell, A.H., Kim, S.T. & Kastan, M.B. Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. *Cancer Res* **62**, 4588-91 (2002).
178. Chen, L., Nievera, C.J., Lee, A.Y. & Wu, X. Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair. *J Biol Chem* **283**, 7713-20 (2008).
179. Yan, J. & Jetten, A.M. RAP80 and RNF8, key players in the recruitment of repair proteins to DNA damage sites. *Cancer Lett* **271**, 179-90 (2008).
180. Uziel, T. *et al.* Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* **22**, 5612-21 (2003).
181. Takeda, S., Nakamura, K., Taniguchi, Y. & Paull, T.T. Ctp1/CtIP and the MRN complex collaborate in the initial steps of homologous recombination. *Mol Cell* **28**, 351-2 (2007).
182. So, S., Davis, A.J. & Chen, D.J. Autophosphorylation at serine 1981 stabilizes ATM at DNA damage sites. *J Cell Biol* **187**, 977-90 (2009).
183. Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. & Bonner, W.M. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* **273**, 5858-68 (1998).
184. Rogakou, E.P., Boon, C., Redon, C. & Bonner, W.M. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol* **146**, 905-16 (1999).
185. Paull, T.T. *et al.* A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* **10**, 886-95 (2000).

186. Yuan, J., Adamski, R. & Chen, J. Focus on histone variant H2AX: to be or not to be. *FEBS Lett* **584**, 3717-24 (2010).
187. Stucki, M. *et al.* MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. *Cell* **123**, 1213-26 (2005).
188. Chowdhury, D. *et al.* gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol Cell* **20**, 801-9 (2005).
189. Luo, K., Yuan, J. & Lou, Z. Oligomerization of MDC1 protein is important for proper DNA damage response. *J Biol Chem* **286**, 28192-9 (2011).
190. Durocher, D. & Jackson, S.P. The FHA domain. *FEBS Lett* **513**, 58-66 (2002).
191. Mailand, N. *et al.* RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell* **131**, 887-900 (2007).
192. Pinato, S. *et al.* RNF168, a new RING finger, MIU-containing protein that modifies chromatin by ubiquitination of histones H2A and H2AX. *BMC Mol Biol* **10**, 55 (2009).
193. Huen, M.S. *et al.* RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. *Cell* **131**, 901-14 (2007).
194. Doil, C. *et al.* RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. *Cell* **136**, 435-46 (2009).
195. Stewart, G.S. *et al.* The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. *Cell* **136**, 420-34 (2009).
196. Yan, J. *et al.* The ubiquitin-interacting motif containing protein RAP80 interacts with BRCA1 and functions in DNA damage repair response. *Cancer Res* **67**, 6647-56 (2007).
197. Cook, P.J. *et al.* Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature* **458**, 591-6 (2009).
198. Xiao, A. *et al.* WSTF regulates the H2A.X DNA damage response via a novel tyrosine kinase activity. *Nature* **457**, 57-62 (2009).
199. Ikura, T. *et al.* DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. *Mol Cell Biol* **27**, 7028-40 (2007).
200. Bekker-Jensen, S., Lukas, C., Melander, F., Bartek, J. & Lukas, J. Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1. *J Cell Biol* **170**, 201-11 (2005).
201. Pei, H. *et al.* MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. *Nature* **470**, 124-8 (2011).
202. Wang, B., Matsuoka, S., Carpenter, P.B. & Elledge, S.J. 53BP1, a mediator of the DNA damage checkpoint. *Science* **298**, 1435-8 (2002).
203. Fernandez-Capetillo, O. *et al.* DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. *Nat Cell Biol* **4**, 993-7 (2002).
204. Ward, I.M., Minn, K., van Deursen, J. & Chen, J. p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. *Mol Cell Biol* **23**, 2556-63 (2003).
205. Lee, J.H., Goodarzi, A.A., Jeggo, P.A. & Paull, T.T. 53BP1 promotes ATM activity through direct interactions with the MRN complex. *EMBO J* **29**, 574-85 (2010).
206. Mao, Z., Bozzella, M., Seluanov, A. & Gorbunova, V. Comparison of nonhomologous end joining and homologous recombination in human cells. *DNA Repair (Amst)* **7**, 1765-71 (2008).

207. Shrivastav, M., De Haro, L.P. & Nickoloff, J.A. Regulation of DNA double-strand break repair pathway choice. *Cell Res* **18**, 134-47 (2008).
208. Chen, Y. *et al.* BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. *Cancer Res* **56**, 3168-72 (1996).
209. Chen, F. *et al.* Cell cycle-dependent protein expression of mammalian homologs of yeast DNA double-strand break repair genes Rad51 and Rad52. *Mutat Res* **384**, 205-11 (1997).
210. Flygare, J., Benson, F. & Hellgren, D. Expression of the human RAD51 gene during the cell cycle in primary human peripheral blood lymphocytes. *Biochim Biophys Acta* **1312**, 231-6 (1996).
211. Chen, B.P. *et al.* Cell cycle dependence of DNA-dependent protein kinase phosphorylation in response to DNA double strand breaks. *J Biol Chem* **280**, 14709-15 (2005).
212. Lee, S.E., Mitchell, R.A., Cheng, A. & Hendrickson, E.A. Evidence for DNA-PK-dependent and -independent DNA double-strand break repair pathways in mammalian cells as a function of the cell cycle. *Mol Cell Biol* **17**, 1425-33 (1997).
213. Ira, G. *et al.* DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature* **431**, 1011-7 (2004).
214. Yun, M.H. & Hiom, K. CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature* **459**, 460-3 (2009).
215. Esashi, F. *et al.* CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature* **434**, 598-604 (2005).
216. Starita, L.M. *et al.* BRCA1-dependent ubiquitination of gamma-tubulin regulates centrosome number. *Mol Cell Biol* **24**, 8457-66 (2004).
217. Shimada, M. & Komatsu, K. Emerging connection between centrosome and DNA repair machinery. *J Radiat Res* **50**, 295-301 (2009).
218. Starita, L.M. & Parvin, J.D. Substrates of the BRCA1-dependent ubiquitin ligase. *Cancer Biol Ther* **5**, 137-41 (2006).
219. Wang, R.H., Yu, H. & Deng, C.X. A requirement for breast-cancer-associated gene 1 (BRCA1) in the spindle checkpoint. *Proc Natl Acad Sci U S A* **101**, 17108-13 (2004).
220. Zhu, Q. *et al.* BRCA1 tumour suppression occurs via heterochromatin-mediated silencing. *Nature* **477**, 179-84 (2011).
221. Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. & Richmond, T.J. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**, 251-60 (1997).
222. Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693-705 (2007).
223. Zhang, Y. Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes Dev* **17**, 2733-40 (2003).
224. Dinant, C., Houtsmuller, A.B. & Vermeulen, W. Chromatin structure and DNA damage repair. *Epigenetics Chromatin* **1**, 9 (2008).
225. Smerdon, M.J. DNA repair and the role of chromatin structure. *Curr Opin Cell Biol* **3**, 422-8 (1991).
226. Sordet, O. *et al.* Hyperphosphorylation of RNA polymerase II in response to topoisomerase I cleavage complexes and its association with transcription- and BRCA1-dependent degradation of topoisomerase I. *J Mol Biol* **381**, 540-9 (2008).

227. Zhang, H. *et al.* BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene* **16**, 1713-21 (1998).
228. Chai, Y.L. *et al.* The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the p21WAF1/CIP1 promoter. *Oncogene* **18**, 263-8 (1999).
229. Vousden, K.H. & Prives, C. Blinded by the Light: The Growing Complexity of p53. *Cell* **137**, 413-31 (2009).
230. MacLachlan, T.K., Takimoto, R. & El-Deiry, W.S. BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets. *Mol Cell Biol* **22**, 4280-92 (2002).
231. Ongusaha, P.P. *et al.* BRCA1 shifts p53-mediated cellular outcomes towards irreversible growth arrest. *Oncogene* **22**, 3749-58 (2003).
232. Aprelikova, O., Pace, A.J., Fang, B., Koller, B.H. & Liu, E.T. BRCA1 is a selective co-activator of 14-3-3 sigma gene transcription in mouse embryonic stem cells. *J Biol Chem* **276**, 25647-50 (2001).
233. Wang, Q., Zhang, H., Kajino, K. & Greene, M.I. BRCA1 binds c-Myc and inhibits its transcriptional and transforming activity in cells. *Oncogene* **17**, 1939-48 (1998).
234. Li, H., Lee, T.H. & Avraham, H. A novel tricomplex of BRCA1, Nmi, and c-Myc inhibits c-Myc-induced human telomerase reverse transcriptase gene (hTERT) promoter activity in breast cancer. *J Biol Chem* **277**, 20965-73 (2002).
235. Kim, N.W. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011-5 (1994).
236. Breslow, R.A., Shay, J.W., Gazdar, A.F. & Srivastava, S. Telomerase and early detection of cancer: a National Cancer Institute workshop. *J Natl Cancer Inst* **89**, 618-23 (1997).
237. Hahn, W.C. Senescence, telomere shortening and telomere maintenance. *Cancer Biol Ther* **1**, 398-400 (2002).
238. de Kok, J.B. *et al.* Real-time quantification of human telomerase reverse transcriptase mRNA in tumors and healthy tissues. *Clin Chem* **46**, 313-8 (2000).
239. Dahlman-Wright, K. *et al.* International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacol Rev* **58**, 773-81 (2006).
240. Lin, C.Y. *et al.* Discovery of estrogen receptor alpha target genes and response elements in breast tumor cells. *Genome Biol* **5**, R66 (2004).
241. Kawai, H., Li, H., Chun, P., Avraham, S. & Avraham, H.K. Direct interaction between BRCA1 and the estrogen receptor regulates vascular endothelial growth factor (VEGF) transcription and secretion in breast cancer cells. *Oncogene* **21**, 7730-9 (2002).
242. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1**, 27-31 (1995).
243. Kim, K.J., Li, B., Houck, K., Winer, J. & Ferrara, N. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. *Growth Factors* **7**, 53-64 (1992).
244. Yoshiji, H., Harris, S.R. & Thorgeirsson, U.P. Vascular endothelial growth factor is essential for initial but not continued in vivo growth of human breast carcinoma cells. *Cancer Res* **57**, 3924-8 (1997).
245. Venkitaraman, A.R. Linking the cellular functions of BRCA genes to cancer pathogenesis and treatment. *Annu Rev Pathol* **4**, 461-87 (2009).

246. Bochar, D.A. *et al.* BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell* **102**, 257-65 (2000).
247. Pao, G.M., Janknecht, R., Ruffner, H., Hunter, T. & Verma, I.M. CBP/p300 interact with and function as transcriptional coactivators of BRCA1. *Proc Natl Acad Sci U S A* **97**, 1020-5 (2000).
248. Ogryzko, V.V., Wong, P. & Howard, B.H. WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. *Mol Cell Biol* **17**, 4877-82 (1997).
249. MacLachlan, T.K. *et al.* BRCA1 effects on the cell cycle and the DNA damage response are linked to altered gene expression. *J Biol Chem* **275**, 2777-85 (2000).
250. Wang, X.W. *et al.* GADD45 induction of a G2/M cell cycle checkpoint. *Proc Natl Acad Sci U S A* **96**, 3706-11 (1999).
251. Yarden, R.I., Pardo-Reoyo, S., Sgagias, M., Cowan, K.H. & Brody, L.C. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat Genet* **30**, 285-9 (2002).
252. Ree, A.H., Bratland, A., Nome, R.V., Stokke, T. & Fodstad, O. Repression of mRNA for the PLK cell cycle gene after DNA damage requires BRCA1. *Oncogene* **22**, 8952-5 (2003).
253. Cantor, S.B. *et al.* BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell* **105**, 149-60 (2001).
254. Wei, L. *et al.* Rapid recruitment of BRCA1 to DNA double-strand breaks is dependent on its association with Ku80. *Mol Cell Biol* **28**, 7380-93 (2008).
255. Kessler, B.M. *et al.* Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic beta-subunits. *Chem Biol* **8**, 913-29 (2001).
256. Affar el, B. *et al.* Essential dosage-dependent functions of the transcription factor yin yang 1 in late embryonic development and cell cycle progression. *Mol Cell Biol* **26**, 3565-81 (2006).
257. Daou, S. *et al.* Crosstalk between O-GlcNAcylation and proteolytic cleavage regulates the host cell factor-1 maturation pathway. *Proc Natl Acad Sci U S A* **108**, 2747-52 (2011).
258. Harper, J.V. Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. *Methods Mol Biol* **296**, 157-66 (2005).
259. Choudhury, A.D., Xu, H. & Baer, R. Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression. *J Biol Chem* **279**, 33909-18 (2004).
260. Kuluncsics, Z., Perdiz, D., Brulay, E., Muel, B. & Sage, E. Wavelength dependence of ultraviolet-induced DNA damage distribution: involvement of direct or indirect mechanisms and possible artefacts. *J Photochem Photobiol B* **49**, 71-80 (1999).
261. Yoon, J.H., Lee, C.S., O'Connor, T.R., Yasui, A. & Pfeifer, G.P. The DNA damage spectrum produced by simulated sunlight. *J Mol Biol* **299**, 681-93 (2000).
262. Wilson, C.A. *et al.* Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. *Nat Genet* **21**, 236-40 (1999).
263. Sankaran, S., Starita, L.M., Simons, A.M. & Parvin, J.D. Identification of domains of BRCA1 critical for the ubiquitin-dependent inhibition of centrosome function. *Cancer Res* **66**, 4100-7 (2006).

264. Hammond-Martel, I. *et al.* PI 3 kinase related kinases-independent proteolysis of BRCA1 regulates Rad51 recruitment during genotoxic stress in human cells. *PLoS One* **5**, e14027 (2010).
265. Fabian, M.R., Sonenberg, N. & Filipowicz, W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* **79**, 351-79 (2010).
266. Lee, Y. *et al.* The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-9 (2003).
267. Cullen, B.R. RNAi the natural way. *Nat Genet* **37**, 1163-5 (2005).
268. Song, J.J., Smith, S.K., Hannon, G.J. & Joshua-Tor, L. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**, 1434-7 (2004).
269. Scully, R. *et al.* Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* **90**, 425-35 (1997).
270. Jin, Y. *et al.* Cell cycle-dependent colocalization of BARD1 and BRCA1 proteins in discrete nuclear domains. *Proc Natl Acad Sci U S A* **94**, 12075-80 (1997).
271. Wu, X. *et al.* Independence of R/M/N focus formation and the presence of intact BRCA1. *Science* **289**, 11 (2000).
272. Pageau, G.J. & Lawrence, J.B. BRCA1 foci in normal S-phase nuclei are linked to interphase centromeres and replication of pericentric heterochromatin. *J Cell Biol* **175**, 693-701 (2006).
273. Bekker-Jensen, S. *et al.* HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes. *Nat Cell Biol* **12**, 80-6; sup pp 1-12 (2010).
274. Adams, J. *et al.* Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* **59**, 2615-22 (1999).
275. Ding, W.X. *et al.* A coordinated action of Bax, PUMA, and p53 promotes MG132-induced mitochondria activation and apoptosis in colon cancer cells. *Mol Cancer Ther* **6**, 1062-9 (2007).
276. Han, Y.H. & Park, W.H. The effects of MAPK inhibitors on a proteasome inhibitor, MG132-induced HeLa cell death in relation to reactive oxygen species and glutathione. *Toxicol Lett* **192**, 134-40 (2010).
277. Jacquemont, C. & Taniguchi, T. Proteasome function is required for DNA damage response and fanconi anemia pathway activation. *Cancer Res* **67**, 7395-405 (2007).
278. Ward, I.M., Minn, K. & Chen, J. UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress. *J Biol Chem* **279**, 9677-80 (2004).
279. Staszewski, O., Nikolova, T. & Kaina, B. Kinetics of gamma-H2AX focus formation upon treatment of cells with UV light and alkylating agents. *Environ Mol Mutagen* **49**, 734-40 (2008).
280. Ward, I.M. & Chen, J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem* **276**, 47759-62 (2001).
281. Liu, J.S., Kuo, S.R. & Melendy, T. Comparison of checkpoint responses triggered by DNA polymerase inhibition versus DNA damaging agents. *Mutat Res* **532**, 215-26 (2003).
282. Nikiforov, A. *et al.* DNA damage-induced accumulation of Rad18 protein at stalled replication forks in mammalian cells involves upstream protein phosphorylation. *Biochem Biophys Res Commun* **323**, 831-7 (2004).

283. Petermann, E., Orta, M.L., Issaeva, N., Schultz, N. & Helleday, T. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol Cell* **37**, 492-502 (2010).
284. Vanoli, F., Fumasoni, M., Szakal, B., Maloisel, L. & Branzei, D. Replication and recombination factors contributing to recombination-dependent bypass of DNA lesions by template switch. *PLoS Genet* **6**, e1001205 (2010).
285. Rouget, R., Auclair, Y., Loignon, M., Affar el, B. & Drobetsky, E.A. A sensitive flow cytometry-based nucleotide excision repair assay unexpectedly reveals that mitogen-activated protein kinase signaling does not regulate the removal of UV-induced DNA damage in human cells. *J Biol Chem* **283**, 5533-41 (2008).
286. Artavanis-Tsakonas, K. *et al.* Characterization and structural studies of the Plasmodium falciparum ubiquitin and Nedd8 hydrolase UCHL3. *J Biol Chem* **285**, 6857-66 (2010).
287. Xirodimas, D.P. *et al.* Ribosomal proteins are targets for the NEDD8 pathway. *EMBO Rep* **9**, 280-6 (2008).
288. Pan, Z.Q., Kentsis, A., Dias, D.C., Yamoah, K. & Wu, K. Nedd8 on cullin: building an expressway to protein destruction. *Oncogene* **23**, 1985-97 (2004).
289. Li, J. *et al.* Differential display identifies overexpression of the USP36 gene, encoding a deubiquitinating enzyme, in ovarian cancer. *Int J Med Sci* **5**, 133-42 (2008).
290. Massoumi, R. CYLD: a deubiquitination enzyme with multiple roles in cancer. *Future Oncol* **7**, 285-97 (2011).
291. Sacco, J.J., Coulson, J.M., Clague, M.J. & Urbe, S. Emerging roles of deubiquitinases in cancer-associated pathways. *IUBMB Life* **62**, 140-57 (2010).
292. Fitzgerald, D.C. *et al.* Tumour necrosis factor-alpha (TNF-alpha) increases nuclear factor kappaB (NFkappaB) activity in and interleukin-8 (IL-8) release from bovine mammary epithelial cells. *Vet Immunol Immunopathol* **116**, 59-68 (2007).
293. Pahl, H.L. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**, 6853-66 (1999).
294. Sherman, M.L., Datta, R., Hallahan, D.E., Weichselbaum, R.R. & Kufe, D.W. Regulation of tumor necrosis factor gene expression by ionizing radiation in human myeloid leukemia cells and peripheral blood monocytes. *J Clin Invest* **87**, 1794-7 (1991).
295. Stegmeier, F. *et al.* The tumor suppressor CYLD regulates entry into mitosis. *Proc Natl Acad Sci U S A* **104**, 8869-74 (2007).
296. Wilkinson, K.D. *et al.* The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* **246**, 670-3 (1989).
297. Osaka, H. *et al.* Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Hum Mol Genet* **12**, 1945-58 (2003).
298. Li, L. *et al.* The tumor suppressor UCHL1 forms a complex with p53/MDM2/ARF to promote p53 signaling and is frequently silenced in nasopharyngeal carcinoma. *Clin Cancer Res* **16**, 2949-58 (2010).
299. Okochi-Takada, E. *et al.* Silencing of the UCHL1 gene in human colorectal and ovarian cancers. *Int J Cancer* **119**, 1338-44 (2006).
300. Wang, W.J. *et al.* Over-expression of ubiquitin carboxy terminal hydrolase-L1 induces apoptosis in breast cancer cells. *Int J Oncol* **33**, 1037-45 (2008).

301. Stevenson, L.F. *et al.* The deubiquitinating enzyme USP2a regulates the p53 pathway by targeting Mdm2. *EMBO J* **26**, 976-86 (2007).
302. Wu, Y.R. *et al.* Ubiquitin specific proteases USP24 and USP40 and ubiquitin thiolesterase UCHL1 polymorphisms have synergic effect on the risk of Parkinson's disease among Taiwanese. *Clin Chim Acta* **411**, 955-8 (2010).
303. Davies, C.W., Paul, L.N., Kim, M.I. & Das, C. Structural and thermodynamic comparison of the catalytic domain of AMSH and AMSH-LP: nearly identical fold but different stability. *J Mol Biol* **413**, 416-29 (2011).
304. Kikuchi, K., Ishii, N., Asao, H. & Sugamura, K. Identification of AMSH-LP containing a Jab1/MPN domain metalloenzyme motif. *Biochem Biophys Res Commun* **306**, 637-43 (2003).
305. McCullough, J., Clague, M.J. & Urbe, S. AMSH is an endosome-associated ubiquitin isopeptidase. *J Cell Biol* **166**, 487-92 (2004).
306. Itoh, F. *et al.* Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. *EMBO J* **20**, 4132-42 (2001).
307. Xu, Z., Zheng, Y., Zhu, Y., Kong, X. & Hu, L. Evidence for OTUD-6B participation in B lymphocytes cell cycle after cytokine stimulation. *PLoS One* **6**, e14514 (2011).
308. Stanisic, V., Malovannaya, A., Qin, J., Lonard, D.M. & O'Malley, B.W. OTU Domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) deubiquitinates estrogen receptor (ER) alpha and affects ERalpha transcriptional activity. *J Biol Chem* **284**, 16135-45 (2009).
309. Tateishi, Y. *et al.* Ligand-dependent switching of ubiquitin-proteasome pathways for estrogen receptor. *EMBO J* **23**, 4813-23 (2004).
310. Ma, Y.X. *et al.* Structural determinants of the BRCA1 : estrogen receptor interaction. *Oncogene* **24**, 1831-46 (2005).
311. Ma, Y. *et al.* BRCA1 regulates acetylation and ubiquitination of estrogen receptor-alpha. *Mol Endocrinol* **24**, 76-90 (2010).
312. Nakada, S. *et al.* Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. *Nature* **466**, 941-6 (2010).
313. Xiao, N. *et al.* Ubiquitin-specific protease 4 (USP4) targets TRAF2 and TRAF6 for deubiquitination and inhibits TNFalpha-induced cancer cell migration. *Biochem J* **441**, 979-86 (2012).
314. Zhang, X., Berger, F.G., Yang, J. & Lu, X. USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. *EMBO J* **30**, 2177-89 (2011).
315. Tzimas, C. *et al.* Human ubiquitin specific protease 31 is a deubiquitinating enzyme implicated in activation of nuclear factor-kappaB. *Cell Signal* **18**, 83-92 (2006).
316. Jiang, M., Chiu, S.Y. & Hsu, W. SUMO-specific protease 2 in Mdm2-mediated regulation of p53. *Cell Death Differ* **18**, 1005-15 (2011).
317. Tran, H., Hamada, F., Schwarz-Romond, T. & Bienz, M. Trabid, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. *Genes Dev* **22**, 528-42 (2008).
318. Shao, G. *et al.* The Rap80-BRCC36 de-ubiquitinating enzyme complex antagonizes RNF8-Ubc13-dependent ubiquitination events at DNA double strand breaks. *Proc Natl Acad Sci U S A* **106**, 3166-71 (2009).

319. Dong, Y. *et al.* Regulation of BRCC, a holoenzyme complex containing BRCA1 and BRCA2, by a signalosome-like subunit and its role in DNA repair. *Mol Cell* **12**, 1087-99 (2003).
320. Chen, X., Arciero, C.A., Wang, C., Broccoli, D. & Godwin, A.K. BRCC36 is essential for ionizing radiation-induced BRCA1 phosphorylation and nuclear foci formation. *Cancer Res* **66**, 5039-46 (2006).
321. Jensen, D.E. *et al.* BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene* **16**, 1097-112 (1998).
322. Eletr, Z.M. & Wilkinson, K.D. An emerging model for BAP1's role in regulating cell cycle progression. *Cell Biochem Biophys* **60**, 3-11 (2011).
323. Scheuermann, J.C. *et al.* Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB. *Nature* **465**, 243-7 (2010).
324. Ventii, K.H. *et al.* BRCA1-associated protein-1 is a tumor suppressor that requires deubiquitinating activity and nuclear localization. *Cancer Res* **68**, 6953-62 (2008).
325. Tyagi, S., Chabes, A.L., Wysocka, J. & Herr, W. E2F activation of S phase promoters via association with HCF-1 and the MLL family of histone H3K4 methyltransferases. *Mol Cell* **27**, 107-19 (2007).
326. Nishikawa, H. *et al.* BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity. *Cancer Res* **69**, 111-9 (2009).
327. Mallery, D.L., Vandenberg, C.J. & Hiom, K. Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO J* **21**, 6755-62 (2002).
328. Machida, Y.J., Machida, Y., Vashisht, A.A., Wohlschlegel, J.A. & Dutta, A. The deubiquitinating enzyme BAP1 regulates cell growth via interaction with HCF-1. *J Biol Chem* **284**, 34179-88 (2009).
329. Misaghi, S. *et al.* Association of C-terminal ubiquitin hydrolase BRCA1-associated protein 1 with cell cycle regulator host cell factor 1. *Mol Cell Biol* **29**, 2181-92 (2009).
330. Pereg, Y. *et al.* Ubiquitin hydrolase Dub3 promotes oncogenic transformation by stabilizing Cdc25A. *Nat Cell Biol* **12**, 400-6 (2010).
331. Nicassio, F. *et al.* Human USP3 is a chromatin modifier required for S phase progression and genome stability. *Curr Biol* **17**, 1972-7 (2007).
332. Wei, N. & Deng, X.W. The COP9 signalosome. *Annu Rev Cell Dev Biol* **19**, 261-86 (2003).
333. Cope, G.A. & Deshaies, R.J. COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. *Cell* **114**, 663-71 (2003).
334. Spataro, V. *et al.* Resistance to diverse drugs and ultraviolet light conferred by overexpression of a novel human 26 S proteasome subunit. *J Biol Chem* **272**, 30470-5 (1997).
335. Yu, H. *et al.* The ubiquitin carboxyl hydrolase BAP1 forms a ternary complex with YY1 and HCF-1 and is a critical regulator of gene expression. *Mol Cell Biol* **30**, 5071-85 (2010).

G) ANNEX

G) ANNEX

Proteins	Antibodies	Company/reference	Catalog #	Application and dilution
BRCA1	MS110 (Ab-1)	Calbiochem	OP92	Western blot 1/100 Immunofluorescence 1/100
BRCA1	I-20	Santa Cruz	Sc-646	Immunoprecipitation 1/25
BRCA1	SD188	*		Western Blot 1/20
BRCA1	Poly	**		Western Blot 1/2000
BARD1	2059C4a	Santa Cruz	Sc-81195	Western blot 1/100
β-actin	Anti-Actin clone 4	Millipore	MAB1501	Western 1/500000
RPA	9H8	Abcam	Ab2175	Immunofluorescence 1/1000
Rad51	H-92	Santa Cruz	Sc-8349	Western blot 1/200 Immunofluorescence 1/500
CDC6	H-304	Santa Cruz	Sc-8341	Western blot 1/200
Caspase-3	H-277	Santa Cruz	Sc-7148	Western blot 1/200
PARP-1	F2	Santa Cruz	Sc-8007	Western blot 1/1000
GFP	B2	Santa Cruz	Sc-9996	Western blot 1/200
Ubiquitin	P4D1	Santa Cruz	Sc-8017	Western blot 1/200
γH2A.X	Anti-phospho-histone H2A.X (ser139) clone JBW301	Millipore	05-636	Immunoflorescence 1/1000
53BP1	H-300	Santa Cruz	Sc-22760	Immunoflorescence 1/1000

*Wilson C.A, Ramos L, Villasenor M.R, Anders K.H, Press M.F et al. (1999) Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinoma. Nat Genet 21: 236-240. [262]

**Sankaran S, Starita L.M, Simons A.M, Parvin J.D (2006). Identification of domains of BRCA1 critical for the ubiquitin dependent inhibition of centrosome function. Cancer Res. 66: 4100-4107. [263]

Table 6: List of antibodies used with their respective dilutions.

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

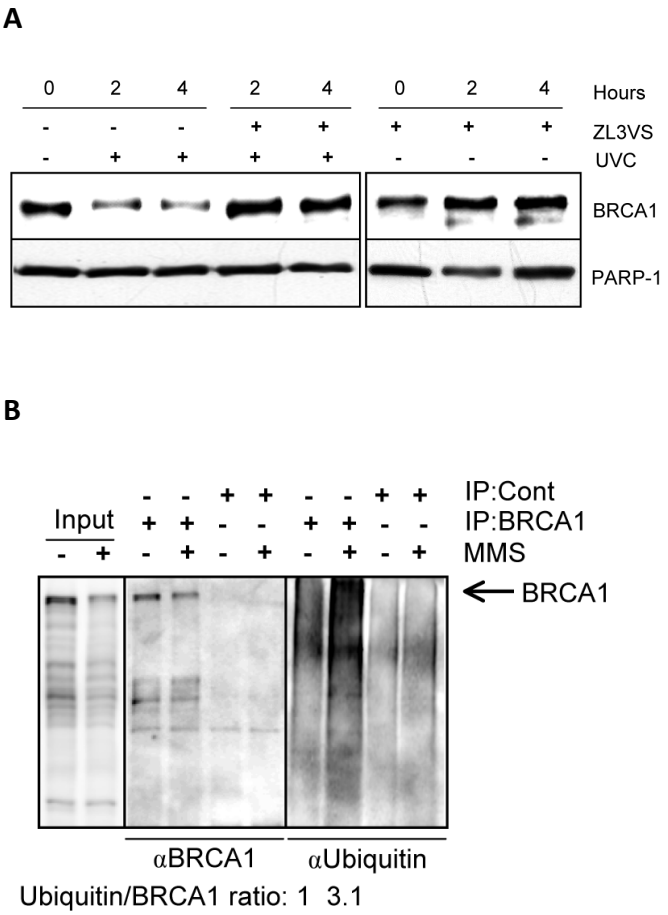


Figure 26: BRCA1 downregulation is mediated by the proteasome.

A) HeLa cells were treated with 20 μM of the proteasome inhibitor ZLV3S for 30 minutes prior to UVC treatment. The cells were harvested at the indicated time for immunoblotting. Detection of PARP-1 was used as a loading control.
B) Immunoprecipitation using HeLa cells with an antibody against BRCA1 or a non-related polyclonal rabbit IgG antibody as a control. Prior to immunoprecipitation, the cells were treated or not with 200 μM of MMS for 3 hours. The immunoprecipitated BRCA1 was then used for immunoblot with anti-BRCA1 or anti-ubiquitin. The ubiquitination of BRCA1 was quantified using the FUJI multi gauge imager.

Reference: Hammond-Martel I (2010) PLoS ONE 5 (11): e14027. [264]

DUB Si	BRCA1 foci	DUB Si	BRCA1 foci	DUB Si	BRCA1 foci	DUB Si	BRCA1 foci
BAP1	24	STAMBP	60	USP19	63	USP42	51
COPS5	52	STAMBPL1	58	USP2	41	USP43	45
CXORF53	45	TNFAIP3	53	USP20	39	USP44	44
CYLD	47	UBL3	54	USP21	54	USP45	48
DUB1A	44	UBL4	46	USP22	57	USP46	45
DUB3	52	UBL5	47	USP24	55	USP47	43
FBXO7	54	UBR1	56	USP25	51	USP48	58
FBXO8	47	UBTD1	52	USP26	51	USP49	53
FLJ14981	60	DC-UBP	62	USP27X	49	USP5	45
JOSD1	52	UCHL1	51	USP28	53	USP50	39
MJD	51	UCHL3	35	USP29	58	USP51	50
MYSM1	45	UCHL5	58	USP3	56	USP52	51
OTUB1	53	UMPK	56	USP30	48	USP53	51
OTUB2	55	UEVLD	52	USP31	47	USP54	51
OTUD1	47	UFD1L	48	USP32	46	USP6	51
OTUD4	53	USP1	49	USP33	51	USP7	48
OTUD5	49	USP10	65	USP34	51	USP8	53
OTUD6B	53	USP11	47	USP35	39	USP9X	51
OTUD7	55	USP12	50	USP36	49	USP9Y	47
ZA20D1	50	USP13	47	USP37	56	C13ORF22	50
PARP11	46	USP14	53	USP38	51	VCPIP1	51
PRPF8	54	USP15	46	USP39	42	YOD1	50
PSMD14	42	USP16	51	USP4	46	ZA20D1	60
SBBI54	41	USP17	57	USP40	53	ZRANB1	51
SENP2	42	USP18	57	USP41	51	Control	52

Table 7: siDUB screen in untreated U2OS cells for BRCA1 foci assembly

The values represent the percentage of cells with BRCA1 foci out of approximately 100 cells.

siDUB	BRCA1 foci	siDUB	BRCA1 foci	siDUB	BRCA1 foci	siDUB	BRCA1 foci
BAP1	49	STAMBP	71	USP19	64	USP42	69
COPS5	39	STAMBPL1	70	USP2	73	USP43	67
CXORF53	38	TNFAIP3	77	USP20	64	USP44	76
CYLD	42	UBL3	75	USP21	55	USP45	71
DUB1A	62	UBL4	75	USP22	73	USP46	68
DUB3	47	UBL5	69	USP24	68	USP47	70
FBXO7	70	UBR1	73	USP25	70	USP48	72
FBXO8	66	UBTD1	78	USP26	76	USP49	71
FLJ14981	67	DC-UBP	69	USP27X	70	USP5	66
JOSD1	65	UCHL1	87	USP28	81	USP50	66
MJD	67	UCHL3	72	USP29	85	USP51	45
MYSM1	72	UCHL5	70	USP3	83	USP52	66
OTUB1	80	UMPK	60	USP30	78	USP53	70
OTUB2	65	UEVLD	61	USP31	78	USP54	67
OTUD1	68	UFD1L	71	USP32	81	USP6	75
OTUD4	69	USP1	63	USP33	52	USP7	70
OTUD5	81	USP10	81	USP34	68	USP8	69
OTUD6B	71	USP11	61	USP35	65	USP9X	64
OTUD7	70	USP12	58	USP36	46	USP9Y	54
ZA20D1	67	USP13	70	USP37	69	C13ORF22	74
PARP11	79	USP14	65	USP38	81	VCPIP1	83
PRPF8	62	USP15	72	USP39	75	YOD1	63
PSMD14	45	USP16	73	USP4	73	ZA20D1	90
SBBI54	60	USP17	61	USP40	68	ZRANB1	68
SENP2	76	USP18	66	USP41	52	Control	71

Table 8: siDUB screen in MMS-treated U2OS cells for BRCA1 foci assembly

The values represent the percentage of cells with BRCA1 foci out of approximately 100 cells.

DUB Si	γH2A.X foci	DUB Si	γH2A.X foci	DUB Si	γH2A.X foci	DUB Si	γH2A.X foci
BAP1	95	STAMBP	97	USP19	95	USP42	100
COPS5	100	STAMBPL1	97	USP2	22	USP43	97
CXORF53	91	TNFAIP3	100	USP20	98	USP44	99
CYLD	93	UBL3	98	USP21	100	USP45	98
DUB1A	98	UBL4	100	USP22	100	USP46	99
DUB3	100	UBL5	100	USP24	71	USP47	99
FBXO7	97	UBR1	98	USP25	98	USP48	97
FBXO8	98	UBTD1	100	USP26	98	USP49	97
FLJ14981	97	DC-UBP	96	USP27X	96	USP5	96
JOSD1	97	UCHL1	100	USP28	97	USP50	98
MJD	100	UCHL3	98	USP29	96	USP51	97
MYSM1	100	UCHL5	100	USP3	98	USP52	92
OTUB1	98	UMPK	100	USP30	93	USP53	100
OTUB2	98	UEVLD	100	USP31	99	USP54	96
OTUD1	98	UFD1L	97	USP32	100	USP6	98
OTUD4	99	USP1	100	USP33	97	USP7	97
OTUD5	100	USP10	100	USP34	100	USP8	100
OTUD6B	98	USP11	100	USP35	97	USP9X	99
OTUD7	100	USP12	100	USP36	100	USP9Y	98
ZA20D1	98	USP13	97	USP37	99	C13ORF22	100
PARP11	98	USP14	97	USP38	99	VCPIP1	97
PRPF8	99	USP15	99	USP39	95	YOD1	95
PSMD14	95	USP16	94	USP4	97	ZA20D1	96
SBBI54	98	USP17	100	USP40	95	ZRANB1	100
SENP2	98	USP18	92	USP41	97	Control	100

Table 9: siDUB screen in MMS-treated U2OS cells for γH2A.X foci assembly

The values represent the percentage of cells with γH2A.X foci out of approximately 100 cells.

DUB Si	BRCA1 foci	DUB Si	BRCA1 foci	DUB Si	BRCA1 foci	DUB Si	BRCA1 foci	DUB Si
BAP1	35	STAMBP	51	USP19	60	USP19	USP42	55
COPS5	41	STAMBPL1	32	USP2	64	USP2	USP43	59
CXORF53	38	TNFAIP3	62	USP20	59	USP20	USP44	56
CYLD	50	UBL3	62	USP21	61	USP21	USP45	58
DUB1A	56	UBL4	57	USP22	59	USP22	USP46	58
DUB3	33	UBL5	40	USP24	60	USP24	USP47	63
FBXO7	66	UBR1	75	USP25	63	USP25	USP48	72
FBXO8	64	UBTD1	73	USP26	58	USP26	USP49	72
FLJ14981	65	DC-UBP	72	USP27X	59	USP27X	USP5	60
JOSD1	63	UCHL1	51	USP28	70	USP28	USP50	58
MJD	61	UCHL3	60	USP29	71	USP29	USP51	66
MYSM1	-*	UCHL5	56	USP3	47	USP3	USP52	55
OTUB1	66	UMPK	61	USP30	58	USP30	USP53	64
OTUB2	55	UEVLD	42	USP31	33	USP31	USP54	56
OTUD1	62	UFD1L	59	USP32	65	USP32	USP6	49
OTUD4	64	USP1	65	USP33	56	USP33	USP7	64
OTUD5	50	USP10	62	USP34	56	USP34	USP8	63
OTUD6B	66	USP11	63	USP35	62	USP35	USP9X	57
OTUD7	48	USP12	68	USP36	56	USP36	USP9Y	59
ZA20D1	63	USP13	61	USP37	61	USP37	C13ORF22	50
PARP11	64	USP14	66	USP38	75	USP38	VCPIP1	60
PRPF8	62	USP15	53	USP39	57	USP39	YOD1	57
PSMD14	41	USP16	55	USP4	61	USP4	ZA20D1	63
SBBI54	63	USP17	64	USP40	64	USP40	ZRANB1	78
SENP2	34	USP18	60	USP41	57	USP41	Control	63

Table 10: siDUB screen in IR-treated U2OS cells for BRCA1 foci assembly

(*: Cells weren't properly permeabilized)

The values represent the percentage of cells with BRCA1 foci out of approximately 100 cells.

DUB Si	RAD51 foci	DUB Si	RAD51 foci	DUB Si	RAD51 foci	DUB Si	RAD51 foci
BAP1	32	STAMBP	25	USP19	53	USP42	52
COPS5	35	STAMBPL1	23	USP2	55	USP43	30
CXORF53	54	TNFAIP3	47	USP20	53	USP44	45
CYLD	48	UBL3	44	USP21	52	USP45	44
DUB1A	51	UBL4	36	USP22	48	USP46	47
DUB3	38	UBL5	43	USP24	41	USP47	59
FBXO7	53	UBR1	50	USP25	51	USP48	54
FBXO8	54	UBTD1	49	USP26	47	USP49	58
FLJ14981	50	DC-UBP	53	USP27X	50	USP5	46
JOSD1	55	UCHL1	62	USP28	46	USP50	52
MJD	53	UCHL3	44	USP29	49	USP51	49
MYSM1	-*	UCHL5	52	USP3	49	USP52	54
OTUB1	33	UMPK	53	USP30	48	USP53	61
OTUB2	44	UEVLD	50	USP31	47	USP54	48
OTUD1	51	UFD1L	54	USP32	46	USP6	46
OTUD4	51	USP1	46	USP33	46	USP7	51
OTUD5	30	USP10	46	USP34	46	USP8	54
OTUD6B	38	USP11	46	USP35	54	USP9X	48
OTUD7	50	USP12	60	USP36	53	USP9Y	55
ZA20D1	46	USP13	54	USP37	53	C13ORF22	44
PARP11	55	USP14	57	USP38	52	VCPIP1	52
PRPF8	46	USP15	53	USP39	52	YOD1	60
PSMD14	31	USP16	55	USP4	33	ZA20D1	50
SBBI54	46	USP17	45	USP40	40	ZRANB1	64
SENP2	44	USP18	45	USP41	51	Control	51

Table 11: siDUB screen IR-treated U2OS Cells for Rad51 foci assembly

(*: Cells weren't properly permeabilized)

The values represent the percentage of cells with Rad51 foci out of approximately 100 cells.

DUB Si	yH2A.X foci	DUB Si	yH2A.X foci	DUB Si	yH2A.X foci	DUB Si	yH2A.X foci
BAP1	87	STAMBP	84	USP19	83	USP42	86
COPS5	88	STAMBPL1	82	USP2	93	USP43	91
CXORF53	90	TNFAIP3	92	USP20	84	USP44	90
CYLD	89	UBL3	91	USP21	82	USP45	86
DUB1A	94	UBL4	89	USP22	77	USP46	88
DUB3	39	UBL5	84	USP24	84	USP47	86
FBXO7	96	UBR1	91	USP25	89	USP48	85
FBXO8	96	UBTD1	83	USP26	80	USP49	86
FLJ14981	98	DC-UBP	85	USP27X	93	USP5	80
JOSD1	95	UCHL1	90	USP28	90	USP50	89
MJD	94	UCHL3	94	USP29	84	USP51	88
MYSM1	91	UCHL5	85	USP3	91	USP52	89
OTUB1	93	UMPK	87	USP30	89	USP53	92
OTUB2	88	UEVLD	85	USP31	91	USP54	80
OTUD1	92	UFD1L	87	USP32	92	USP6	90
OTUD4	91	USP1	91	USP33	95	USP7	88
OTUD5	80	USP10	94	USP34	95	USP8	89
OTUD6B	85	USP11	85	USP35	90	USP9X	92
OTUD7	89	USP12	91	USP36	89	USP9Y	84
ZA20D1	95	USP13	93	USP37	86	C13ORF22	86
PARP11	87	USP14	87	USP38	89	VCPIP1	89
PRPF8	93	USP15	93	USP39	82	YOD1	94
PSMD14	92	USP16	91	USP4	89	ZA20D1	94
SBBI54	77	USP17	87	USP40	85	ZRANB1	97
SENP2	87	USP18	85	USP41	85	Control	90

Table 12: siDUB screen in IR-treated U2OS cells for γH2A.X foci assembly

The values represent the percentage of cells with γH2A.X foci out of approximately 100 cells.

DUB si	53BP1foci	DUB si	53BP1 foci	DUB si	53BP1 foci	DUB si	53BP1 foci
BAP1	75	STAMBP	80	USP19	80	USP42	80
COPS5	77	STAMBPL1	79	USP2	80	USP43	76
CXORF53	85	TNFAIP3	83	USP20	72	USP44	81
CYLD	85	UBL3	79	USP21	84	USP45	80
DUB1A	78	UBL4	77	USP22	68	USP46	75
DUB3	55	UBL5	78	USP24	73	USP47	74
FBXO7	73	UBR1	70	USP25	75	USP48	74
FBXO8	81	UBTD1	74	USP26	68	USP49	76
FLJ14981	80	DC-UBP	69	USP27X	81	USP5	64
JOSD1	85	UCHL1	66	USP28	75	USP50	80
MJD	80	UCHL3	74	USP29	79	USP51	80
MYSM1	83	UCHL5	80	USP3	81	USP52	81
OTUB1	80	UMPK	81	USP30	75	USP53	75
OTUB2	81	UEVLD	85	USP31	90	USP54	81
OTUD1	86	UFD1L	89	USP32	82	USP6	74
OTUD4	84	USP1	85	USP33	78	USP7	71
OTUD5	81	USP10	93	USP34	78	USP8	78
OTUD6B	81	USP11	86	USP35	73	USP9X	84
OTUD7	79	USP12	85	USP36	68	USP9Y	78
ZA20D1	86	USP13	84	USP37	77	C13ORF22	83
PARP11	76	USP14	80	USP38	69	VCPIP1	74
PRPF8	83	USP15	83	USP39	72	YOD1	79
PSMD14	85	USP16	85	USP4	81	ZA20D1	78
SBBI54	69	USP17	76	USP40	83	ZRANB1	83
SENP2	80	USP18	75	USP41	78	Control	81

Table 13: siDUB Screen in IR-treated U2OS cells for 53BP1 foci assembly

The values represent the percentage of cells with 53BP1 foci out of approximately 100 cells.

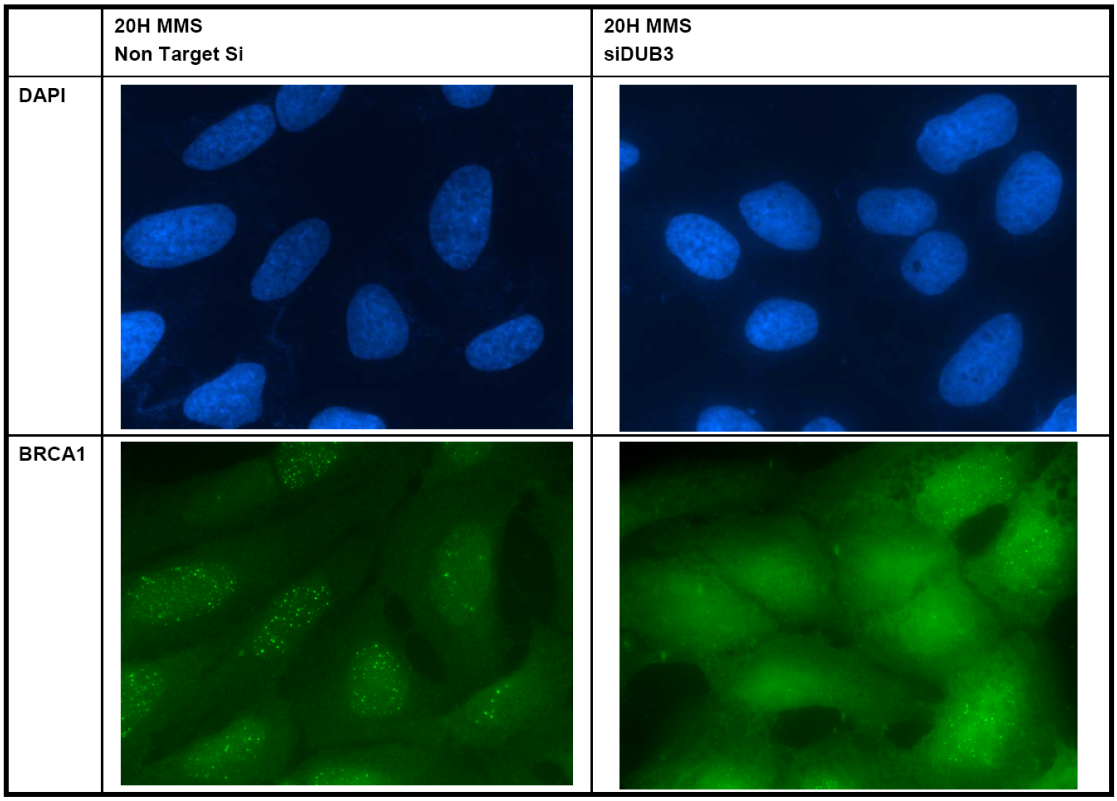
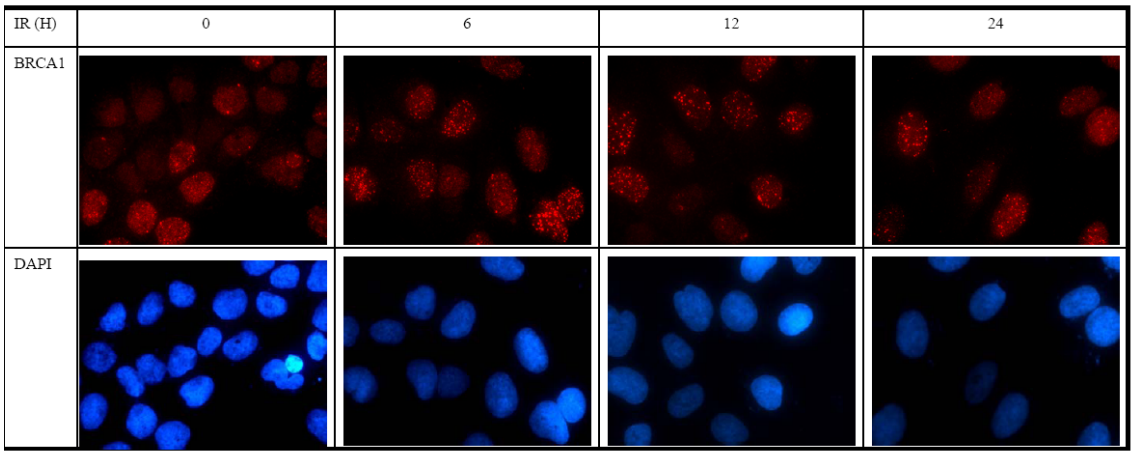


Figure 27: BRCA1 foci assembly in MMS-treated U2OS cells following DUB3 depletion.

An immunostaining for BRCA1 foci after siDUB3 in MMS-treated U2OS cells is used as an example to represent a hit.



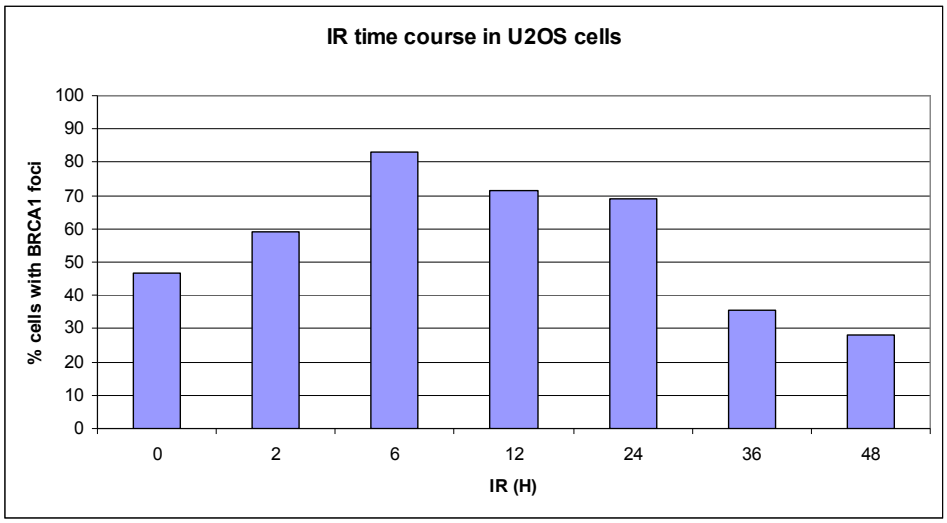


Figure 28: Immunofluorescence for BRCA1 foci assembly in IR-treated U2OS cells

U2OS cells were treated with IR and fixed at the indicated time for immunofluorescence detecting BRCA1. Approximately 100 cells were counted to determine the percentage of cells with BRCA1 foci and were shown as a histogram.