Proteomics and Functional Investigation of SUMO and Ubiquitin E3 ligases

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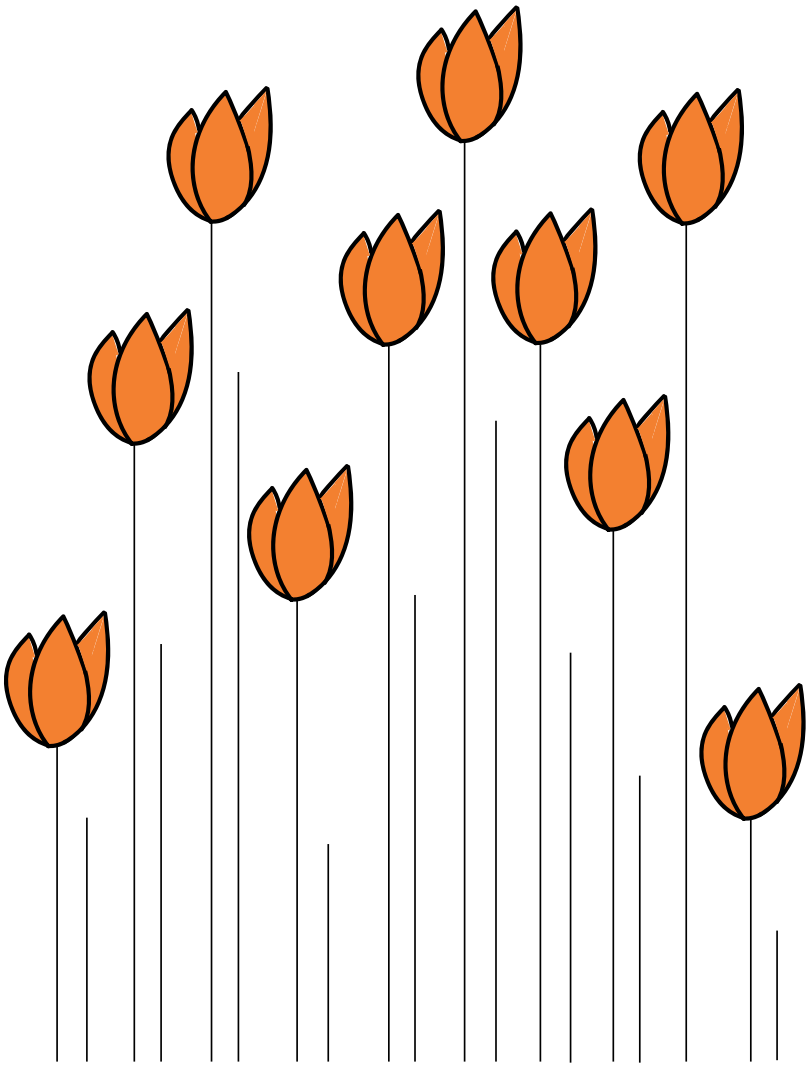

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Unveiling BRCA1-BARD1 ubiquitin ligase heterodimer. DNA repair, Ubiquitin and Cancer

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Abstract

Double strands breaks (DSBs) are the major source of genetic instability. Cells are able to sense the damage and develop a quick and accurate DNA damage response (DDR), which normally ends fixing the DSB. One of the fastest ways to give a response is by post-translational modification (PTM) of proteins. Ubiquitination is a PTM that governs the DDR, from the beginning to the end. E3 ubiquitin ligases are key players during the DDR and their dysregulation is associated to cancer. How different E3 ligases work together to regulate the spread, the repair pathway choice and the termination of the DDR is graphically depicted in this review. We focus on BRCA1/BARD1 as a multifunctional E3 ligase with crucial roles in DNA damage repair and tumor suppression. Here, we decipher the, yet controversial, role of BRCA1/BARD1 E3 ligase activity in homologous recombination and the possible roles on DNA replication and anti-tumorigenesis. Finally, we discuss novel strategies to target the ubiquitination machinery during the DDR and the future directions in the field.

Keywords: DNA damage; Ubiquitin; E3 ligases; BRCA1/BARD1 heterodimer

1. INTRODUCTION

The maintenance of genome integrity is crucial to avoid genetic alterations, from chromosome rearrangements to point mutations, that are associated with pathological disorders, premature aging, inherited diseases and cancer (1). DNA is constantly threatened by exogenous agents such as ionizing radiation (IR), mutagenic chemicals or UV light, as well as endogenous agents such as free radicals, single stranded DNA (ssDNA) and DNA replication problems, resulting in more than 70,000 lesions per cell per day (2). DNA replication is frequently challenged with obstructions of the DNA polymerase (DNA secondary structures, R-loops, etc) and transcription-replication conflicts that could cause replication fork stalling or collapse. Failure to restart stalled replication forks and difficulties resolving single strand breaks (SSB), can lead to double strand breaks (DSBs), which are the most harmful DNA lesions.

Fortunately, cells are able to sense the DNA damage and elaborate a fast and accurate response to solve the lesion. During this process ubiquitination is crucial not only to recruit downstream proteins to DNA damage sites, but also to repress transcription, choose the repair pathway and control the lifespan of proteins during the repair. As key player with E3 ligase activity in the repair of DSB, BRCA1/BARD1 heterodimer is known as tumor suppressor and germline mutations in either BRCA1 or BARD1 lead to development of breast and ovarian cancer (3). However, its E3 activity is not well-defined neither during DSB repair nor as tumor suppressor.

In this review, we will focus on ubiquitination as the key PTM that governs the DDR; the interplay of E3 ligases to achieve proper DNA repair; and the role of BRCA1/BARD1 E3 activity in homologous recombination, replication and tumor suppression.

2. UBIQUITINATION REGULATING THE DDR

In order to repair DSBs, cells are equipped with a plethora of conserved DNA damage sensing and repair mechanisms that combined are known as the DNA damage response (DDR) (13). DDR is tightly regulated by PTMs, including phosphorylation, SUMOylation, methylation and ubiquitination (14, 15). The cross-talk between PTMs can manage the whole DDR. The damage is sensed by kinases which produce phosphorylation cascades in seconds. The continuity of the DDR relies on the recruitment of proteins to DNA damage sites through polyubiquitination chains and scaffold proteins. The choice of repair pathway is influenced by histone modifications, as one modification not only leads to a specific repair pathway but inhibits another one. Finally, the shutdown of the response is also managed by PTMs, where in all mentioned steps, ubiquitination and E3 ligases play a crucial role to successfully repair the DNA damage.

2.1 EARLY STAGE

The first step to sense DNA damage and initiate the DDR is the recruitment of proteins to DNA damage sites, such as DSBs. ATM kinase is a main player in sensing the damage. It is responsible for downstream substrates activation, such as p53, BRCA1 and 53BP1 which will lead to DNA repair, cell cycle progression or apoptosis (16). ATM phosphorylates p53 for its recruitment to chromatin and also the E3 ligase that regulates p53 stability, mouse double minute 2 (MDM2). MDM2 controls p53 levels by proteasomal degradation and its overexpression is related to several cancers (17, 18). ATM-mediated phosphorylation of MDM2 upon DNA damage, results in the

inhibition of its E3 activity against p53. Thus MDM2 phosphorylation allows p53 stabilization at DNA damage sites (19).

Another player recognizing DSBs is the MRN complex (Mre11, Rad50 and NBS1) which is responsible for the initial DNA end resection at DSBs. ATM associates with the MRN complex to promote Histone H2AX phosphorylation (γH2AX) that will be propagated along the DSB. This histone phosphorylation functions as DNA damage mark, and several proteins are recruited to damage sites using γH2AX as a platform (20). One of the proteins that is recruited to DNA damage sites through γH2AX is Mediator of DNA damage Checkpoint protein 1 (MDC1), that interacts with γH2AX by its BRCT domain. Once it is recruited, ATM phosphorylates MDC1, which is necessary for the recruitment of one of the first ubiquitin E3 ligases, RNF8 (21).

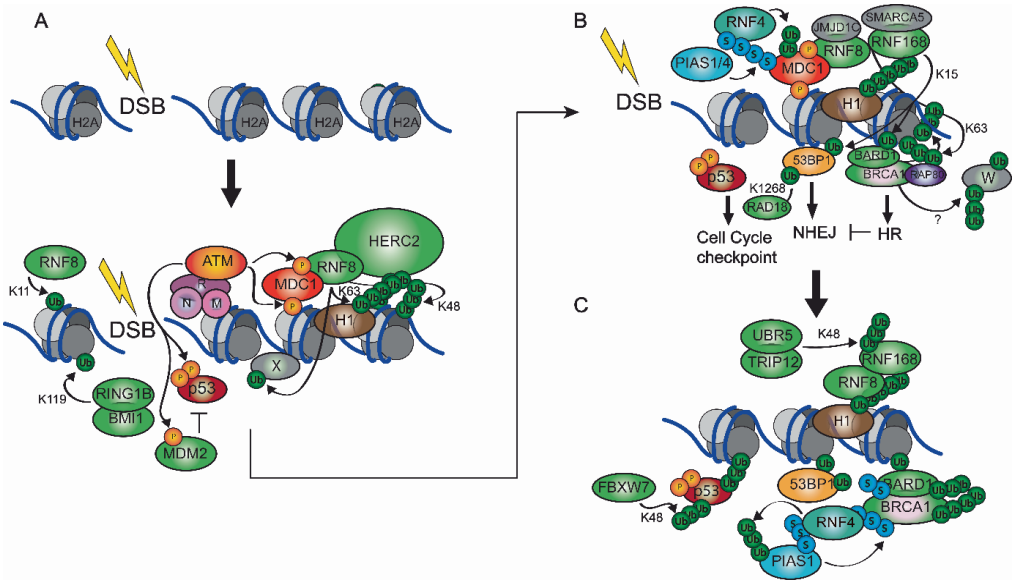


Figure 1. E3 ligases in DDR. DDR after DSBs displaying the role of several E3 ligases (green) with kinases (red) and SUMO E3 ligases (blue). **A.** Early stage where ATM and MRN complex recognize the DBS, ATM phosphorylates H2A and MDM2, it also promotes p53 and MDC1 recruitment to DSBs. MDM2 phosphorylation inhibits p53 degradation, leading to p53 stabilization at DSBs. MDC1 gets phosphorylated by ATM and promotes RNF8 recruitment, which promotes histone ubiquitination leading to RNF168 recruitment. **B.** RNF8-RNF168 stimulates additional histone ubiquitination necessary for the recruitment of downstream proteins such as 53BP1 and BRCA1/BARD1, which will lead to repair pathway choice and cell fate. **C.** Ending of the response, where UBR5 and TIP12 E3 ligases target RNF168 for degradation. STUbL RNF4 labels MDC1 and the SUMO machinery for its proteasomal degradation and E3 ligase FBXW7 targets p53 for degradation after DSBs repair.

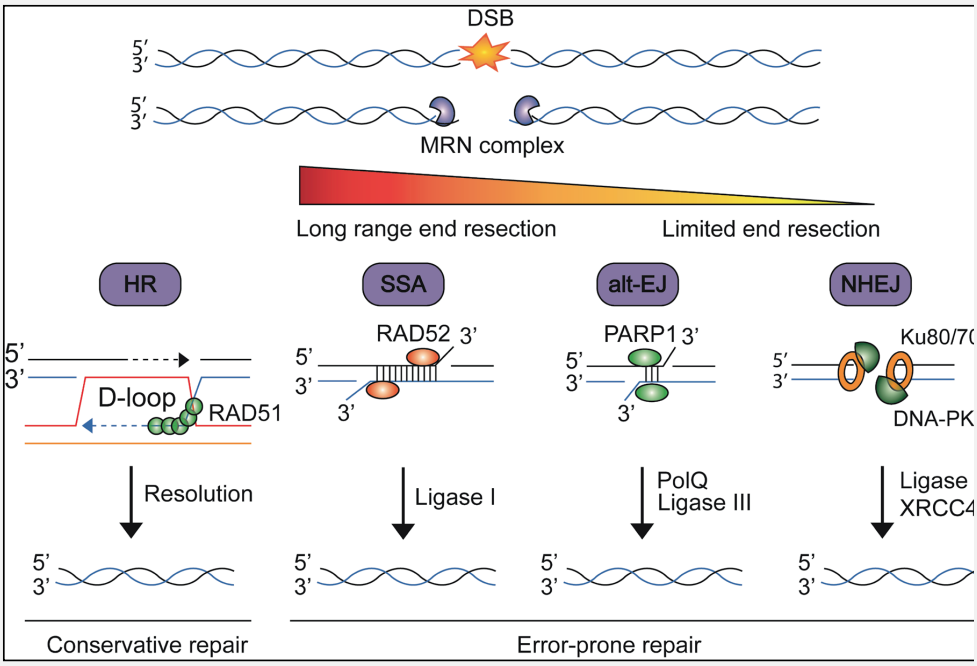
To assure proper DNA repair, RNF8 regulates DNA damage-induced transcription inhibition by K11 linkages on damaged chromatin, protecting damage sites from transcription-repair conflicts (22). Another E3 ligase with a possible role also in genome silencing after DNA damage is E3 BMI1-

RING1B, which is a subunit of the Polycomb Repressive Complex 1 (PRC1), and accumulates at DSB sites to locally increase H2A K119 monoubiquitination. It is recruited after H2AX phosphorylation and might also have a role during the DDR, as it remains at DNA damage sites for 8h post-damage. (23, 24). On the other hand, upon MDC1 interaction, RNF8 poly-K63 ubiquitinates H1 type linker histones, which is responsible for RNF168 recruitment through its motif interacting with ubiquitin (MIU) to amplify the signal (**Figure 1A**). However, spatiotemporal recruitment experiments and the identification of new ubiquitination substrates would be key to spot every E3 ligase and better understand how this ubiquitination network is formed around DNA damage sites.

Once RNF168 is recruited, together with RNF8, it stimulates additional histone ubiquitination and promote the accumulation of downstream proteins such as 53BP1 and BRCA1/BARD1 at DNA damage sites, which will lead to the repair pathway choice (**Figure 1B**) (**BOX 1. DNA repair pathways**) (25).

BOX 1. DNA repair pathways.

Once DNA damage is sensed and the DDR is active and propagated, the DSB must be repaired. Cells have developed several conserved but mechanistically different DSBs repair pathways, including Homologous Recombination (HR), non-homologous end joining (NHEJ), alternative end joining (alt-EJ) and single-strand annealing (SSA).



HR is an error-free repair pathway as it can faithfully restore the original configuration of the broken DNA molecule using the intact sister chromatid as template for repair. Therefore, HR is restricted to S and G2 phase of the cell cycle and requires DNA resection. In contrast, NHEJ, alt-EJ and SSA are error-prone repair pathways. Classical-NHEJ (cNHEJ) is considered the default mechanism for DSB repair in a cell cycle independent manner. Generally, cNHEJ does not require end-trimming and the resolution of the DNA damage typically comprises the deletion or insertion of a few nucleotides. Alt-EJ and SSA require DNA end resection and, in the case of SSA, no limit of end-resection has been determined yet. However, these mechanisms are prone to generate deletions and chromosomes translocations (8). The nucleotide depletion/insertion of the repair pathway can be monitored by employing CRISPR technologies with consequent sequencing analysis, giving rise to different mutational signatures (9).

2.2 MID-LATE STAGE

Before going through mitosis, DNA damage should be resolved and the DDR must come to an end. An efficient way to close a signaling pathway is by targeting its key regulators. One key player in the response, is RNF168. Two E3 ligases, UBR6 and TRIP12, cooperate together to efficiently control the downstream RNF168 events by targeting RNF168 to proteasomal degradation.

Depletion of these two E3 ligases results in supra-physiological accumulation of RNF168 and downstream DDR factors, compromising proper DNA repair (26).

The Sumo Targeting Ubiquitin Ligase (STUbL) RNF4 also plays an important role in this tightly regulated DDR, controlling the residence time of MDC1, which gets SUMOylated by PIAS1/4 and is subsequently labelled for proteasomal degradation by RNF4, controlling the response in a middle stage (27). Persistence of MDC1 prevents downstream signaling through the HR pathway. Therefore, MDC1 removal is required prior to CtIP and RAD51 recruitment (**Figure 3**) (28). In previous work, RNF168 was also found as RNF4 target for proteasomal degradation (29, 30) involving RNF4 in the regulation of RNF168. Together with the fact that BRCA1/BARD1 is SUMOylated upon DNA damage by PIAS1/4 and that RNF4 targets the SUMOylation machinery for proteasomal degradation (29, 30), RNF4 might be also a key factor in the closure of the DDR.

Finally, other proteins like ubiquitin-selective chaperone/ segregase (VCP/p97) have a role regulating the spatiotemporal localization of the recruited chromatin-associated proteins (31) (**Figure 1C**). The DDR is orchestrated by crosstalk between PTMs and it is tightly regulated in space and time. We display the role of main E3 ligases in DDR (**Figure 1**) (**Table 1**). However, more and more E3 ligases are emerging in the DDR field, such as Pellino 1, which appears to interact with phosphorylated p53 (32) (**BOX 2. Other E3 ligases in DDR**). Additionally, new roles are being contributed to the E3 ligases we mentioned above. For example, RNF168 has been recently involved in the recruitment of SLX4 for inter-strand crosslink (ICL) damage repair (33). This denotes that E3 ligases are key regulators in the DDR and their dysregulation may lead to cancer and other diseases.

3. BRCA1/BARD1 AS UBIQUITIN E3 LIGASE

The breast cancer susceptibility gene 1 (BRCA1) and its obligated partner BRCA1-associated RING domain protein 1 (BARD1) form a heterodimer through a four-helix bundle flanking their Really Interesting New Gene (RING) domains (34). Despite BRCA1 is a large protein that participates in multiple cellular activities through forming different complexes with other proteins (35), the E3 ligase activity coming from BARD1 interaction is the only known intrinsic enzymatic activity of this heterodimer. In vitro experiments might show independent E3 ligase activity for each partner, although the formation of the heterodimer exhibits substantially greater E3 ligase activity (36, 37). However, it is generally accepted that BARD1 does not possess inherent E3 ligase activity as it lacks the alpha-helix required for binding to the E2 (38). The residual BARD1-E3 activity might come from the presence of either endogenous BRCA1 or another E3 after immunoprecipitation from cell lysates (37). Later, cryo-EM structures showed that BRCA1 RING domain preferentially binds the E2 while BARD1 RING domain shows higher affinity to the substrate during H2A ubiquitination (**Figure 2**) (38, 39).

Table 1. E3 ligases during the DDR.

E3 Ligase	DDR Stage	Target	Linkage	Function	Ref.
RNF8	Early	H2A/H2AX H1	K11 K63	Transcription inhibition Recruitment	(22) (21)
BMI1/PRC1	Early	H2A	Mono	Genome silencing	(24)
MDM2	Early	P53	?	Cell cycle	(2)
HERC2	Early	H2A/H2AX P53	K63	Recruitment Cell cycle	(1) (2)
RNF168	Medium	H2A/H2AX	K63 Mono	Recruitment DNA signalling BRCA1/BARD1	(21) (43) (47)
BRCA1/BARD1	Medium	H2A/H2AX mH2A	Mono	HR Cellular senescence	(39) (105)
RAD18	Medium	53BP1	Mono	NHEJ	(4)
UBR6	Late	RNF168	K48?	DDR regulation	(26)
TRIP12	Late	RNF168	K48?	DDR regulation	(26)
FBXW7	Late	P53	K48	Cell cycle and DDR	(5)
PELLINO1	Late	?	?	Cell cycle and DDR	(32)
RNF4	Medium Late	MDC1 RNF168	K48? K48?	DDR regulation DDR regulation	(28) (29)

3.1 BRCA1/BARD1 IN REPAIR PATHWAY CHOICE

BRCA1 and 53BP1 are essentially engaged in a tug of war that determines commitments to two different DSBs repair pathways, HR or NHEJ, where the cell cycle plays a crucial role (40) (**BOX 1. DNA Repair Pathways**). BRCA1-RAP80 interacts through its UIMs with K63-linked ubiquitin facilitating the recruitment to DNA damage sites.

Furthermore, knocking down either RAP80, RNF8 or RNF168 prevents BRCA1/BARD1 recruitment to DSBs (41-43). A recent study, using RAP80 CRISPR knock outs (RAP80-KO), found that RAP80-BRCA1 complex is dispensable for the initial recruitment of BRCA1 to DSB and the BRCA1 RING domain is critical for the recruitment in RAP80 deficient cells (44). IR-induced foci localization showed that the BRCA1 recruitment to DSB was completely dependent on RNF8 and partially dependent on RNF168, but displayed a near-normal recruitment in 7 independent RAP80-KO cell lines. Previous data also corroborate the importance of BRCA1 RING domain as mutation of K70/K71 in BRCA1 specifically disrupts the ability of the RING domain to bind to the nucleosome acidic path (45). Likewise, this result is supported by a recent study where RNF168 seems responsible for localizing the BRCA1-PALB2 complex in DSBs (46). These findings propose two possible ways of BRCA1 recruitment, being RNF168-mediated monoubiquitination on H2AK13/15 the predominant pathway, while BRCA1-RAP80 complex contributes as backup pathway.

BOX 2. Other E3 ligases in DDR

Another E3 ligase that aids RNF8 for initiating the recruitment of downstream proteins, is the large HECT-type ligase (HERC2), which interacts with RNF8 to promote downstream ubiquitination at DSB sites. Most of the ubiquitination chains formed by RNF8 and HERC2 are on K63 (1). Recent studies propose HERC2 E3 as regulator of the p53-MDM2 pathway. HERC2 might form a complex with MDM2 that ubiquitinates p53 for proteasomal degradation. Upon DNA damage and consequent MDM2 phosphorylation, the complex is dissociated and p53 is active at DSBs (2).

RNF8-mediated K63 polyubiquitylation also leads to the recruitment of another ubiquitin E3 ligase, RAD18, in a ubiquitin binding domain (UBD) dependent manner. RAD18 mediates RAD9 recruitment through its RING and Zinc finger domains, which might play a role in DSB repair and downstream activation of checkpoints (3). Another possible role of RAD18 in DSB is the monoubiquitination of 53BP1 at K1268, which may retain 53BP1 at DNA damage sites during G1-phase promoting NHEJ repair pathway (4) (**Figure 1B**).

Recently, the role of FBXW7 E3 ligase has been reported to ensure proper DNA repair. ATM is responsible for p53 phosphorylation at S33 and S37 in response to DSBs, as mentioned above. This phosphorylation is required for the interaction with substrate recognition component of FBXW7 that mediates p53 ubiquitination for proteasome degradation once it has been recruited to DNA damaged sites (5) (**Figure 1C**). For regulating cell cycle checkpoints and inducing apoptosis when the DNA damage cannot be solved, p53 is known as the “guardian of the genome”, thus it is not surprising that p53 levels must be precisely regulated under DSBs, letting DNA be repaired before continuing cell cycle progression. This mechanism is poorly understood compared with the widely investigated role of MDM2 targeting p53 for proteasomal degradation (6,7).

Recently, it has been shown how both BARD1 and 53BP1 can bind the same mono H2AK15Ub (**Figure 1B**), but subsequent post-replication histone modifications might govern the DSB repair choice (47). As shown before, the DDR is strongly regulated, and it is not surprising that other mechanisms collaborate to regulate BRCA1 and 53BP1 recruitment. It was reported that the TIP60 complex competes with RNF168 for H2AK15 modification. Acetylation by TIP60 inhibits RNF168 H2AK15 ubiquitination, disrupting the recruitment of 53BP1 and BRCA1/BARD1 to DSBs (48).

Additionally, not only H2A modification is involved in the recruitment of these proteins, but histone 4 (H4) methylation at lysine 20 (H4K20m) has been revealed as a key factor for BRCA1/BARD1 recruitment (47, 49). During G1 phase of the cell cycle, H4K20 is methylated and BRCA1/BARD1 cannot be recruited to DSBs. However, H4K20 is unmethylated right after replication allowing BRCA1/BARD1 recruitment and HR performance during S phase. In concordance with this findings, cryo-EM experiments showed how the Ankyrin and tandem BRCT (BUDR) domains of BARD1 can adopt a compact fold and bind nucleasomal histones, DNA and the monoubiquitin attached to H2A amino-terminal K13/15, to promote ubiquitination of the flexible carboxy-terminal tails of H2A and H2AX (**Figure 2**) (50). BRCA1/BARD1 not only binds and ubiquitinates H2A at K125/127/129 (50-52), but also blocks other ubiquitination events on H2A as K63 linkages, which are responsible for 53BP1 recruitment (50, 53). Deubiquitinating enzymes are also involved in this recruitment. BRCA1/BARD1-mediated H2A ubiquitination seems to be regulated by the deubiquitinating enzyme USP48, which acts as modulator antagonizing BRCA1/BARD1 E3 activity. Loss of USP48 resulted in further 53BP1 repositioning from the break site and extending resection lengths (51). The possible role of BRCA1/BARD1-mediated H2A ubiquitylation may reside in the eviction of 53BP1, which is an agonist of HR and promotes NHEJ repair, and continuation of the HR repair pathway (51, 54).

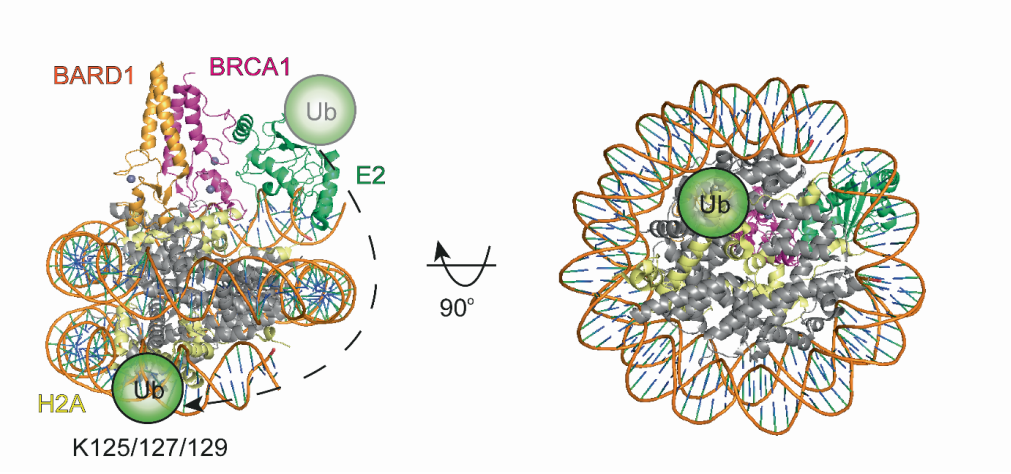


Figure 2. BRCA1-BARD1 RING domains interacting with the nucleosome. Structure obtained from PDB:7JZV. BRCA1 interacts with the E2 enzyme UbcH5c and BARD1 directs H2A ubiquitination at K125/127/129.

3.2 BRCA1/BARD1 IN HR

The first role of BRCA1/BARD1 in HR was related to the interaction between BRCA1 and the recombinase RAD51, which colocalized after ionizing radiation (IR) (55). Following upstream events of the HR pathway (**Figure 3**), BRCA1 has been related to DNA end resection. BRCA1 can form a complex with CtIP and MRN in a cell cycle dependent manner. CtIP associates with BRCA1 through the BRCT domains whereas MRN interacts with the N terminus of BRCA1. This complex promotes the essential steps of DNA resection by opposing the block on resection by 53BP1 and its effector proteins (56). The major evidence of E3 ligase activity involved in this process was observed in BARD1 knock down cells complemented with either a BARD1-WT or BARD1-R99E mutant version. Cell complemented with the BARD1-R99E showed decreased numbers, size and intensity of RPA foci compared with cells complemented with BARD1-WT (54). Additionally, it was reported that BRCA1 is required for CtIP ubiquitination upon DNA damage for G2/M checkpoint control (57). However, rescue experiments including a BRCA1 catalytic dead mutant could indicate whether CtIP ubiquitination depends on BRCA1 E3 activity.

In early years, most investigators assumed that the E3 activity of BRCA1/BARD1 would also be essential for BRCA1/BARD1-mediated HR. However, in 2008 it was reported that the HR function of BRCA1-I26A cells was indistinguishable from isogenic BRCA1-WT cells, as measured by RAD51 foci formation and recombination of a chromosomally-integrated HR reporter constructs (58). In 2016, these conclusions were challenged by the Morris laboratory, who reported that cells expressing the BARD1-R99E mutation, which also disrupts the E3 activity of BRCA1/BARD1 without impairing the heterodimer formation, are both HR deficient and PARPi hypersensitive (54).

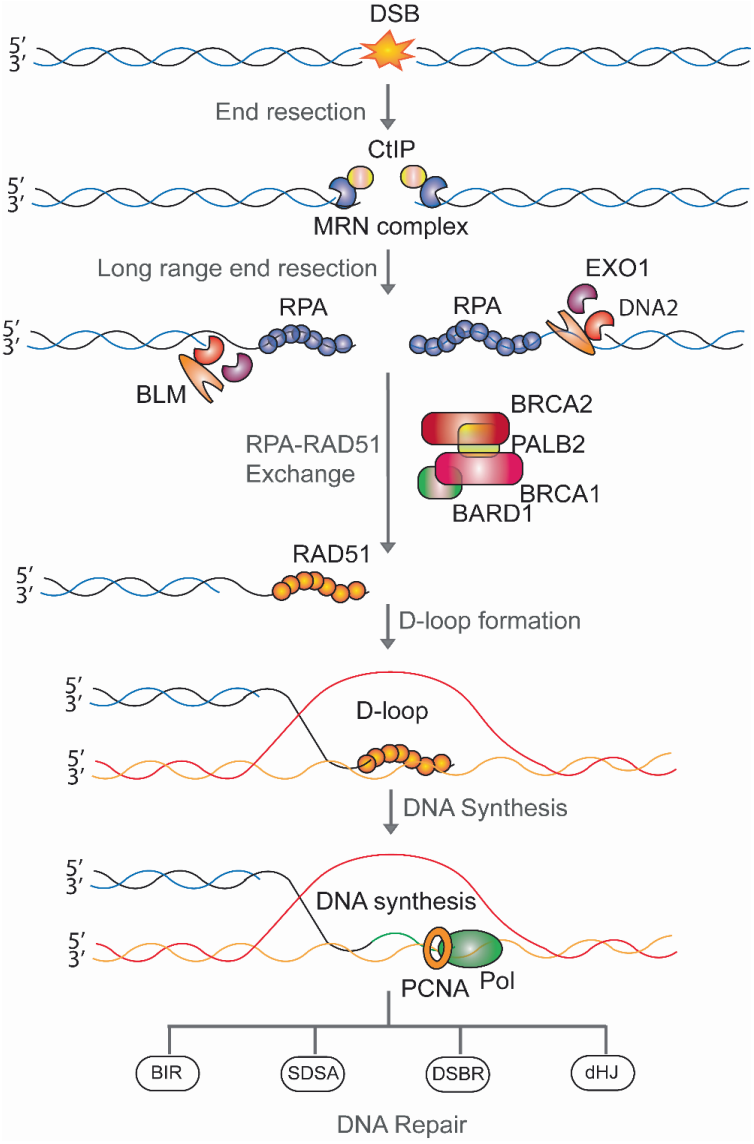


Figure 3. Homologous Recombination Pathway. Once the double strand break (DSB) is produced, BRCA1 interacts with CtIP and the MRN complex to initiate DNA end resection. Exonuclease 1 (EXO1), endonuclease DNA2 and the helicase BLM promote long range end resection. This process is also controlled by the BRCA1-ABRAXAS complex. Replication protein A (RPA) coats ssDNA for subsequent exchange with the recombinase RAD51. This process is mediated by the BRCA1-PALB2-BRCA2 complex. RAD51 will procced to DNA invasion and the formation of the D-loop. Then, DNA polymerase together with PCNA will synthesize new DNA for final resolution by synthesis-dependent single-strand annealing (SDSA), canonical double-strand break repair (DSBR), double Holliday junction (dHJ) resolution or dissolution and break-induced DNA replication (BIR).

Additionally, BRCA1-BARD1 E3 activity was associated to HR by the link between ubiquitination of histone H2A and IR-induced RAD51 foci formation. In the absence of BARD1, there was no presence of RAD51 foci formation, but the foci accumulation was rescued with a H2A-Ub variant and not with a H2A-WT or a Ub-H2A form, suggesting that BRCA1/BARD1-mediated H2A ubiquitination was essential for the progression of the HR pathway (54). In support, a more detailed mechanism was published using cryo-EM (50). BRCA1/BARD1 binds the N-terminus of H2A to produce C-terminus H2A ubiquitination, which seems to be important for the pathway choice and HR continuation (**Figure 4A**). However, HR deficiencies and PARPi hypersensitivity do not appear to be reproducible. Other laboratories observed no effect of the BARD1-R99E mutation on PARPi sensitivity (46, 49). Likewise, cells expressing another E3-impaired mutant (BRCA1-3A) were found to be fully competent for PARPi resistance and IR-induced RAD51 foci formation (44).

In their recent study, Sherker et al. (44), also reported that HR was fully proficient in cells expressing the BRCA1-3A mutant, while HR is abrogated in RAP80-null cells that express BRCA1-3A. Although the interaction between RAP80 and BRCA1/BARD1-mediated ubiquitination needs further validation and the relevant substrates remain unclear, this result might explain why one laboratory observed a requirement for the E3 activity in HR (54) while most laboratories do not (44, 46, 49, 58). Nonetheless, since the RAP80 complex is broadly expressed across mammalian tissues, loss of RAP80 functions seems to be either an artificial or very limited condition. Thus, it remains unclear whether BRCA1/BARD1-mediated ubiquitination is relevant to HR during either normal or malignant development. It may be possible to identify a physiological role by further analysis of BRCA1-I26A mice. Unlike BRCA1-C61G mice, which die early during embryogenesis, BRCA1-I26A mice only exhibit modest developmental defects, such as reduced body weight and male sterility due to a late block in spermatogenesis. These defects may point to the missing physiological functions of BRCA1/BARD1-mediated ubiquitination. Therefore, identifying BRCA1/BARD1-mediated ubiquitination targets would be a crucial step forward to resolving the enigmatic role of this E3 ligase.

3.3 BRCA1/BARD1 E3 LIGASE IN TUMOR SUPRESSION

BRCA1 is a well-known tumor suppressor protein (59) and since the RING domain is required for both its interaction with BARD1 and its E3 activity, the function of the enzymatic activity has been studied using carefully-designed separation-of-function mutations that specifically ablate the ligase activity without impairing heterodimer formation (**Figure 4A**).

Mutations affecting the RING domain of the heterodimer can be found in both partners and divided in two groups. On one hand, BARD1 L44R and BRCA1 C61G mutations disrupt the formation of the heterodimer leading not only to E3 ligase activity depletion but also to BRCA1 instability, resulting in its proteasomal degradation (54, 60) (**Figure 4A**). These mutations have been found in tumors (61, 62) and showed a tumorigenic profile in mice studies. BRCA1-C61G mice showed increased breast tumor formation similar to BRCA1 deficient mice (63). In human cells, BARD1-L44R and BRCA1-C61G shared similar HR and genotoxic stress phenotypes compared to BARD1 and BRCA1 deficient cells (54).

On the other hand, BARD1-R99E and BRCA1-I26A RING domain mutations disrupt the interaction with the E2 and abrogate the E3 ligase activity while keeping the heterodimer assembly

(38, 54) (**Figure 4A**). These mutations have not been found in tumors yet, and mice studies showed that the E3 ligase activity is dispensable for the suppression of tumorigenesis. In contrast to BRCA1-C61G mice that failed to suppress tumor development, BRCA1-I26A mice were able to suppress tumor formation (64). This conclusion is supported by previous and recent work in mouse embryonic stem cells and human cells, where the BRCA1-I26A mutation presented similar HR and genotoxic stress phenotypes compared to BRCA1-WT (46, 49, 58). Together, these studies argued that heterodimer formation, but not BRCA1/BARD1-mediated ubiquitination, is essential for tumor suppression.

Nevertheless, mutations in BARD1 (C53W/C71Y/C83R) that impair H2A ubiquitination have been identified in families afflicted with breast cancer (65). These mutations alter three zinc-binding residues in the BARD1 RING domain but allow the formation of the BRCA1/BARD1 heterodimer. Previous work suggested that BRCA1/BARD1-mediated H2A ubiquitination was involved in tumor suppression by the maintenance of the global heterochromatin integrity (66). By chromatin immunoprecipitation (ChIP), BRCA1 was observed to bind satellite DNA regions, and its deficiency was accompanied by de-repression of normally silenced genes and loss of H2A monoubiquitination in a murine model. Satellite DNA regions are normally transcriptionally repressed and α-satellite RNAs overexpression has been related to genomic instability and breast cancer development in mice (67). BRCA1/BARD1 E3 activity on H2A was shown by rescuing BRCA1 deficient cells with ectopic expression of H2A-Ub, which not only restored silencing but also proliferative and HR defects. Additionally, BRCA1 deficient cells were also reconstituted with either a BRCA1-WT or with a BRCA1-I26A mutant version. While BRCA1-WT reconstituted cells were able to rescue the ubiquitinated H2A accumulation, the BRCA1-I26A mutant failed to significantly enrich ubiquitinated H2A at satellite repeats, suggesting that H2A ubiquitination is specific for BRCA1/BARD1 and has an essential role in heterochromatin integrity and tumor suppression.

The main controversy comes from the non-tumorigenic role of BRCA1/BARD1 E3 activity and the tumorigenic role of BRCA1/BARD1-mediated H2A ubiquitination (**Figure 4A**). It would be possible that other E3 ligases could overcome histone H2A ubiquitination after BRCA1/BARD1 depletion or malfunctioning. Although there has been much speculation about the BRCA1/BARD1 E3 activity role in tumor suppression, to date only the BRCA1-I26A mutant has been examined in animals, which is the most appropriate setting to experimentally assess tumor suppression. Surprisingly, homozygous BRCA1-I26A mice displayed a very mild phenotype, unlike homozygous null or tumor-associated alleles mice, most of which suffer embryonic lethality. Likewise, a conditional mouse model of human triple-negative breast cancer carrying BRCA1-I26A retained the ability to suppress mammary tumor formation, in contrast to BRCA1-S1598F homozygous mice (63, 64). Since 2011, this result has not been challenged experimentally despite the data in human and mice cells proposing an antitumoral role of BRCA1/BARD1 E3 activity. Therefore, more studies employing mice models or organoids with well-defined BRCA1/BARD1 mutants are required to fully understand the potential role as tumor suppressor.

3.4 BRCA1-BARD1 E3 ACTIVITY IN REPLICATION

BRCA1/BARD1 has crucial roles in the repair and restart of stalled and damaged DNA replication forks and in their protection against nucleolytic attack. The first time BRCA1/BARD1 was associated with replication was in 1997 when breast cancer cells were subjected to hydroxyurea (HU) treatment, which inhibits ribonucleotide reductase, leading to depletion of nucleotides causing replication stress and fork stalling. In this study, BRCA1 and BARD1 colocalized with RAD51 and PCNA upon HU treatment, suggesting that BRCA1/BARD1 are present in stalled replication forks (68). Later, mass spectrometry studies employing isolation of proteins on nascent DNA (iPOND), placed BRCA1/BARD1 at ongoing and stalled replication forks upon HU treatment (69, 70). DNA fiber experiments showed a role of BRCA1/BARD1 preventing replication fork degradation also after HU treatment (71). Whether the E3 ligase activity of BRCA1/BARD1 has a role during fork protection is still uncertain. BRCA1 contains a S114-P115 regulatory region, and BARD1 contains a RAD51 binding region. Disruption of these regions leads to defective fork protection and have been reported in cancer patients. Cells supplemented with a BARD1 F133A/D135A/A136E mutant, which abrogates the RAD51 binding site, exhibited defective fork protection phenotypes upon HU treatment. However, cells complemented with a BARD1-I26A mutant, which is deficient in E3 ligase activity, did not present replication fork protection problems, similarly to cells complemented with BARD1-WT (72).

However, not only a decrease of the nucleotide pool affects DNA replication. DNA polymerase can encounter difficulties during DNA replication such as replication barriers (**Figure 4B**). A well-known barrier is a RNA-DNA hybrid with an appended displaced ssDNA known as R-loop. BRCA1/BARD1 interacts with senataxin (SETX) and participates in the resolution of R-loops structures (73). The role of BRCA1 in R-loop resolution and prevention has been reviewed and more recent studies arise in this field (74, 75), but there is yet no relationship between the BRCA1/BARD1 E3 activity with R-loops. Another replication barrier is the G-quadruplex DNA structure that is formed at G-rich sites. G-quadruplex structures present strong impediments to replication fork progression. BRCA1 deficient cells have shown hypersensitivity to compounds stabilizing these DNA structures, suggesting that BRCA1/BARD1 promotes restart of stalled replication forks at G-quadruplex DNA structures (76). It could also be possible that the stabilization of the G-quadruplex leads to an accumulation of ssDNA gaps during replication. The excess of ssDNA might be toxic for BRCA1 deficient cells independently of its role in HR or replication fork restart, similarly to PARPi treatments (77) (**Figure 4B**). In line, mass-spectrometry data revealed a possible role of BRCA1 in replication fork protection by neutralizing RNA satellite overexpression, which leads to replication problems and chromosome breaks (67). Unfortunately, the E3 activity of BRCA1/BARD1 has not been interrogated during these replication difficulties, leaving unclear the relationship between BRCA1/BARD1 E3 activity and its role in compromised DNA replication. Addressing this topic would be key for understanding BRCA1/BARD1 E3 activity in the future.

Nevertheless, as mentioned above, BRCA1 has been placed on ongoing replication forks, meaning that it could have a role during this process. It is possible that BRCA1/BARD1 E3 activity could be involved in replication fork homeostasis by ubiquitination of key players. Recently, PCNA was found to be ubiquitinated during normal S-phase progression. Human cells carrying PCNA

K164R (PCNA-KR) mutation, being K164 the major ubiquitination site on PCNA, showed an increased ssDNA gap formation and degradation of stalled replication forks. Additionally, BRCA1 knock down in PCNAKR cell lines resulted in hypersensitivity to PARP inhibitors (78). These results suggest that lack of ubiquitination on replication forks, which includes BRCA1/BARD1 deficient and PCNA-KR mutant cells, is associated with ssDNA gap formation and replication fork protection (**Figure 4B**).

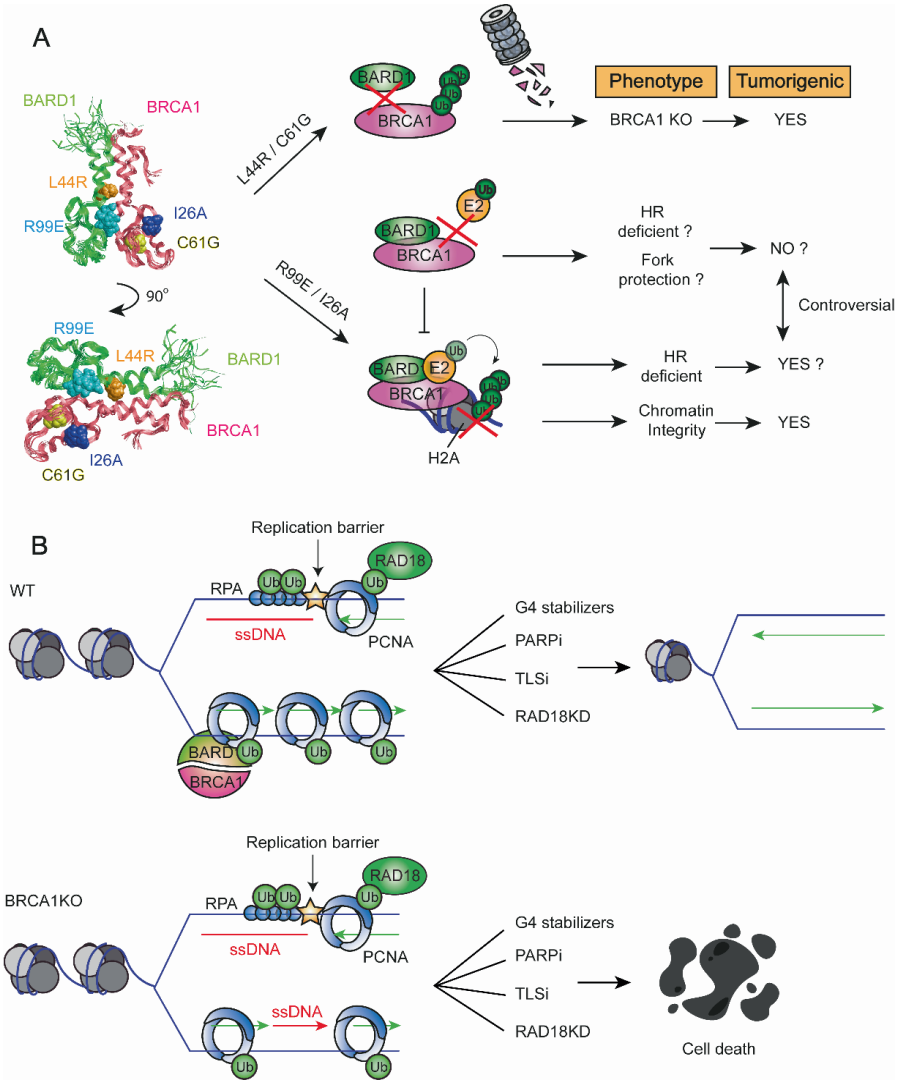


Figure 4. BRCA1/BARD1 in homologous recombination and replication. A. Solution structure of the BRCA1/BARD1 RING domain heterodimer was obtained from PDB (1JM7). Both L44R and C61G mutations in BARD1 and BRCA1 respectively disrupt the formation of the heterodimer resulting in BRCA1 instability and its subsequent proteasomal degradation. These mutations lead to a BRCA1KO phenotype being tumorigenic. BARD1 R99E and BRCA1 I26A mutations abrogate the E3 ligase activity but allow the formation of the heterodimer. These mutations have not been found in cancer patients and mice carrying these

mutations suppressed the formation of tumors. Therefore, they are no tumorigenic. However, R99E and I26A mutations in BARD1 and BRCA1 respectively, influence histone H2A ubiquitination, which impairment has been found in cancer patients and it is therefore tumorigenic. BRCA1/BARD1-mediated histone H2A ubiquitination has also been related to functional HR and chromatin integrity. **B.** Replication forks encounter a replication barrier. WT cells are able to survive G4 stabilizers, PARPi, TLSi and the knock down of the RAD18 E3 ligase. However, BRCA1 deficient cells accumulate ssDNA gaps under normal grow conditions and are unable to overcome the excessive replication stress upon inhibitors or disruption of PCNA ubiquitination, leading BRCA1 deficient cancer cells to death.

According to this data, a recent study revealed that BRCA1 deficient cells accumulate ssDNA gaps and this can be exploited therapeutically using PARP inhibitors (77). Authors suggest that PARPi sensitivity in BRCA1 deficient cells derives from ssDNA replication gaps and not due to BRCA1 function during HR or fork protection. How to take advantage of these ssDNA gaps to overcome BRCA1 deficient cancer cells is a current topic in the breast cancer field. Nayak and colleges found that TLS inhibition using a REV1 inhibitor caused cell death in cancer cells. This finding suggests that cancer cells rely on gap suppression during DNA replication (79). In agreement, Taglialatela et al., found that BRCA1 deficient cancer cells need TLS polymerases to maintain viability. TLS depends on PCNA ubiquitination, which is mediated by RAD18 E3 ligase. Therefore, it is not surprising that RAD18 knock down in BRCA1 deficient cells will lead to cell death in a similar way as using TLS inhibitors (80) (**Figure 4B)**.

However, the biological mechanism underlaying why BRCA1 deficient cells accumulate ssDNA gaps is still unclear. Again, the E3 activity of the heterodimer has not been studied in this context yet, and it would be key for future research. Afterall, there is still no evidence of BRCA1/BARD1 E3 activity involved in DNA replication. Therefore, experiments using BRCA1 and BARD1 mutants disrupting the E3 ligase activity will be one of the future directions in the field.

4. TARGETING THE UBIQUITINATION MACHINERY FOR CANCER TREATMENT

Virtually every cellular process is regulated by ubiquitination and its deregulation is associated to pathological disorders and cancer (81). Ubiquitination is carried out by an enzymatic cascade, in which Ub is activated by an activating enzyme (E1), which hydrolyzes ATP to form a thioester bond with ubiquitin. Then, ubiquitin is transferred via thioester-like complex to the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase enzyme (E3) mediates the conjugation of the ubiquitin moiety to either a lysine residue or the extreme amino terminus of the targeted protein (82-85). There are two known E1 enzymes, UBA1 and UBA6, nonetheless just UBA1 seems to charge the 99% of cellular ubiquitin. Therefore, targeting UBA1 will inhibit the majority of the ubiquitination events. There is already one commercial UBA1 inhibitor, TAK-243. This inhibitor forms a TAK-243-ubiquitin adduct that drastically decrease the formation of cellular ubiquitin conjugates, affecting cell cycle progression and DNA repair, leading to cancer cell death (86). This inhibitor, used at a tolerated dose, has shown promising results in mice bearing human xenograft tumors and in preclinical evaluation of acute myeloid leukemia (AML) (86, 87).

The complexity of the ubiquitination cascade scales up when we look at the numbers of the E2s and E3s enzymes, with 38 E2s and more than 600 E3s (88, 89). Potent inhibitors and new strategies have been developed targeting E2s enzymes. CC0651 is an allosteric inhibitor of CDC34,

an E2 involved in cell proliferation. CDC34 inhibition by CC0651 exhibited the accumulation of its ubiquitination targets and inhibition of cell proliferation (90). Recently, a new strategy concerning protein-based reagents, called ubiquitin variants (UbVs), can be used to develop protein-based inhibitor against E2s. The designed UbVs bind UBE2K and block both, ubiquitin charging and E3 catalyzed ubiquitin transfer to the target protein (91).

The proteome profile is constantly changing and the changes during cancer disease can be tracked. Consequently, controlling the protein turnover by inhibiting upregulated E3 ligases is a key factor to disturb cancer cells. However, one of the major limitations to properly control the protein levels of an E3 ligase substrate, is the identification of the targets for the E3 ligase under consideration. Fortunately, some targets for specific E3s have been elucidated, and the ability of these E3s to form K48 linkages on the target protein for its proteasomal degradation has identified these E3s as potential targets for cancer treatments (92-94). A major example of E3 inhibitors is *nutlin*, the first small-molecule inhibitor of the p53-MDM2 E3 interaction (95). P53 is inactivated in 50% of cancers, and in some cases this is due to MDM2 overexpression. Advanced MDM2 inhibitors are in clinical trials for solid tumours, haematological neoplasms, liposarcomas, soft tissue sarcoma and AML (96).

Recently, two new small molecules with anticancer potential have been reported as Culling-RING E3 ligases (CRLs) inhibitors (97). These inhibitors seem to impact the Culling-E2 interaction, inhibiting the ubiquitination process. Moreover, the compounds exhibited in vivo antitumoral activity in AML MV4-11 xenograft mouse models. These inhibitors could have great potential in tumors with low expression of CRLs. Another way to make use of the ubiquitin proteasome system (UPS) as cancer vulnerability, is targeting deubiquitinating enzymes (DUBs) (98). This is the case of TAK-659, a DUB inhibitor in clinical trials, which targets USP10 and induces degradation of the spleen tyrosine kinase (SYK). SYK seems to be critical for AML transformation and maintenance in AML patients, and its degradation results in death of AML cancer cells (99). Nevertheless, not only E3s involved in proteasomal degradation are associated with cancer. As it was illustrated above, cells have developed signaling mechanisms to end the DDR. However, we can try to control the response with the use of inhibitors that target upstream players, as is the case for the early recruited BMI1-RING1B ligase which ubiquitinates H2A K119. PRT4165 inhibits BMI1-RING1B-mediated H2A ubiquitination in vitro and in vivo (100). The use of this inhibitor revealed not only its potential use as cancer treatment but also supports the early role of this E3 ligase in the DDR.

The association between E3 deregulation and cancer has been previously reviewed and several E3s with non-degradative or still yet to know substrates appear as potential targets for cancer therapy (81, 101, 102). The BRCA1/BARD1 E3 role in DNA damage and cell cycle checkpoints regulation has been linked to cancer development (103). BRCA1/BARD1 mediates monoubiquitination, degradative and non-degradative polyubiquitination of its targets, meaning signaling purposes in multiple cellular processes (**Table 2**). However, very few targets have been validated as direct targets for BRCA1/BARD1. There are several considerations to keep in mind when validating the ubiquitination targets: the use of catalytically dead mutants that still can use a different E2 ligase (104). Depletion and overexpression of an E3 ligase can completely change the ubiquitinated proteome of a cell and lead to artificial protein ubiquitination. Finally, the overexpression of a substrate could lead to its ubiquitination for the mere reason of being in

excess within the cell. Up to date, there is only one well-described ubiquitination target for BRCA1/BARD1 and it is the histone H2A.

Besides the canonical H2A as well-defined BRCA1/BARD1 ubiquitination target, the histone variant mH2A has emerged as solid ubiquitination target (105). In addition to its role in senescence, the ubiquitination of mH2A could be related to the repair pathway choice and the Alt-EJ repair pathway, due to its recently described roles in these cellular processes (106). It would be beneficial to develop BRCA1/BARD1 inhibitors as they could be used not only as chemotherapy strategy but also to study the BRCA1/BARD1 function during the DDR. However, in order to develop successful inhibitors, it will be crucial to identify and carefully validate its targets. In this way, some inhibitors could work blocking the interaction between BRCA1/BARD1 and a specific substrate.

Table 2. BRCA1/BARD1 Ubiquitination targets.

Substrate	Cellular function	Linkage	Reference
Histone H2A	DNA damage repair Heterochromatin integrity	Mono	(39, 50, 52)
Macro H2A1 (H2AFY)	Cellular senescence Alt-EJ repair	Mono	(105) (106)
Cyclin B	Cell cycle regulation	Poly (K6)	(112)
CtIP	DNA damage repair	Poly	(57)
Merlin (NF2)	Hippo growth signaling	Poly (K63)	(113)
Claspin	DNA damage repair	-	(114)
RNApol II	DNA damage repair	Poly	(115)

5. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The DDR is tightly regulated in time and space, and a large set of new players has arisen in a short period of time. To completely understand the whole DDR and be able to take advantage of it when there is disease as cancer, years of research will be needed. In the meantime, we dissected the DDR from an ubiquitination point of view, where E3 ligases are key regulators. Controlling the ubiquitination signaling of the DDR could influence the whole response. In the case of E3 ligases, finding their ubiquitination substrates discloses their function. Lots of effort have been done to elucidate BRCA1/BARD1 ubiquitination substrates and completely understand its E3 ligase function (**Table 2**). In the last decade, the proteomic field has evolved notoriously and mass-spectrometry (MS) approaches has been used to find BRCA1/BARD1 targets (105, 107-111). However, the validation of targets from proteomic screenings is very challenging.

Different strategies can be used to find potential substrates for BRCA1/BARD1. To investigate the role of BRCA1/BARD1 in biological functions, the employment of BRCA1 mutants combined

with MS approaches could lead to the identification of its interactors and ubiquitination substrates. The development of organoids harboring BRCA1/BARD1 null and E3 dead mutants could contribute to unveil the role of this E3 ligase. Finally, the development of inhibitors interrupting the E3 activity might be of interest to address the E3 role of BRCA1/BARD1 and finding its targets by MS strategies.

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