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Ubiquitination – An Evolving Role in DNA Repair

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1. Introduction

Upon DNA damage due to endogenous or exogenous causes, chromatin is dynamically modified, especially through posttranslational modifications (PTMs) of histones together with noncoding RNA expression. Both procedures modify accessibility of genes and regulatory genomic loci by protein factors and enzymes involved in gene expression as well as DNA repair processes. In addition, PTMs of proteins involved in genome integrity seem to play important roles in regulating their functions and protein–protein interactions (PPI). The most studied PTMs involved in DNA repair machinery function are histone phosphorylations, methylations/demethylations and to a lesser extent acetylations, allowing/prohibiting accessibility to double-strand break recognition and binding of factors/enzymes. Protein ubiquitination – the covalent link of the small protein ubiquitin (Ub) to lysine residues of a target protein – was classically related to protein degradation, ensuring structural integrity control and/or protein turnover rate. This procedure involves the addition of multiple ubiquitin molecules in a specific manner, which targets the polyubiquitinated protein to the proteasome for degradation. In recent years, accumulating data unveiled a role for nondegrading ubiquitination of proteins involved in DNA repair pathways and cell fate decisions [1,2]. A well-documented overview depicting the exceptional importance of ubiquitination in the restoration of genotoxic insults was carried out by a number of reviews, clearly pointing out ubiquitination and DNA damage response/repair interrelation [3]. The issue comprehensively covers practically all principal aspects of Ub function in the field. Therefore, many issues of the ubiquitin landscape at DNA double-strand breaks (DSBs) have been highlighted. As described, many challenges and puzzles remain to be solved regarding the intimate relationship between the DNA repair machinery and nondegrading ubiquitin signaling at DNA DSBs and the surrounding chromatin (Fig. 1) [3].

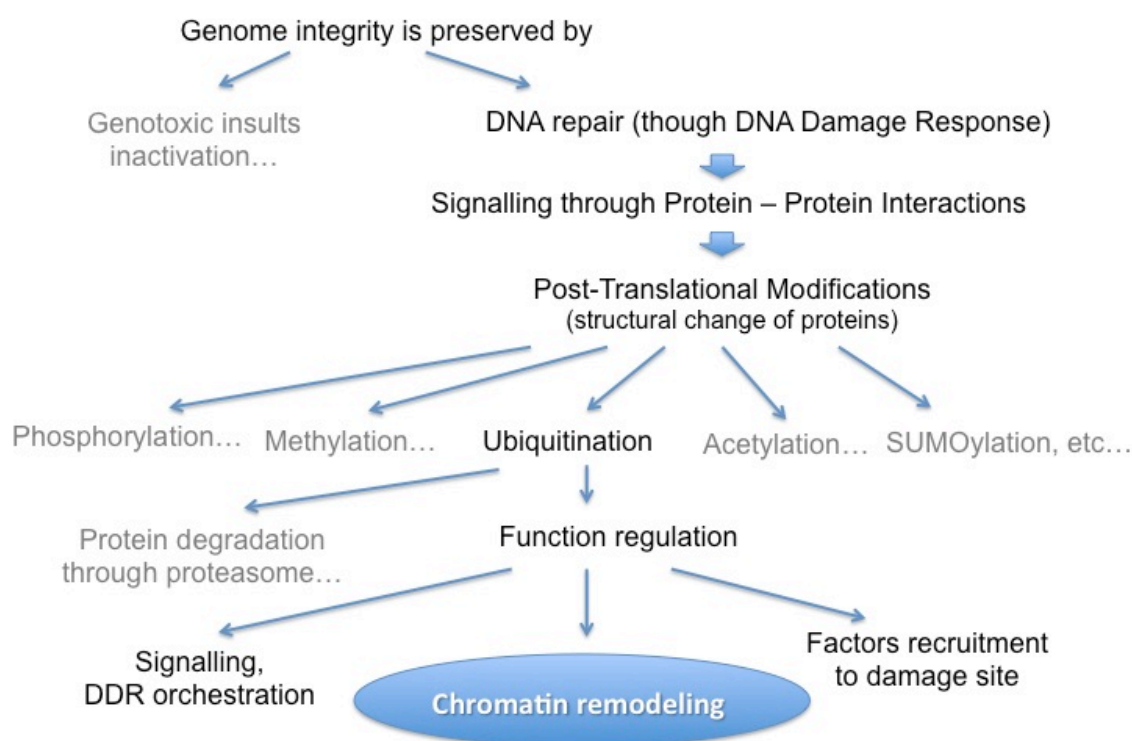


Figure 1. A simplified scheme of ubiquitination involvement in DNA repair through chromatin remodeling. (Details in the text.)

Consequently, the current chapter's objectives are focused on clarifying aspects of ubiquitination involvement in DNA repair regulation as a PTM and not as a signal for degradation and more specifically on chromatin remodeling at the site of DNA damage and its vicinity. Chromatin relaxation and/or histone removal controls, in turn, the accessibility of damaged DNA molecules to the DNA damage response (DDR) machinery and repair processes. In addition, abolishment of ubiquitin add-on, to specific lysine residues of histones H2A and H2B may also play an additional role in malignancy. Clarification of the mechanisms and the pathways involved, apart from contributing to the deeper understanding of basic mechanisms governing genome integrity, may also lead to the development of innovative therapeutic approaches against major cancer types and shed light on aging phenomena related to DNA repair deficiencies.

2. Ubiquitin as a PTM

Ubiquitin (Ub) is a small (76 amino-acid residues, 8.5 kDa molecular mass) regulatory protein expressed in all cell types (ubiquitously) of eukaryotic organisms. It is highly conserved among eukaryotic species: for example, human and yeast ubiquitin share 96% sequence identity.

Ubiquitin is covalently attached to a large range of cellular proteins by specific enzymatic reactions referred to as the ubiquitination system. The ubiquitination reaction is an ATP- and

Mg²⁺-dependent process where the carboxyl group of the C-terminal glycine residue of ubiquitin (G76) is the moiety conjugated to substrate lysine residues [4]. Ub performs its myriad functions through conjugation to a large range of target proteins. A variety of different modifications can occur. Key features include its C-terminal tail and the seven lysine residues of the protein. Ubiquitination may exert its function via multiple ways. As depicted in Fig. 2, ubiquitination was primarily attributed to signal proteins for degradation via the proteasome but it may also alter protein cellular location, affect protein activity, and promote or prevent protein–protein interactions [5-7]. More specifically, protein modifications by ubiquitination may be distinct. Either a single ubiquitin molecule (monoubiquitination) or a chain of ubiquitins (polyubiquitination) can be added. The ubiquitin is ligated on the target protein through one of its seven lysine residues. These “linking” lysine residues are defined by a “K” (one-letter lysine symbol) and a number, referring to K position on the ubiquitin molecule, from the amino-terminal to the carboxy-terminal edge of the protein. Initially, a ubiquitin molecule is bonded by its C-terminus (G76) to usually the ϵ -amino group of a specific lysine residue (e.g., K48, K29, K63) of the target protein. Polyubiquitination occurs when the C-terminus of another ubiquitin, is linked again to a lysine residue (e.g., again K48 or K29) on the previously added ubiquitin molecule, forming a chain. This process is repeated several times, leading to the addition of several ubiquitins on a certain protein molecule. While polyubiquitination mostly on K48 and K29, is related to degradation via the proteasome (referred to as the “molecular kiss of death”), other polyubiquitinations (e.g., on K63, K11, K6) and monoubiquitinations may regulate several cellular processes such as endocytic trafficking, inflammation, gene expression, and DNA repair (Fig. 2) [8]. Recent publications also relate ubiquitination to stem cell differentiation [9].

The most extensively studied ubiquitin chains are the K48-linked ones, which signal proteins to the proteasome for degradation and recycling [10]. This discovery was honored by the Nobel Prize for chemistry in 2004. The ubiquitin-proteasome system is implicated through protein homeostasis in practically all aspects of cellular processes including DNA repair [11-12]. A nice example of DNA repair regulation through ubiquitination by UBR3 E3 ligase and subsequent protein degradation is the control of APE1 (Ref-1), a protein involved in DNA repair (mostly excision repair of abasic sites) and regulation of transcription [13]. On the other hand, proteins ubiquitinated in K63 are primarily involved in protein–protein interactions (PPI) and in turn in cascade signaling and chromatin remodeling. The process may involve both, mono- or polyubiquitination. Overall, protein structural modification through ubiquitination may thus affect its localization, activity, and/or stability together with interactions with partner molecules.

Ubiquitination is carried out in three main steps performed by the concerted action of respective types of enzymes organized in multiple levels of specificity. The activation is performed by a family of ubiquitin-activating enzymes termed as E1 ligases, the conjugation by a group of ubiquitin-conjugating enzymes (E2 ligases), and finally the ligation on the target protein by the ubiquitin ligases of E3 type. This sequential cascade binds ubiquitin to lysine residues on the protein substrate via an isopeptide bond or to the amino group of the protein's N-terminus via a peptide bond [10,14]. Among the ligases, the E2 types act as key mediators

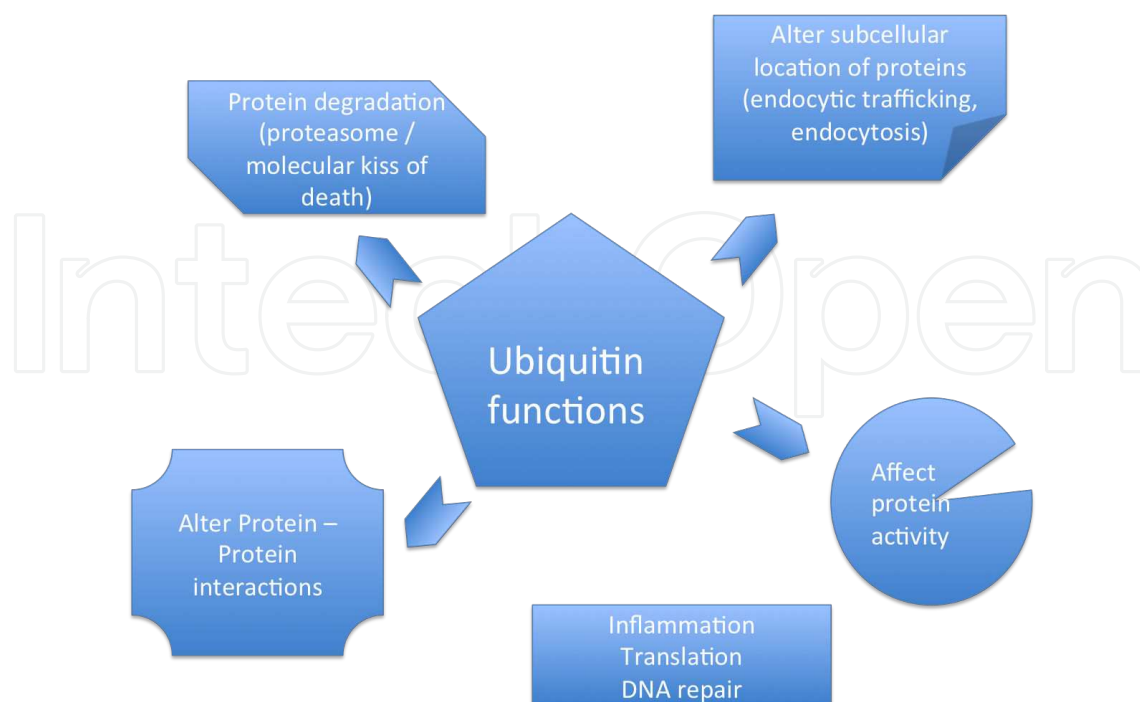


Figure 2. Schematic illustration overviewing ubiquitin functions in the cell.

of chain assembly, thus being able to govern the switch from ubiquitin chain initiation to elongation. By this activity, E2 ligases regulate the processivity of chain formation and establish the topology of assembled chains, thereby determining the consequences of ubiquitination for the modified proteins [15]. Addition of the activated ubiquitin on the target protein substrates is performed by a variety of enzymes collectively termed as E3 ubiquitin ligases. The human genome codes for only two E1s, about 35 E2s and strikingly, more than 500 E3 type enzymes.

Ubiquitination comprises a dynamic phenomenon; therefore, Ub removal enzymes termed as deubiquitinases have also been detected. A number of specific deubiquitinases (DUBs) recognizing both the Ub position and the target protein have been characterized. DUBs' action reverses the ubiquitination-induced function and serves as a rapid mechanism to adjust cellular responses to stimuli by the fine-tuning of Ub-driven complex formation. A characteristic example of DUB activity in relation to DDR signalling cascade is the fine regulation of the E3 ubiquitin ligases RNF8-RNF168 activity. At the sites of DSBs, lysine63-linked ubiquitin chains are built at the damage sites, through the activity of RNF8-RNF168 complex, driving the effective assembly of DNA repair factors for proper control and repair. RNF168 has a short half-life and upon DNA damage is stabilized by the DUB USP34. Abolishment of USP34 activity results in rapid degradation of RNF168 that in turn, through attenuated DSB-associated ubiquitination, results in defective recruitment of BRCA1 and 53BP1 at the damage sites and compromised cell survival following ionizing radiation [16].

In addition, a number of protein molecules termed Cullin Family proteins, which serve as scaffolds to complex formation of E3 Ub ligases with RING proteins adaptor proteins, and

substrate recognition receptors, add another degree of complexity in the ubiquitin embroidery of cellular functions [17].

Overall, ubiquitin addition/removal on a specific K residue of a target protein is a highly regulated and finely tuned process involved in regulation of many crucial cellular pathways. During DDR and genome repair, nondegradative protein ubiquitination seems to be implicating in a number of procedures including sensing/signalling, chromatin remodelling and factor recruitment to damage points.

3. Ubiquitination in DNA damage response

DNA damage is sensed by a number of DNA interacting proteins, mainly histone subunits. The primary signal produced appears to be the phosphorylation of H2Ax (γ H2AX), activating in turn ATM/ATR kinases, which serve as sensors and through a series of target molecules phosphorylation the DNA damage alert is transduced to appropriate pathways toward activating DDR mediators and effectors (Fig. 3). One of the primary mediators activated by ATM/ATR kinases is BRCA1, which in turn through its multiple roles orchestrates DDR [18]. BRCA1 dynamically interacts with numerous protein partners and according to these interactions (cell cycle stage dependent) is involved in cell cycle regulation, transcription coupled repair, and repair processes [19]. Non-degradative ubiquitination of histones and a number of DDR factors is also a crucial event upon DNA damage (Fig. 4) and is sensed by a number of DNA interacting proteins, mainly histone subunits and chromatin remodelers [16].

Upon triggering the DNA damage response by introduction of a DNA double strand break, the initial response step includes histone variant H2AX molecules rapid phosphorylation at the γ position (γ H2AX) along chromatin tracks flanking the DSB. Phosphorylation is performed by kinases ATM, ATR, and DNA-PK [20]. H2AX phosphorylation facilitates in turn the accumulation of DNA damage response regulators, Mdc1/NFBD1 [21,22], RNF8, and RNF168. RNF8 and RNF168 are RING-type E3 ubiquitin ligases, which catalyze the K63-linked polyubiquitin chain formation on histones H2A and H2AX [23-26]. RAP80 is then recognized and recruited to the K63-linked polyubiquitinated histones driven by its ubiquitin interaction motif [27-29]. Recruitment of RAP80 allows docking of BRCA1. Moreover, induction of the intact IR-induced G2/M checkpoint is also dependent on RAP80 and its interaction with K63-linked polyubiquitin chains on H2A and H2AX [27-30]. Abolishment of histone ubiquitination enzymes by knockdown experiments impairs DSB-associated polyubiquitination of H2A and H2AX and inhibits retention of 53BP1 and BRCA1 at the DSB sites, thus resulting in sensitization of the corresponding cells to ionizing radiation [23-26, 31].

Remarkably, the quantity and stoichiometry of ubiquitinated factors at the site of the lesion and the flanking area appear to direct the cell toward selecting the appropriate repair pathway. Recent findings elegantly showed that the stoichiometry of the ubiquitin-binding proteins RAD18 and RNF168 are related to the selection of either error-prone Non Homologous End Joining (NHEJ) or the high-fidelity Homologous Recombination (HR) pathway in IR-treated cells (Fig. 5) [32]. More specifically, the hierarchical assembly of ubiquitin-related factors that

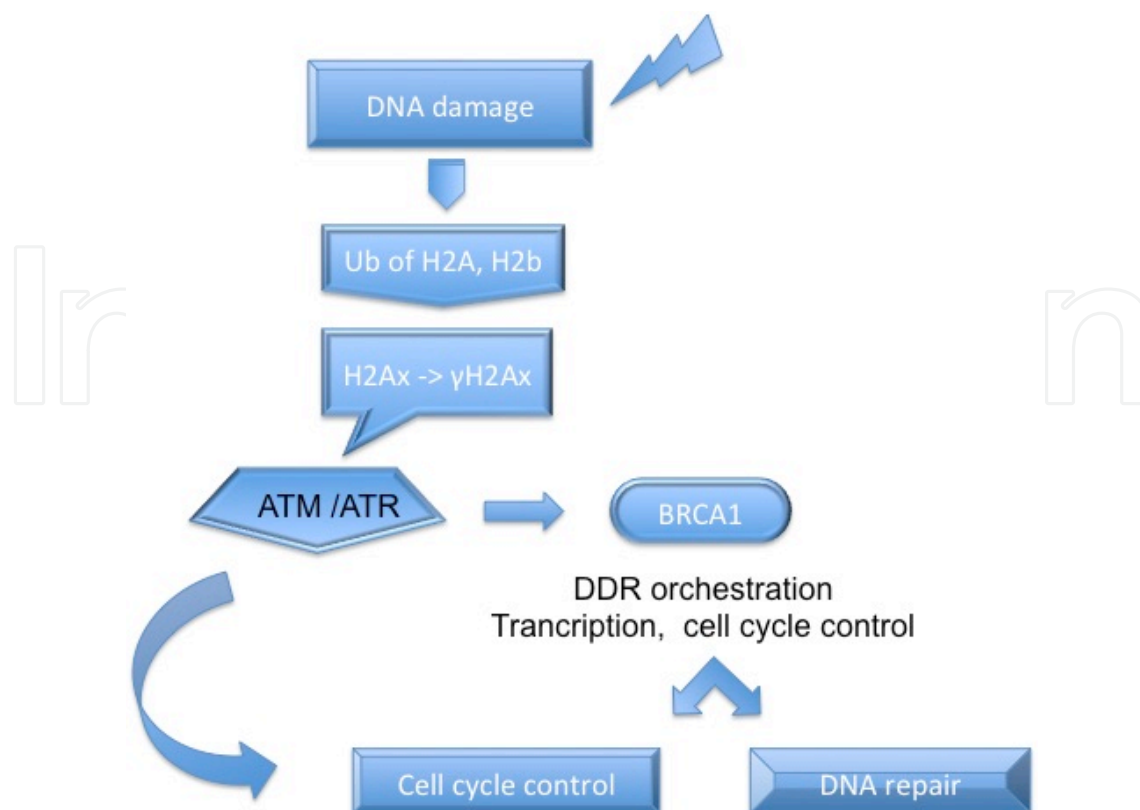


Figure 3. Schematic representation of the initial cascade of reactions following DNA damage. Ubiquitination of DNA packaging proteins (H2A and H2B) result in chromatin remodeling, enabling the binding of signaling and repair factors.

begin with RNF8 assembly is further enhanced by RNF168, facilitating the association of 53BP1 and the Ub ligases BRCA1 and RAD18. As 53BP1 blocks the resection of broken DNA strands, it suppresses HR in favor of the NHEJ pathway. RAD18 overexpression dramatically impairs 53BP1 and in turn favors RAP80–BRCA1 binding to lesion sites, following IR, without affecting damage signaling, repair, or radiosensitivity. In this case, the HR pathway is promoted [32]. In accordance with these data, it seems that the key selection point of the repair pathway, the RAD18 E3 ligase, when monoubiquitinated (RAD18-ub1), does not interact with SNF2 histone linker plant homeodomain RING helicase (SHPRH) nor with helicase-like transcription factor, two downstream E3 ligases required for the promotion of error-free bypass of lesions during genome duplication. Interestingly, the RAD18-ub1 form by its zinc finger domain, binds to nonubiquitinated RAD18, thus inhibiting RAD18's function and resulting in fine-tuning of the ratio of ubiquitinated versus nonubiquitinated forms of RAD18 in the nucleus [33]. It is of interest that ubiquitination not only prevents RAD18 from localizing to the damage site but also, through ubiquitination of the proliferating cell nuclear antigen (PCNA) [34], a factor facilitating DNA replication, suppresses mutagenesis [35]. These data are concomitant with a model where monoubiquitination controls RAD18 function by sequestering active (non-ubiquitinated) RAD18 molecules. The damage-triggered removal of the ubiquitin load by one or more DUBs favors the switch from RAD18-ub1–RAD18 complex to RAD18–SHPRH complex formation required for high-fidelity lesion bypass during DNA replication [33].

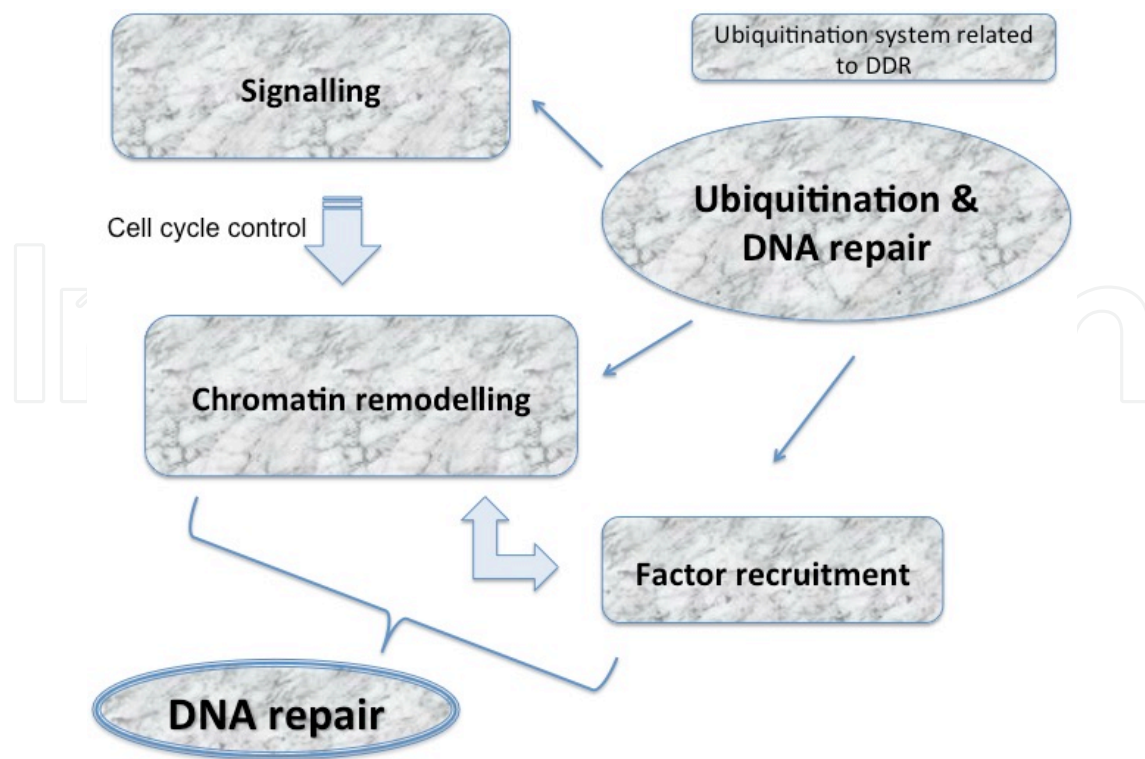


Figure 4. Nondegradative ubiquitination of DNA damage response and repair factors together with chromatin structure modification is a dynamic process essential for genome restoration and cell viability. Ubiquitination has been detected in signaling molecules, mediators, and effectors of DDR as well as histone subunits and histone interacting factors. Deregulation of this cascade is implicated in malignancies.

Therefore, it seems that ubiquitin-family (ubiquitin and ubiquitin-like molecules like SUMO) modifications regulate damage-induced template switching. Moreover, the ratio of ubiquitinated to nonubiquitinated RAD18 appears to be modified during cell cycle progression, thus serving as a posttranslational mechanism controlling RAD18 activity, while it may also be implicated in abnormal cell state conditions and malignancies.

An elegant example of ubiquitination regulation of DNA repair per se comprises the modification of substrate recognition and activities of FBH1 helicase. Single-molecule sorting revealed that ubiquitination affects FBH1 interaction with the RAD51 nucleoprotein filament – the major recombinase of the HR pathway, without perturbing its translocase and helicase activities [36].

Replication Protein A (RPA) complex is another factor involved in the HR pathway (repair based on replicated sister chromatid sequence) by polymerizing onto single-stranded DNA (ssDNA) and coordinating the recruitment and exchange of factors involved in DNA replication, recombination, and repair. The RPA-ssDNA platform also activates the master ATR kinase during replication stress. The RPA complex is regulated by a number of post-translational modifications, one of which is ubiquitination. RPA ubiquitination results in modulation of its interactions with partner proteins, a critical function in the maintenance of genome stability through the error-free HR process [37].

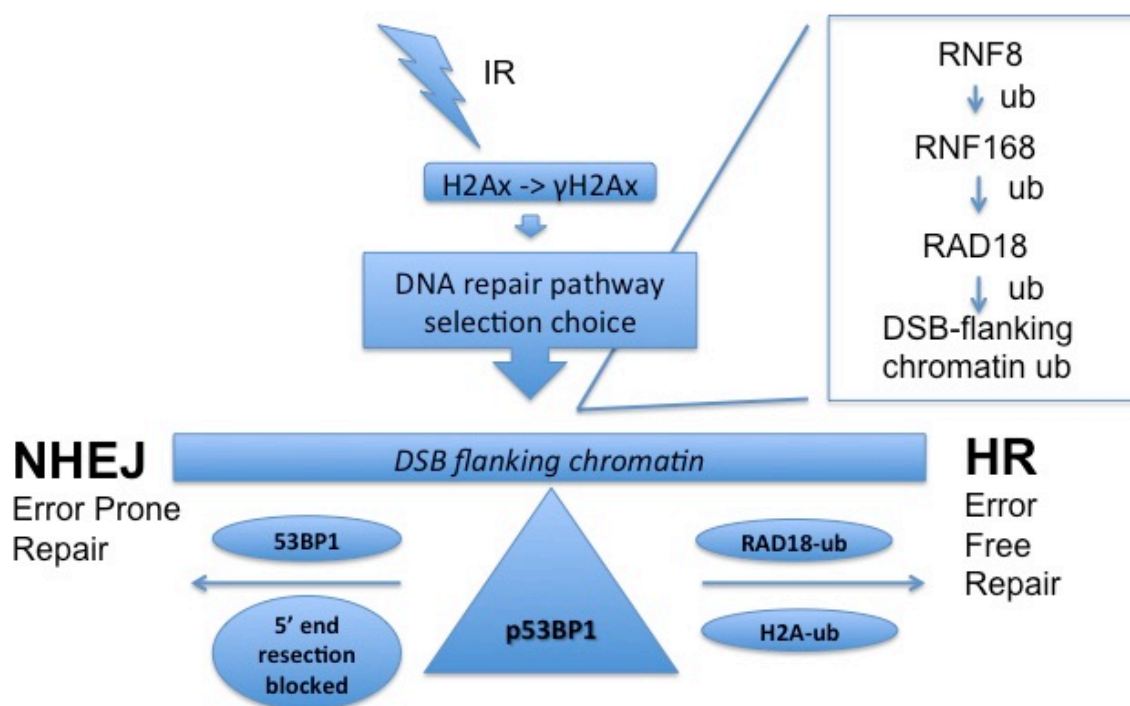


Figure 5. Stoichiometry of Ub-binding proteins RAD18 / RNF168 associated with IR-induced foci influences 53BP1 association and subsequent selection of the repair pathway.

4. Ubiquitination & chromatin remodeling in DNA repair

Chromatin consists of functional units of DNA packed in solid nucleoprotein structures, the nucleosomes. Nucleosomes are composed of the four core histone proteins (H2A, H2B, H3, and H4) wrapped by 147 base-pairs (bp) of DNA. Two nucleosomes are separated by linker DNA, ranging between 20 and 80 bp in length. Nucleosomes, apart from packing the large eukaryotic genomes into the limited volume of the nucleus, are also responsible for the DNA accessibility by interacting with DNA binding factors and modifying enzymes. Monoubiquitination of histone H2B shows a genome-wide distribution in different organisms and is probably related to hetero-/euchromatin determination as well as gene expression profiles.

DNA damage induces structural changes in chromatin, serving as the initial signal for DDR sensors. In order to repair the nuclear DNA, multiple regulated processes facilitate the exposure of DNA at the lesion point and its vicinity. As the DNA repair machinery requires direct access to DNA, nucleosomes should either loosen, move, or be removed from the damaged area. Recruitment of enzymes and factors enabling repair is thus facilitated/allowed by structural changes in histones – the protein components of nucleosomes [38-42]. In general, the local chromatin architecture is mainly driven by nucleosome remodelers and histone and DNA modifiers. Ubiquitination of histones and histone binding factors results in critical chromatin rearrangements either genome-wide or at the local scale, enabling accessibility to factors controlling important biological processes like transcription, genome duplication, chromatin condensation, and DNA repair. Regarding DDR, ubiquitination of several DNA repair machinery components enables interactions with factors recruited from other cellular

pathways during DNA damage [43-45]. A threatened genome is an extremely stressful and emergency condition for the cell requiring rapid responses. These responses are definitely required for prompt lesion restoration and cell viability [46].

Histones H2A, H2AX and H2B are monoubiquitinated (ub1) at K119 (H2Aub1, H2AXub1) and at K120 (H2Bub1) respectively, at the sites of DNA damage, a reaction catalyzed by RING1B/BMI1 and by a prominent E3 RING finger ubiquitin ligase RNF20/RNF40 [47-54]. H2Bub1 pertains the ability to physically disrupt chromatin strands (due to significant increase in dimension), adopting a more open chromatin structure, accessible to DNA repair proteins, thus facilitating the repair processes, as was shown by *in vitro* studies. Roles of signal recruitment, histone cross-talk and methylation influencing of H3 are also attributed to H2Bub1. Besides, recruitment of DNA repair machine proteins involved in both NHEJ and HR repair pathways to the DSB requires the activity of RNF20 and histone H2B monoubiquitination [52]. It is assumed that the H2B monoubiquitination machinery is temporarily recruited to damage sites. The locally produced H2Bub1 is in turn required for timely recruitment of DSB repair mediators and co-mediators, resulting in DSB repair. This phenomenon represents a crossroad between the DDR pathway and chromatin structure, and represents an example for the intercommunication and tight co-operation of pathways required to ensure genome integrity.

DDR and repair are urgent stress responses of the cell consuming vast amount of energy and recruiting protein components related to various normal functioning pathways in order to address the challenge. Therefore, a dynamic equilibrium of ubiquitination addition/removal is required at the chromatin level and the key player, the RNF20-RNF40 complex, is in dialogue with a number of deubiquitinases (including USP7, USP22, and USP44 enzymes). The active regulation of histone ubiquitination also by DUBs USP3 and K63-Ub DUB BRCC36 plays a critical role in efficient DDR and DNA repair pathways [55]. Apart from the RNF20-RNF40 complex, other key protein factors involved in DNA repair, like BRCA1, may also act as ubiquitin ligases, although this field still remains obscure [19].

Overall, it is hypothesized that the monoubiquitination/deubiquitination interplay of histones H2A and H2B regulates chromatin condensation, thereby facilitating recognition and binding of the repair machinery at the DNA damage site [52] and the restoration of chromatin structure upon damage repair. Despite intensive studies, the underlying mechanisms still remain elusive.

More extensive studies of the role of ubiquitination of histones have attracted particular interest, especially since H2Bub1 presents low to undetectable levels in many cancer types, including breast, colorectal, and lung. Approaches adopted include crosslinking combined with pull-down assays and similar high-throughput/directed strategies and functional assays. Based on these observations, members of the pathway-regulating H2Bub1 may represent promising therapeutic targets for malignancies and aging-related syndromes.

More intensive studies on elucidating mechanisms governing general histone modification induced by DNA damage in health and disease are expected to shed light in DNA repair and chromatin structure intercommunication along with the ability to explore and design pioneer treatment schemes for both cancer and other DNA-impairment-related diseases.

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