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Protein Ubiquitination in IR-Induced DNA Damage Response

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1. Introduction

Ionizing radiation is a major source of double-strand DNA breaks, the most detrimental form of DNA damage that, if left unrepaired, leads to genomic instability and cancer. Eukaryotic cells have evolved a DNA damage response (DDR), an intricate network that can be rapidly activated to response to such challenge. Mutations in genes that encode DDR proteins are associated with many genetic diseases, suggesting the importance of DDR in maintaining genomic integrity (*1-5*).

DDR is a sophisticated signal transduction network comprised of proteins that are capable of sensing DNA damage, amplifying the signals and executing various cellular functions that have direct impact on cell fate. Of great importance for the understanding of DDR are series of studies that aim to identify proteome-wide substrates of checkpoint kinase ATM/ATR. These unbiased screens greatly expanded the DDR landscape and revealed the involvement of a broad spectrum of pathways that were not previously known (*6, 7*). One of the most intriguing observations made in these studies is the over-representation of proteins involved in ubiquitin proteasome system (UPS) pathways.

Ubiquitin (Ub) is a 76 amino acid protein that can be post-translationally covalently attached to the lysine residues of target proteins(*8*). Ubiquitin can influence the function of its target proteins by altering their enzymatic activity, abundance or localization (*9-14*). In this chapter, we will focus mainly on the role of ubiquitination in DDR signaling and cell cycle regulation. Following a brief introduction to principles of ubiquitination and ubiquitin-like modification, we will present an overview of enzymes and substrates that have been identified over the last two decades of intensive research. Finally, we will introduce recent advances in application of affinity purification and mass spectrometric-based identification of ubiquitinated proteins.

2. Principle of protein modification by ubiquitin and ubiquitin-like modification

2.1 Conjugation of Ub and UBLs through the three enzyme cascade

Ubiquitin is a highly conserved small protein that is expressed in all the eukaryotic cells and across different organisms. Ubiquitin was initially purified by Gideon Goldstein and co-workers from bovine thymus (*15*). The discovery of ubiquitin function in protein

degradation was later made by Irvin Rose, Avram Hershko and his student Aaron Cieshanover when they were studying the ATP-dependent degradation of the tyrosine aminotransferase enzyme. Rose, Hershko and Ciechanover were awarded the 2004 Nobel Prize in Chemistry for their discovery (*16-19*).

Protein ubiquitination is an ATP-dependent reversible post-translational modification (PTM) that results in the formation of an isopeptide bond between carboxyl terminal of glycine (Gly) on ubiquitin and the epsilon amine of the lysine or, less commonly, the amino terminus or cysteine of the target protein (*8, 13, 17, 20-23*). Ubiquitination is carried out by a set of three enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) (*8, 18, 22*). Initially, ubiquitin is 'activated' (see below) in a two-step reaction by ubiquitin activating enzyme E1. The ubiquitin molecules are then transferred from E1 to the active site cysteine of an ubiquitin-conjugating enzyme E2 via a trans (thio) esterification reaction. Finally, an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin is established with the help of an ubiquitin ligase (E3), which recognizes and binds the target substrate and E2, thereby catalyzing the ubiquitin transfer from E2 to target protein **(Figure 1A)** (*8, 12-14, 21*). Human genome encodes two E1 activating enzymes, approximately 50 E2s and over 700 E3 ligases (*24, 25*).

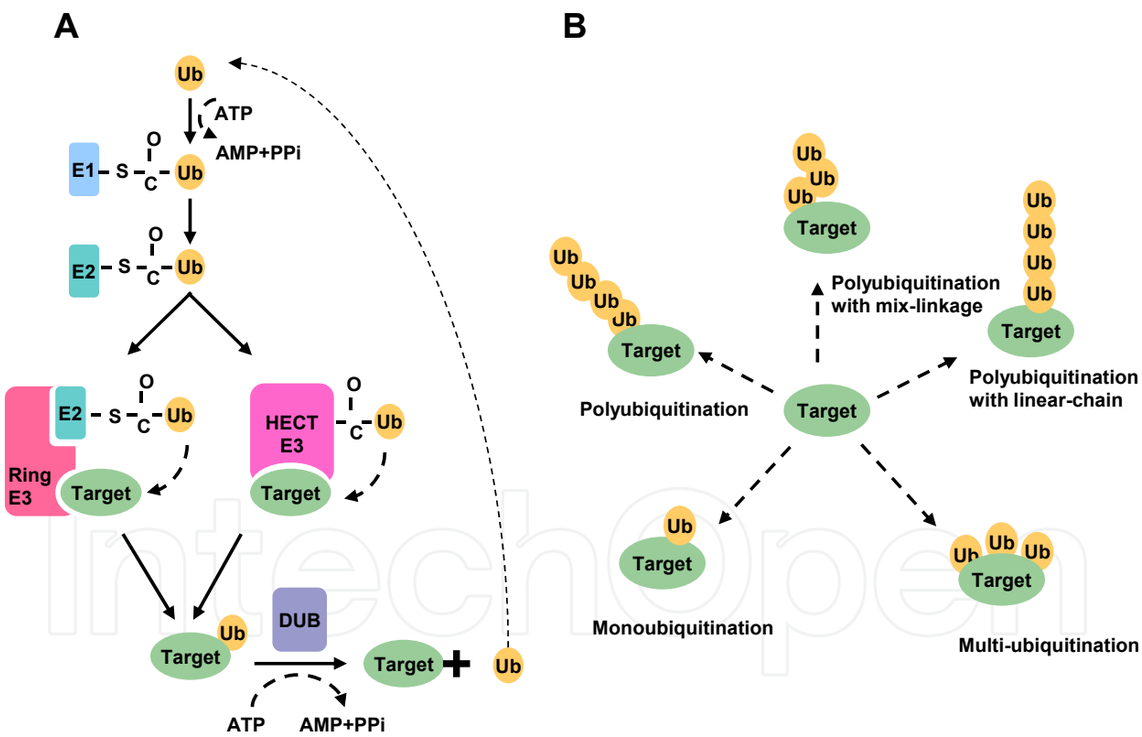

Fig. 1. An Overview of the Ubiquitination cascade and patterns. A) Schematic representation of the ubiquitination cascade as it is mediated by the E1, E2 and E3 enzymes and removed by DUB. B) Schematic representation of diferent ubiquination patterns.

The E1 activity represents an essential step during ubiquitin and ubiquitin-like protein (Ubl) conjugation, and the general mechanism of the E1-catalyzed reaction is well established (*17, 24-27*). In the first step of ubiquitin activation, the E1 enzyme catalyzes the adenylation of the Ub or Ubl C-terminus in an ATP-dependent process. In the second step, E1 forms a

thioester between a conserved catalytic cysteine and the Ub or Ubl (*17, 18*). It has been long believed that a single E1 (Ube1) is responsible for activation and conjugation of all Ubls including ubiquitin, Nedd8 and SUMO. Recently, a divergent E1 in vertebrates and sea urchin, Uba6, was discovered. Human Uba6 charges a previously uncharacterized E2 (Use1) and specifically activates ubiquitin, but not other UBLs, *in vitro* and *in vivo* (*28, 29*).

Members of the ubiquitin-conjugating enzymes (E2s) family have highly conserved ubiquitin-conjugating catalytic (UBC) domain that provides a binding platform for E1, E3 and activated Ub/Ubl. Although E2s were initially thought to only be "ubiquitin carriers", recent studies revealed that E2s play an active role in determining ubiquitin chain length and linkage specificity(*30*). For instance, UBE2T specifically catalyzes the monoubiquitination of FANCD2, but not polyubiquitin chain formation due to lack of of extension activity(*30, 31*). UBE2N (Ubc13) mediates elongation of K63- specific polyubiquitin chains, yet it cannot initiate the ubiquitination (*30, 32-34*). UBE2C (UbcH10) is involved in the formation of K11-linked polyubiquitins, while UBE2K, UBE2R1 and UBE2G2 build the K48-linked ubiquitin chain (*30*).

In contrast to the E2s, whose catalytic sites are well conserved among its members, E3 ligases are structurally diverse and can be classified into two broad groups on the basis of their ubiquitin transfer mechanisms: HECT-type and adaptor-type. HECT-type E3 ligases are characterized by the HECT (Homologous to E6-associated protein C-Terminus) domain, which can form thioester bond with ubiquitin via an evolutionally conserved cysteine residue and play a direct catalytic role in final transfer of ubiquitin to a substrate (*35-37*).

On the other hand, adapter-type E3 ligases, including RING (Really Interesting New Gene) , U-box (modified RING motif without the full complement of Zn^{+2} – binding residues) domain containing E3 ligases, never form a covalent bond with ubiquitin (*38*). Rather, they function as scaffold proteins that facilitate the transfer of ubiquitin by recruiting ubiquitin conjugated E2 and substrate proteins into close proximity. The RING domain is a cysteine-rich sequence motif that is coordinated with two zinc atoms in "cross-brace" manner. Although U-box domain has very similar structure to RING domain, , the major difference between them is that U-box E3 ligase uses hydrogen bonds instead of zinc atoms (*38*).

Most the RING/U-box domain containing E3 ligases such as BRCA1, MDM2, RNF8 are multi-domain proteins with the exception of the Cullin–RING Ligases (CRL). Multi-domain E3 ligases are capable of catalyzing the reaction by themselves, albeit with relatively low efficiencies. The ligase activity is greatly enhanced by binding to a partner E3, e.g. BRCA1 with BARD1 and MDM2 with MDM4 (*39, 40*). On the other hand, the CRL are multi-components ubiquitin ligases and are arranged into elongated structure by a cullin scaffold protein and a catalytic RING subunit, RBX1 or RBX2 . Human genome encodes at least seven different cullin proteins: CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5 and CUL7 and two 'cullin homology domain' containing proteins: Parkin-like cytoplasmic protein (PARC) and subunit of the APC/C complex (APC2) (*41-44*). N-terminal domain of cullin binds substrate receptor, which holds an F box, SOCS box, VHL box or BTB box domain, directly or indirectly through adaptor proteins, such as SKP1 in SCF. C-terminal domain of cullin interacts with RBX RING domain, which brings to E2 ubiquitin conjugate enzyme into complex and promotes ubiquitin transfer (*41, 42*).

2.2 Different ubiquitination patterns have distinct functions

Attachment of single ubiquitin molecule on target protein (monoubiquitination) is sufficient to regulate various cellular processes such as endocytosis, DNA repair and replication,

histone function, and transcription (*45-48*). Moreover, targeted proteins can be monoubiquitinated on several lysine residues, resulting in multi-ubiquitination (*49-51*). Alternatively, the targeted protein can be modified with a chain of ubiquitin molecules (polyubiquitination) **(Figure 1B)**. Since ubiquitin protein has seven lysine (K) residues (K6, K11, K27, K29, K33, K48 and K63) within its sequence, ubiquitin itself can be a target for ubiquitination. Since either lysine residue in ubiquitin can partake in chain formation, the reactions can produce structurally and functionally distinct polyubiquitin chains(*49-52*). K48-linked polyubiquitin chain with at least four ubiquitin molecules is well characterized as a signal for degradation by the proteasome (*53-55*). K63-linked polyubiquitin chains play crucial roles in DNA repair, protein trafficking and inflammation, and K6-linked chains are involved in DNA repair (*47, 52, 56-60*). The anaphase-promoting complex (APC/C) catalyzed K11 polyubiquitin chain also acts as efficient proteosomal targeting signal *in vitro* and *in vivo* (*61-63*). Another layer of complexity is introduced when several different lysines in the ubiquitin are conjugated and form mix-linkage or forked chains (*52*). There is also evidence for the linear polyubiquitin chain in which the C-terminal glycine of ubiquitin is bound to the α-amino group of the N-terminal methionine of another ubiquitin (*64*). Detailed characterization of these diverse ubiquitination patterns still awaits and will further extend our understanding of the functions of ubiquitin in the cell.

2.3 Principles of protein SUMOylation

Following the discovery of ubiquitin function, several structurally similar ubiquitin-like proteins (Ubls) have been identified as protein modifiers. Although Ubls do not share high sequence similarity, they all possess essentially the same three dimensional structure, and appear to be attached to substrates via similar sequential enzymatic cascade (*65*). Small Ubiquitin related MOdifier (SUMO)-protein is the best studied Ubl and functions as a critical regulator of many cellular processes, including nuclear transport, transcription, degradation, DNA repair and cell cycle control (*66-72*). SUMO conjugation to substrates occurs through a sequential enzymatic cascade involving a SUMO-specific E1 activating enzyme (SAE1/SAE2 heterodimer), an E2 conjugating enzyme (UBC9) and E3 ligases such as MMS21, PIAS1-4, RanBP2 or TOPORS (*70, 73, 74*). Similar to ubiquitin, the C-terminal glycine residue of SUMO is conjugated to lysine side chain of the target protein via an isopeptide bond, frequently, on the consensus sequence ψKxE (where ψ corresponds to a large hydrophobic amino acid, and x is any amino acid) (*70, 75, 76*). In mammals, three expressed forms of SUMO proteins have been well characterized: SUMO1, -2 and -3. SUMO2 and -3 have 95% homology with each other, so they are considered functionally equivalent and often referred to as SUMO2/3(*70, 75, 76*). Although SUMO2/3 is only 50% identical in sequence to SUMO1, all SUMO proteins share a common tertiary structure known as the ubiquitin superfold (*70, 71, 75*). Consistent with sequence divergences, SUMO1 and SUMO2/3 have distinct substrates specificity and also differ in their ability to form SUMO chains. SUMO2/3 possesses SUMO consensus sequence at their N-terminus and has the ability to form poly-SUMO chains, wheras SUMO1 lacks this consensus motif and is able to terminate the chain on SUMO2/3 polymers (*70, 71, 74-76*).

2.4 Reversing the ubiquitination and ubiquitin like modifications

Ubiquitination can be reversed by deubiquitination enzymes (DUBs). DUBs play four major roles in ubiquitin metabolism. First, DUBs engage in activation and processing of the

ubiquitin precursor into mature ubiquitin monomers, probably co-translationally. Second, DUBs recycle the ubiquitin that was accidentally removed during the ubiquitination process. Third, DUBs restore monoubiquitin molecules from the unanchored polyubiquitin chains that is synthesized or released during the ubiquitination process. Finally, DUBs act as antagonist to E3 ligases by reversing the ubiquitination or ubiquitin-like modification of target proteins (*77, 78*).

Human genome encodes approximately 100 deubiquitinases and they can be divided into five major families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteses (USP), ovarian tumour proteases (OTUs), Ataxin-3/Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMM/MPN+). The UCH, USP, OTU and Ataxin-3/Josephin families belong to Cys proteases, whereas the JAMM/MPN+ family members are zinc metalloproteases (*79*).

Protein SUMOylation is also a highly dynamic reversible process. The de-conjugation enzymes, also known as SUMO-specific proteases (SENP), cleave the isopeptide bond between SUMO and the target protein and are also responsible for processing of SUMO precursors into mature forms (*80*). In mammalian cell, SENP family contains six proteins (SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7) (*81*). SENPs can be classified into three subgroups according to their functions. SENP1 and -2 can cleave both SUMO1 and SUMO2/3 from substrates and are involved in cellular maturation of the SUMO proteins. SENP3 and -5 remove the monomeric SUMO2/3 from substrates and SENP6 and -7 perform the editing and deconjugating of poly-SUMO-2/3 chains (*82, 83*).

3. An overview of DNA damage response signaling and modifications by ubiquitin and ubiquitin-like molecules

Exposure to endogenous and exogenous genotoxic stress triggers signaling pathways that allow the cell to slow down cell cycle progression and repair the damaged DNA. The framework of DDR can be defined in three categories: 1) sensors that continuously scan chromatin to detect the damage sites; 2) mediators or transducers that spread the signal, and 3) effectors that receive the signal and coordinate the response (*84*). Of crucial importance is the activation of the central checkpoint kinase Ataxia Telangiectasia Mutated (ATM) and ATM and Rad3-related kinase (ATR) that results in the phosphorylation of large number of substrates that execute diverse functions (*6, 7*). This is by far the most extensively studied pathway of the DDR.

Accumulating evidence suggest that phosphorylation is intimately coupled with other post-translational modifications including those by ubiquitination and ubiquitin-like molecules. Consistent with this hypothesis, proteins involved in ubiquitin proteasome systems are over-represented among the ATM/ATR substrates identified from proteomics screen (*6, 7*). Importantly, functional studies demonstrate that several E3 ligases acts at various stages of DNA damage response, including chromosome structure remodeling, signal amplification and repair, and their deregulation is associated with genetic diseases (*1, 25, 85-87*). In next section, we will describe the role of E3 ligases in DNA damage response pathway.

4. Modification by non-degradable Ubls in DNA damage signaling

The initial response to DSB is the sensing of DNA damage by multifunctional protein complexes that are able to bind directly to DNA. They include the MRE11/RAD50/NBS1

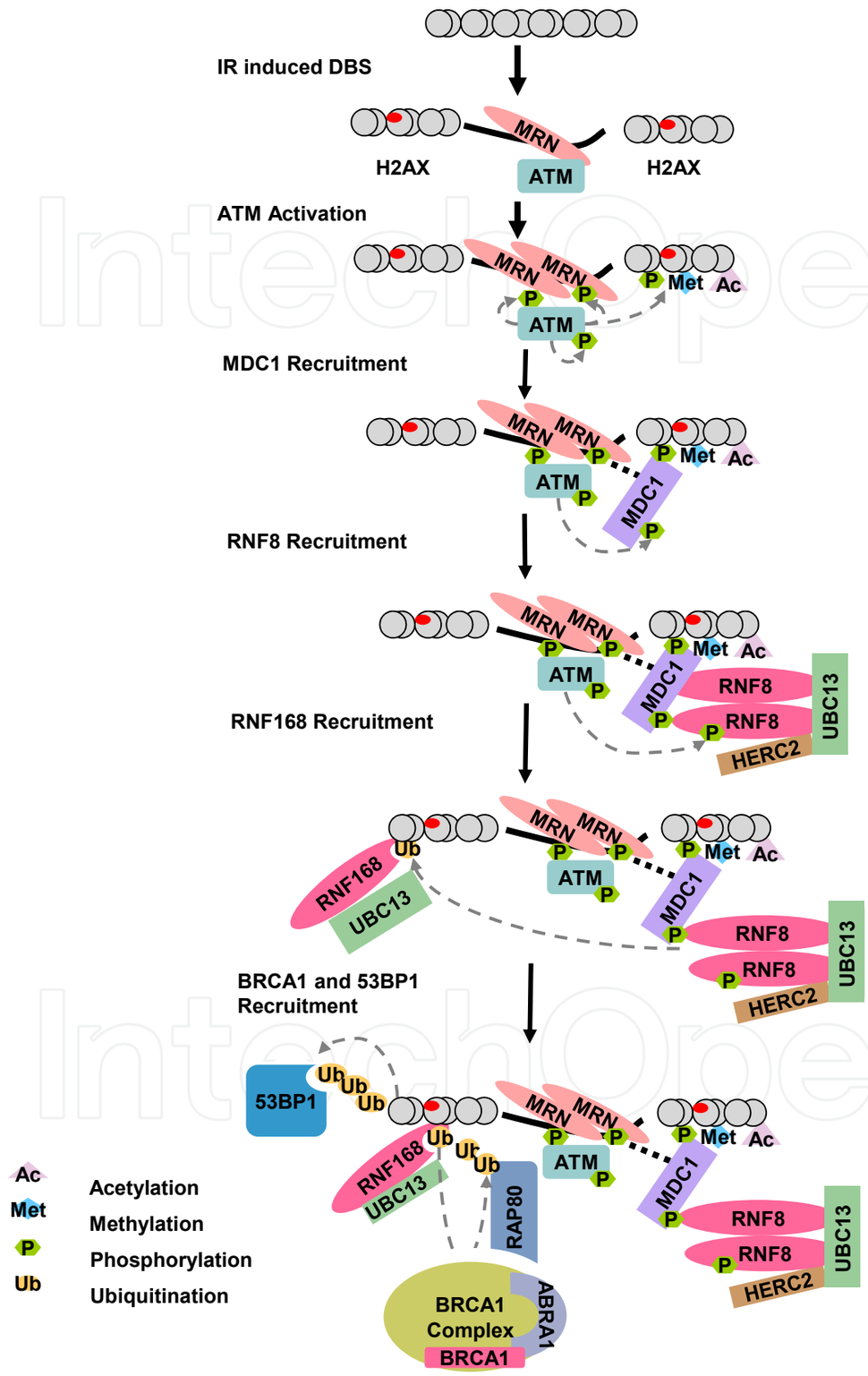

Fig. 2. Schematic representation of RNF8/RNF-168- mediatred ubiquitination at DSB and recruitment of DDR factors.

(M/R/N) complex, the Ku70/80/DNA-PKcs and perhaps ATM kinase itself (*88, 89*). Binding of M/R/N and Ku70/80 complexes onto DNA leads to the recruitment and activation of the Ser/Thr kinases ATM and DNA dependent kinase catalytic subunit (DNA-PKcs), respectively. Both kinases belong to Phosphatidylinositol -3-Kinase-like-Kinses (PIKK) family of serine–threonine kinases with sequence similarity to Phosphatidylinositol-3 Kinases (PI3Ks) and phoshorylate the histone variant H2AX on Ser139 residue (γ–H2AX) (*90, 91*). This phoshorylation marks the DNA damage sites and facilitates the recruitment of Mediator of DNA damage Checkpoint (MDC1). This initiates amplification of DDR signal by promoting the accumulation P53 Binding Protein 1(53BP1), RING Finger Protein 8 (RNF8), RING Finger Protein 168 (RNF168), RAP80 and Breast cancer susceptibility gene 1 (BRCA1) at damage sites (*92-97*). MDC1 and γ-H2AX dependent localization of RNF8 and RNF168 E3 ligases and ubiquitination of γ–H2AX are responsible for organizing the recruitment of BRCA1, 53BP1 and other DDR complexes and higher-order chromatin remodeling to facilitate local exposure of constitutive chromatin marks which leads to amplification of signal **(Figure 2)** (*58, 59, 96, 98*).

4.1 E3 ligases that are involved in the histone ubiquitination in the initial DNA damage signaling

RNF8 and RNF168 are substrates of checkpoint kinases that are recruited to sites of DNA damage

Following MDC1 phosphrorylation, RNF8 appears to be the first of the several E3s recruited to sites of DNA damage. RNF8 recognizes phosphorylated TQXF motifs on MDC1 through its FHA domain and cooperates with UBC13 to initiate the transfer of ubiquitins to histones (*58, 94, 96, 98*). Since UBC13 catalyzes the elongation of K63- specific polyubiquitin chain, RNF8/UBC13 mediated ubiquitination does not involve protein degradation (*58, 94, 96, 98, 99*). Importantly, accumulation of RNF8 on DNA damage sites is required for the recruitment of checkpoint mediators 53BP1 and BRCA1, suggesting that RNF8 lies upstream of these proteins. Recent studies also show that RNF8/UBC13-mediated ubiquitination contributes the recruitment of BRCA1 and 53BP1 to damage site, but it is not sufficient for persistent assembly of these factors (*58, 96, 100, 101*). Another RING type ubiquitin ligase, RNF168, recognizes RNF8/UBC13-mediated H2A and γ-H2AX ubiquitination by its Motif Interacting with Ubiquitin (MIU) domains to amplify and spread the regulatory K63-polyubiquitination on H2A and γ-H2AX (*1, 59*). In turn, RNF168-dependent ubiquitylation promotes the retention of BRCA1 and 53BP1 at DSB sites (*59*).

The importance of RNF8-mediated pathway in DNA damage response was demonstrated by the observation that biallelic mutations in RNF168 are associated with radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties (RIDDLE) syndrome (*1*), a disorder associated with defective DSB repair that shares some clinical features with other genome instability syndromes such as ataxia-telangiectasia (*102*). Cells derived from a RIDDLE patient are defective in DNA damage-induced ubiquitylation and exhibit impaired localization of 53BP1 and BRCA1 to DSBs, while MDC1 and NBS1, similarly to RNF8 and RNF168 depleted cells, remain unaffected (*1, 59, 102*).

HERC2 facilitates K63-polyubiquitination by stabilizing RNF8-UBC13 interaction

Recently, another ubiquitin ligase, HERC2 was shown to be involved in non-proteolytic histone polyubiquitination at DNA lesions (*103*). HERC2 interacts with the FHA domain of

RNF8 in phosphorylation-dependent manner. DNA damage-dependent phosphorylation of HERC2 on T4827 by PIKKs provides the docking site for FHA domain of RNF8 and allows the formation of a ternary MDC1-RNF8-HERC2 complex at DNA lesions (*103*). The exact function of HERC2 at DNA lesions is not entirely understood but it is suggested that HERC2 mediates and stabilizes the RNF8–UBC13 interaction and stimulates the K63-polyubiquitination. Another study showed that HERC2 promotes BRCA1 polyubiquitination thereby targeting BRCA1 for proteasome degradation (*104*). These findings are consistent with the overexpression of HERC2 in breast epithelial and carcinoma cells, implicating its role in breast cancer carcinogenesis.

BMI/RING1 E3 ligase contributes to γH2AX ubiquitination in a RNF8-independent manner

The polycomb repressive complex 1, which contains BMI1, RING1, and RING2, functions as an E3-ubuiquitin ligase. BMI1 and RING2 are recruited to sites of DSB where they contribute to the ubiquitination of γ-H2AX (*105*). In the absence of BMI1, ubiquitination-dependent recruitment of several proteins, including 53BP1, BRCA1, and RAP80, are impaired (*105, 106*). In contrast to RNF8, recruitment of BMI1 does not require H2AX, but depends on the FHA and BRCT domains of NBS1. Loss of BMI1 sensitizes cells to ionizing radiation to the same extent as loss of RNF8, and simultaneous depletion of both proteins revealed an additive increase in radiation sensitivity. Thus, it appears that RNF8 and BMI represent two distinct recruitment pathways for 53BP1 and BRCA1.

RNF20 and RNF40 regulate H2B mono-ubiquitination and homologous recombination

Recently, monoubiquitination of histone 2B (H2B), which is associated with transcription elongation, is shown to be a part of DDR in an ATM-dependent manner and required for timely damage repair (*107-109*). Heterodimeric RING-finger proteins RNF20 and RNF40, which are orthologs of the budding yeast protein Bre1 together with the ubiquitin-conjugating enzyme Rad6, catalyze H2B monoubiquitylation on lysine120 in the mammalian cells and on lysine 123 in yeast (*110*). Importantly, RNF20 and RNF40 physically interact with ATM and NBS1 (a member of the MRN complex) and are phosphorylated by ATM, which is essential for their recruitments to DNA damage site(*108, 111*). Either depletion of RNF20 /RNF40 or the mutation of H2B monoubiquitination sites lead to a significant decrease in RPA, RAD51 and BRCA1 localization on DSBs and interfere with Homologous Recombinational Repair (HRR) in human cells. Since the defect in HR causes the genomic instability by accelerating cancer-promoting mutations, dysfunctional RNF20/RNF40 increase the susceptibility to cancer (*112*).

4.2 E3s that are recruited to DSBs by ubiquitinated histones

Recruitment of BRCA1-A complex to DNA damage sites by RNF8/RNF168-mediated histone ubiquitination

The mechanism by which RNF8/RNF168 mediated-ubiquitination facilitates the recruitment of DDR factors is only partially resolved. RNF8/RNF168 can add K63-polyubiquitin chains on H2A and γ-H2AX that, in turn, act as molecular landing pads for downstream DDR factors with ubiquitin binding domains. The BRCA1 /BARD1 complex is one of the E3 complexes that are recruited to DNA damage sites in γ-H2AX-ubiquitination dependent manner.

The product of tumor suppressor gene BRCA1 plays a critical role in maintenance of genomic integrity by regulating DNA damage-induced cell cycle checkpoint activation, repair, chromatin remodeling, as well as transcriptional regulation and apoptosis (*5, 113*). BRCA1 protein contains two BRCT motifs at its C-terminus and RING domain at N-terminus (*114*). BRCT motif of BCRA1 functions as a phospho-protein binding domain and is required for its translocation and accumulation at DNA damage sites (*115-117*). RING domain of BRCA1 interacts RING domain of BARD1 (BRCA1-Associated Ring Domain protein 1) as a heterodimer and operates as an E3 ubiquitin ligase (*39, 118, 119*). The BRCA1/BARD1 complex can assemble with UBCH5C E2 ligase to promote the K6 ubiquitin chain *in vitro* (*120*). BRCA1/BARD1 heterodimer also forms at least four non-overlapping endogenous complexes with distinct functions. The "BRCA1-A" complex contains BRE/BRCC3/NBA1, FAM175A (Abraxas), FAM175B (Abraxas brother 1), and RAP80 (*100, 121, 122*). The RAP80 (Receptor Associated Protein 80 or UIMC1) is the component within BRCA1-A complex and is responsible for this recruitment of this complex onto the damage site by recognizing the K63 polyubiquitin chain through its ubiquitin interacting motifs (UIM) (*123, 124*).

Aside from RAP80 and BRCA1/BARD1, many components of the BRCA1-A complex possess structural domains implicating them in ubiquitination pathway. BRCC36 has a JAM domain characteristic of lysine 63-specific deubiquitinating enzymes, and Abraxas and BRE contain predicted Mpr-1/Pad1 N-terminal (MPN$^-$) and ubiquitin E2 variant (UEV) domains respectively (*122, 125-128*). Possible targets of BRCC36 include RAP80 and di-ubiquitinated H2A/γH2AX. BRCC36 DUB activity and localization are regulated via its interactions within the context of each protein complex. In the BRCA1-A complex, ABRA1 and BRE are essential for BRCC36 DUB activity and localization at DNA damage site (*100, 122, 127, 128*).

Although the exact function of BRCC36 deubiqutination at DNA damage site is not known, it has been suggested that BRCC36 catalyzes the deubiquitination of chromatin-associated proteins that lead to the chromatin remodeling and amplification of damage signal. In addition, deubiqutination by BRCC36 may contribute to the switch-off mechanism after the damage is repaired, which is required to resume normal DNA replication and cell proliferation. It is also speculated that BRCC36-driven deubiquitination not only removes the K63 polyubiquitin chain, but it may also promote BRCA1- dependent K6 ubiquitination.

Rad18 is another E3 ubiquitin ligase that accumulates at DSB sites in a manner dependent on RNF8

Rad18 is well-known for its function in DNA damage bypass and post-replication repair in yeast and vertebrates, where it promotes monoubiquitination of proliferating cell nuclear antigen (PCNA) at stalled replication forks (*129*). It was shown recently that RAD18 also participates in the signaling and homologous repair of DSB (*130*). The UBZ-like zinc finger domain on RAD18 preferentially binds to K63-linked Ub chains *in vitro*, and is required for Rad18 IR-induced foci (IRIF) formation, suggesting that RAD18 may be recruited to DSB through binding of its zinc finger domain to ubiquitinated proteins. RAD18 functions as an adaptor to facilitate homologous recombination through direct interaction with the recombinase RAD51C, although this function does not require its E3 ligase activity(*129-132*). Thus, the substrates of RAD18 in this process remain unknown.

4.3 SUMOylation system

Recent studies showed that the SUMO-conjugating system is operating in parallel and in cooperation with the ubiquitin-conjugating system for the assembly of 53BP1 and BRCA1.

SUMO1, SUMO2/3 and SUMO E3 ligases PIAS1 and PIAS4, as well as the E2 UBC9 also accumulate at DSB sites. Moreover, both 53BP1 and BRCA1 are possible substrates for SUMOylation, and the E3 Ub ligase activity of BRCA1 may also be subjected to regulation by SUMOylation (*73*).

5. Poly-ub-dependent protein degradation in cell cycle checkpoint control

Damaged DNA can lead to fixation of potentially harmful mutations during cell division. To this end, cells evolved checkpoints that can stop the cell cycle or slow down its pace to allow damage to be repaired. Two E3 ligases, the SCF and APC/C complexes that regulate normal cell cycle progression, also control cell cycle arrest. Another well-studied DNA damage checkpoint protein that is subjected to regulation by polyubiquitination-dependent degradation is the tumor suppressor protein p53, which regulates a wide variety of cellular processes, including transcription, apoptosis and senescence. Under normal growth conditions, p53 is kept at low levels by multiple E3 ligases through rapid turn-over; in response to DNA damage, p53 is rapidly stabilized and its level is maintained. Over the years, an increasing number of E3s and DUBs have been shown to be involved in this response. In this section, we will introduce the E3 ligases and DUBs that regulate p53 and substrates that are subjected to SCF and APC/C-mediated degradation for cell cycle controls.

5.1 The E3s and Dubs that keep the master tumor suppressor p53 under tight regulation

Tumor suppressor protein p53, also known as "the guardian of the genome", is a transcription factor that plays essential roles in multicellular organisms to regulate cell cycle progression, apoptosis, and senescence in response to DNA damage (*133-135*). Loss of function mutations in p53 are found in more than 50% of the tumors. In unstressed cell, p53 protein level is kept low predominantly through the ubiquitin-mediated proteosomal degradation. Several E3 ligases and their associated factors are involved in p53 ubiquitination and directly affect p53 levels, sub-cellular localization, and activity (*136, 137*). In addition, deubiquitination enzymes such as Herpes virus-Associated Ubiquitin Specific Protease (HAUSP, also known as USP7), and other ubiquitin-like (UBL) proteins also have impact on p53 abundance and activity (*136, 137*).

The identification of the human papilloma virus (HPV) E6-associated cellular protein E6AP was the first indication for the ubiquitination-dependent p53 regulation (*138*). Shortly after, RING type ubiquitin ligase Murine Double Minute 2 (MDM2) was characterized as the p53 E3 ligase that regulates its stability by promoting the formation of polyubiquitin chains on p53 and targeting it for ubiquitin-dependent proteasomal degradation (*139-141*). The importance of MDM2 in p53 regulation is highlighted by the *Mdm*2 knock-out mice studies. The embryonic lethality of *Mdm2*-knockout mice, which is due to p53 induced apoptosis, is rescued by deletion of p53 (*142, 143*).

The MDM2 analogous MDM4 or MDMX, has emerged as another key regulator of p53. *Mdm4* was identified as a novel *p53* interacting protein in mouse and as a negative regulator of p53 by inhibiting p53 transcription activity (*144*). Importantly, MDM4 can form a heterodimer with MDM2 through their RING domains (*145, 146*) and while MDM4 lacks intrinsic ubiquitin-ligase activity, it can stimulate MDM2–dependent p53 ubiquitination and degradation *in vivo* (*40*). MDM4 also stabilizes MDM2, presumably by reducing MDM2

homodimers and thereby restricting MDM2 autoubiquitination activity (*146*). Like *Mdm2*, *Mdm4* deficiency in mice leads to early embryonic lethality due to cell arrest, and the phenotype is rescued by p53 loss and partially rescued by the loss of cyclin-dependent kinase inhibitor 1A (Cdk1a, the gene that encodes p21 which regulates proliferation) (*147, 148*).

The six key lysine residues (K370, K372, K373, K381, K382, and K386) located at the most C-terminus of p53 are well-characterized ubiqutination sites for MDM2 *in vitro* (*149*). However, knock-in mice studies, where all the lysine residues are replaced with arginines (p53-6KR mutant) *in vivo*, have shown that p53 expression levels are similar to wild type p53, implying that while these lysines are critical to regulate p53 function, they are not sufficient for degradation and alternative lysine residues may be involved in p53 stability (*150, 151*). Recently, it was shown that p53 could be ubiqutinated in the DNA binding domain *in vitro* and deletion mutant of DNA binding domain stabilizes p53 by decreasing its ubiqutination (*152*).

Many stress factors, such as DNA damage and oncogene activation, lead to p53 stabilization and increase in its transcriptional activity to regulate checkpoints especially the G1/S and apoptosis. In a widely accepted model, rapid p53 stabilization during early response to DNA damage occurs through phosphorylation of p53 at Ser15 and Ser20 and/or acetylation of p53 at C-terminal lysine residues instead of ubiqutination by DNA damage responsive enzymes, such as ATM/ATR, DNA-PK, CHK1, CHK2 and p300. These post-translational modifications (PTMs) cause p53 to dissociate from its negative regulators MDM2 and MDM4 and enhance the p53 transcriptional activity (*137, 153*). ATM also stimulates the phosphorylation of MDM2 and MDM4 at their C-terminus, which leads to their rapid degradation. ATM also activates another kinase c-Abl, to phosphorylate MDM2 at tyrosine 394 and MDM4 at tyrosine 99, which decrease their ability to inhibit p53 (*154-157*). Tumor suppressor ARF (known as p14ARF in humans and p19ARF in mouse) contributes to p53 stabilization by sequestering MDM2 and thus preventing MDM2 from physically interacting with p53 (*158*).

Active form of p53 is also known to be a transcription factor for its own major negative regulator MDM2 and lead to the accumulation of MDM2 by creating a negative feedback loop(*159, 160*). On the other hand, MDM2 contributes to the positive feedback loop to p53 stability by stimulating its and MDM4 ubiquitination for degradation (*161, 162*). Recent study also shows that another E3 ubiquitin ligase, RFWD3 (RING finger and WD repeat domain 3) positively regulates p53 stability as part of the late response to DNA damage. RFWD3, which is phosphorylated by ATM/ATR, is a potent E3 ligase for p53 that promotes its oligo-ubiquitination in vitro. RFWD3 forms a complex with MDM2 and p53, restricts MDM2 polyubiquitination activity in a way that p53 becomes resistant to 26S proteosomal degradation (*163*).

MDM2 also mediates ubiquitin-like modifications such as SUMOylation and Neddylation of p53 (*164, 165*). PIAS family of E3 ligases mediates p53 sumoylation at lysine 386 (K386) in vitro and MDM2 can fulfill this in an ARF-dependent manner. MDM2/ARF mediated p53 SUMOylation is associated with relocalization of p53 to subnuclear structures and modest increase in p53 transcriptional activity, the biological consequences of this phenomenon are not completely clear (*72, 165, 166*).

Although MDM2 is a major ubiquitin ligase of p53, other ubiquitin ligases also play a role in p53 ubiqutination, as p53 protein is still degraded at certain levels in Mdm2 deficient mice. Several p53 ubiquitin ligases have been identified including ARFBP1, COP1, E4F1, Ubc13,

Pirh2, TOPORS and WWP1 (*136, 137*) **(Figure 3).** These ubiquitin ligases can be categorized into two groups according to their ability to built K48 linked-polyubiquitin chain and non-proteasomal ubiquitin chain. The first group, which includes Pirh2, COP1, CARP1, CARP2 and ARFBP1, regulates the p53 protein level through the proteasomal degradation with the exception of TOROS, whose function is unknown (*166-171*). The main function of the proteins in second group (WWP1, MSL2 and E2 enzyme Ubc13) is to regulate the p53 localization (*172-174*).

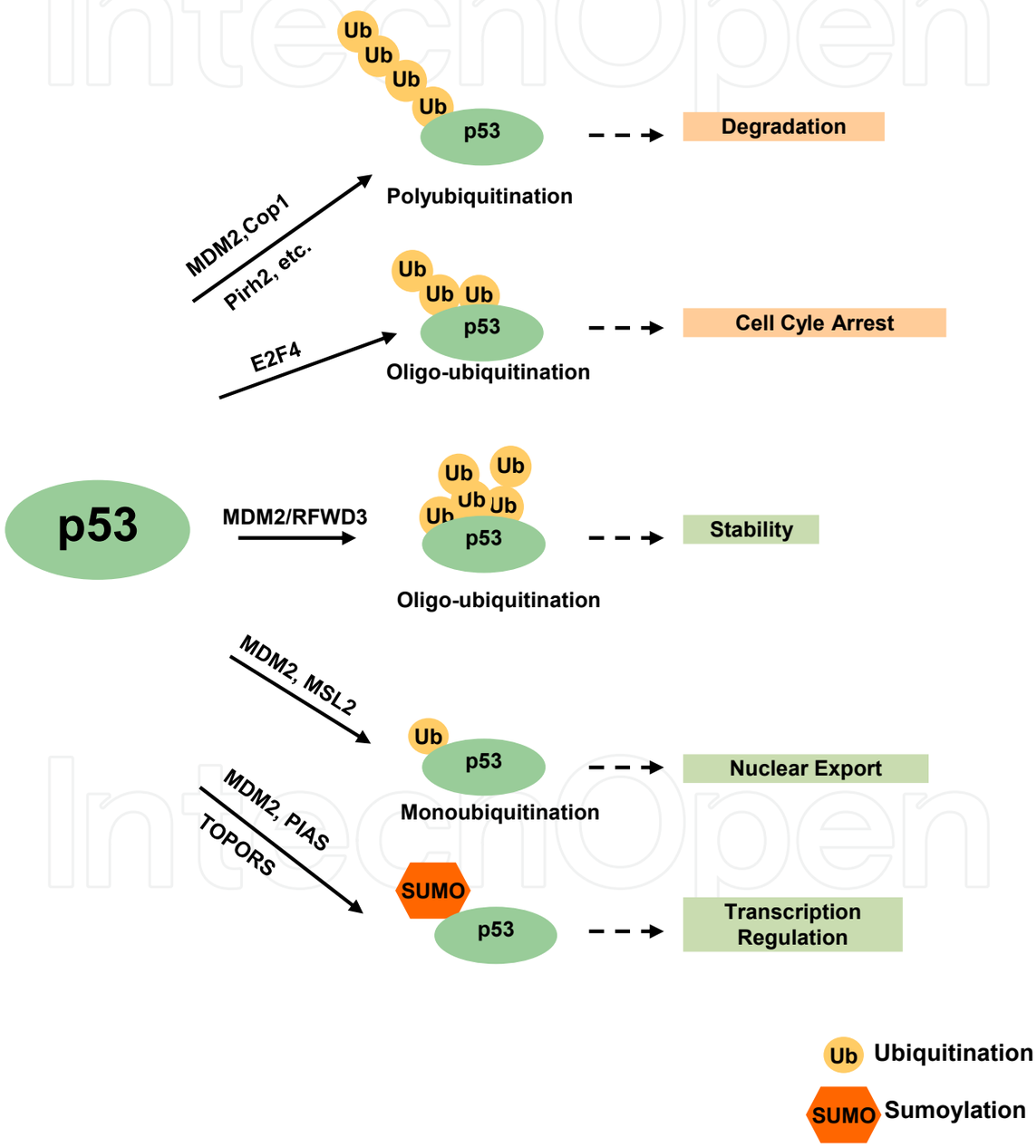

Fig. 3. MDM2-dependent and independent p53 ubiquitinationation patterns and their distinct functions in p53 biology.

In addition, transcription factor E4F1 was shown to ubiquitinate p53 mainly through the K48-linkage (*175*). E4F1 is considered as an atypical E3 ligase because it does not contain a HECT or RING domain. However, it has SUMO E3 ligase RanBP2 catalytic domain homologous region (aa 30-80), which provides its E3 ligase activity (*66, 175, 176*). E4F1 can catalyze the formation of mono-, di-, and tri-ubiqutination on the lysine cluster K319–K321 of p53 that are distinct from those targeted by MDM2 and these oligo-ubiquitin chains neither target p53 for degradation nor change the p53 nuclear/cytoplasmic ratio. E4F1-dependent ubiquitination instead increases p53 localization to the chromatin fraction selectively in p21 and cyclin G promoter region and regulates transcription of p53 target genes that are required for cell cycle arrest, but not of those involved in apoptosis. Furthermore, E4F1 mediated K320 ubiquitination competes for acetylation of K320 by PCAF that induce apoptosis in response to DNA damage (*175, 177*). This suggests that E4F1 dependent ubiquitination inhibits the PCAF-mediated acetylation of p53 and triggers an anti-apoptotic program in response DNA damage.

Ubiquitination is dynamic and reversible process through ubiquitin ligases and deubiquitinases (DUBs). Like ubiquitin ligases, deubiquitination enzymes (DUBs) regulate the fate and function of ubiquitin-conjugated proteins and have been implicated in several important pathways including cell growth and differentiation, development, oncogenesis and transcriptional regulation. DUBs activity is especially critical and tightly regulated when ubiquitination is used as a signal for non-proteasomal process and play an important role in p53 stability and activity. The first DUB shown to regulate p53 stability was HAUSP (*178*). HAUSP was originally characterized as Herpes virus-associated cellular factor and shown to interact and deubiquitinate p53 in vitro and in vivo (*178-180*). HAUSP belongs to the Ub-specific processing protease (UBP) subfamily of DUBs and contains conserved core enzymatic domain with characteristic Cys and His motifs (*181*). Beyond the catalytic domain, HAUSP has N-terminal and C-terminal extensions with no significant similarity to other members of UBP family and these extensions are important for substrate recognition and subcellular localization. HAUSP interacts and modulates not only p53 but also MDM2 and MDM4 protein level and function has greatly increased the complexity of its role within the p53-MDM2 pathway (*182, 183*). While overexpression of HAUSP results in the stabilization of p53 through de-ubiquitination and partial knockdown of HAUSP decreased p53 protein level, complete ablation of HAUSP led to an increase in p53 levels due to increase in MDM2 ubiquitination and proteasomal degradation (*184*). In the absence of stress, HAUSP is involved in stabilizing MDM2 and MDM4 by removing the ubiquitin molecules, however, MDM2 and MDM4 phosphorylated by ATM as a response to DNA damage and this phosphorylation inhibits MDM2 or MDM4 interaction with HAUSP and causes the MDM2 and MDM4 degradation in favor of p53 activation (*185*).

Besides the regulation of protein stability, DUBs have been shown to regulate chromatin structure and to facilitate both gene activation and gene silencing. HAUSP also stimulates p53 DNA binding activity and involves in its transcription function (*186*).

Another deubiquitinating enzyme USP2a was associated with MDM2 *in vivo* and shown to deubiquitinate MDM2, without reversing MDM2-mediated ubiquitination of p53. Ectopic expression of USP2a therefore leads to an increase in the levels of MDM2 and promotes p53 degradation. Downregulation of USP2a protein level with siRNA results in destabilization of MDM2 and thus elevated p53 protein expression and upregulation of p53 target genes. MDM2 specific deubiquitin ligase USP2a contributes to repression of p53 activity *in vivo* (*187*).

5.2 Substrates of CRLs that regulate cell cycle arrest

Many cellular factors that orchestrate cell cycle progression under normal conditions, including numerous UPS proteins, also play key roles in regulating damage-induced cell cycle arrest. Their functions in normal cell and in damage-induced cell mainly manage through synthesis-degradation and phosphorylation-dephosphorylation manner. Two well-studied E3 ligase families, the SCF and APC/C complexes polyubiquitinate these cellular factors and target them for proteasome-mediated degradation. Both SCF and APC/C are CRL-type E3 ligases composed of a scaffold protein (Cullins and APC2), a RING domain containing protein that mediates its interaction with E2 (RBX1 and APC11), adaptor proteins and co-activators that determine substrate specificity (F-box proteins, CDC20 or CDH1) (*188*). Importantly, activation of these E3 ligases is closely coupled with the activation of checkpoint kinases, which phosphorylate their substrates and facilitate the recognition by the F-box proteins in the SCF complex.

Chk1, a substrate of ATR/ATM, is an effector kinase that is part of both checkpoint initiation and termination

CHK1 is essential for cell viability and is phosphorylated and activated by ATR/ATM in response to replication block and DNA damage. Activation of CHK1 leads to the phosphorylation of a plethora of substrates including Cdc25A, Claspin, Kap1, which are critical for the activation of intra-S and G2/M checkpoint (*189*). CHK1 phosphorylation also promotes its own degradation mediated by the SCF E3 ligase cullin 4A/DDB1 or cullin 1 CUL1/Fbx6 (*190*). Therefore, CHK1 activity is fine-tuned by a negative feed-back loop to ensure timely termination of checkpoint signal and resuming cell cycle progression. In addition, the adaptor protein Claspin that facilitates ATR-mediated Chk1 phosphorylation, also contributes to down-regulation of Chk1 activity through its own proteolysis (*191*) (see below).

CDC25A is phosphorylated and degraded to prevent G1-S transition

Cdc25A is a phosphatase that is an essential regulator for CDK (Cyclin Dependent Kinase) activity during cell cycle progression (*189*). By removing the inhibitory phosphorylation of CDKs, Cdc25A drives cell cycle from G1 to S, and elevated Cdc25 levels resulting from their stabilization and persistence during cell cycle periods have significant oncogenic potential. In unperturbed cells, Cdc25A protein is maintained at modest levels during S and early G2 by the action of the ubiquitin ligase $SCF^{\beta TrCP}$, whose recognition of Cdc25A depends upon phosphorylation by a number of kinases (*192, 193*). At the end of mitosis and through G1, Cdc25A is eliminated via the APC/CCdh1 ubiquitin ligase. DNA damage induction of Chk1 activity increases the priming phosphorylation of Cdc25A, leading to greater $SCF^{\beta TrCP}$-induced degradation (*194*). Moreover, Cdc25A can also be stabilized by the ubiquitin hydrolase Dub3, which removes the polyubiquitin modifications that mark Cdc25A for proteasomal degradation (*195*). Importantly, Dub3 was able to transform NIH-3T3 cells and cooperated with H-Ras to promote growth and its overexpression is responsible for an abnormally high level of Cdc25A in a subset of human breast cancers (*195*).

CDT1 and Cdc6 are degraded upon DNA replication block to prevent replication

CDT1 is an essential protein required for licensing of replication origin in G1 and is degraded in S-phase to prevent re-replication and following DNA damage to prevent late origin firing (*196, 197*). In unperturbed cells, phosphorylation of CDT1 by cyclin-dependent kinase promotes its binding to and ubiquitination by SCF-Skp2 E3 ubiquitin ligase (*198-202*).

However, this pathway is not essential for the degradation of CDT1. It contains a second degradation signal at its N-terminus that is active after DNA damage, and is dependent on the loading of PCNA. Its ubiquitination and degradation requires CDT2 and DDB1, which are components of a CUL4 ubiquitin ligase (*200, 203*).

DNA damage-dependent ubiquitination of CDC6 has been attributed to two ubiquitin ligases, depending on the cell type and source of DNA damage. In p53-proficient cells treated with ionizing radiation, CDC6 is down-regulated through ubiquitination by the CDH1-associated form of the anaphase promoting complex, APC/C (*204*). In response to other forms of DNA damage, CDC6 is ubiquitinated by the HUWE1 enzyme irrespective of the p53 status of the cells (*205*).

Claspin is phosphorylated by CHK1 and PLK1, which mediate its degradation by $SCF^{\beta TrCP}$ and is involved in G2/M checkpoint-recovery from replication block

Claspin is an adaptor protein that facilitates ATR-mediated phosphorylation and activation of CHK1 in response to replication arrest and replication stress during normal cell cycle progression as a result of replication stress (*191, 206*). Claspin level is regulated throughout the cell cycle by ubiquitin mediated proteolysis. Two E3 ubiquitin ligases, the SKP1-CUL1-βTrCP and APC/CDH1 contribute to Claspin degradation (*207, 208*). Claspin is phosphorylated in a canonical DSGΦxSDSGFxS degron sequence, which is typical of $SCF^{\beta TrCP}$ substrates. Phosphorylation of Claspin is mediated by PLK1 and is essential for binding to βTrCP (*191, 209*). Thus, degradation of Claspin is essential for entering mitosis during normal growth and termination of checkpoint signaling to allow resumption of cell cycle. Claspin is also destabilized by the anaphase-promoting complex (APC) and thus remains unstable in G1. Whether APC/C-CDH1 also regulates Claspin stability after DNA damage is not clear.

Ubiquitination of Claspin is counteracted by 2 ubiquitin hydrolases. USP7 interacts with Claspin in vivo and is required to maintain steady-state levels of Claspin (*208*). Consistent with its role in stabilizing Claspin, USP7 also controls the recovery from replication arrest, presumably through regulating CHK1 phosphorylation. However, USP7 has no effect on APC/C-CDH1-mediated Claspin turn-over. USP28, a major DUB for maintaining 53BP1 protein level, has also been implicated in stabilization of several checkpoint proteins including that of Claspin (*210*).

6. Identification of new ubiquitinated proteins involved in DNA damage response

The key to a better understanding of how ubiquitination regulates biological processes is the identification of ubiquitinated substrates. An analogy can be drawn from recent systematic screens that identify multiple proteins that are phosphorylated by checkpoint kinase ATM/ATR. Taking advantage of a unique affinity of these kinases towards SQ motifs and the technical advancements in mass spectrometry, thousands of phosphorylated proteins were affinity purified with antibodies that recognize various pSQ motifs through "cross-reactivity" and identified by mass spectrometry (*6, 7*). Information derived from these studies tremendously expanded the landscape of DNA damage response pathways and provided valuable clues for follow-up functional characterization of these pathways. Unfortunately, such approach cannot be effectively applied to ubiquitination studies, as there is little information on any consensus motif that directs E3 ligases to their targets. Even

less is known about how E3 activity is regulated after exposure to DNA damaging agents. Nevertheless, it is expected that improved sensitivity of mass spectrometry and new bioinformatics tools will allow for quantitative analysis of the ubiquitination events that correlate with changes that take place after DNA damage. In this section, we summarize recent proteome-wide approaches for affinity purification and mass spectrometry identification of ubiquitinated proteins.

6.1 Affinity-based purification coupled with Mass spectrometric analysis

Affinity purification of ubiquitinated proteins

The first and essential step in the identification of ubiquitinated substrates on a proteome-wide scale is enrichment by affinity purification; as the abundance of ubiquitinated species is generally low. Currently, there are at least three different strategies for large-scale ubiquitination profiling (Figure 4B-4D); Epitope Tagged Ubiquitin (Ub) expression systems (Figure 4B), Tandem Ubiquitin-Binding Domain (UBD) affinity purification (Figure 4C), Linkage-Specific Poly-Ubiqutin Antibody purification (Figure 4D), and Ubiquitination Signature Remnant Antibody purification (*211*).

Epitope tagged Ub strategy was initially applied to profile yeast ubiquitome by using an engineered yeast strain, which expresses epitope-tagged ubiquitin at endogenous level in ubiqutin null background. (*212*). Totally 110 ubiquitination sites were identified by using a LCQ mass spectrometer (*212*). A similar approach was adapted in the mammalian system, in which a tandem His-biotin-tagged Ub was stably expressed in HeLa cells for efficient substrate isolation. In this experiment, ~ 50 ubiquitination sites were identified with a LTQ-Orbitrap mass spectrometer (*213*). Recently, the identification of ~750 lysine ubiquitination sites from two human cell lines that stably express HA-Ub was reported, which stands, by far, as the largest collection of ubiquitination sites (*202*).

Major advantages of epitope tagged Ub strategy are to purify the substrates under protein denaturing conditions and to eliminate contaminant non-substrate proteins. However, it remains uncertain to which extent tagging Ub over-expression may interfere with normal cellular functions (*214, 215*). Substitution the endogenous Ub lysine mutants would allow to investigate linkage-specific substrates, but the presence of four Ub loci in the mammalian genome is discouraging to establish ubiquitin null cell lines and to analyze the substrates in that background. Recently, engineered cell line that stably expresses an exogenous K63R mutant Ub with a tetracycline-inducible RNAi that eliminates endogenous Ubiquitin expression was generated (*216*). This method may partially solve this problem and facilitates the profiling of linkage specific ubiquitination substrates in mammalian cells.

High-affinity Ub antibodies, once successfully generated, are powerful tools for ubiquitination profiling of endogenous substrates. Previous attempts to generate high affinity Ub antibody from mice and rabbits were unsuccessful due to sequence conservation among species. Recently, several linkage-specific monoclonal antibodies have been selected from phage display libraries and have been shown to be useful for immunoprecipitation and western blot (*217, 218*). Whether or not these antibodies can efficiently profile linkage-specific substrates remains unclear.

The Gly-Gly remnant antibody was generated against the Gly-Gly signature peptides that generated after trypsin digestion of ubiquitinated proteins (*198, 201*). Since it recognizes specifically the modified peptides, it is more efficient for ubiquitination site profiling than

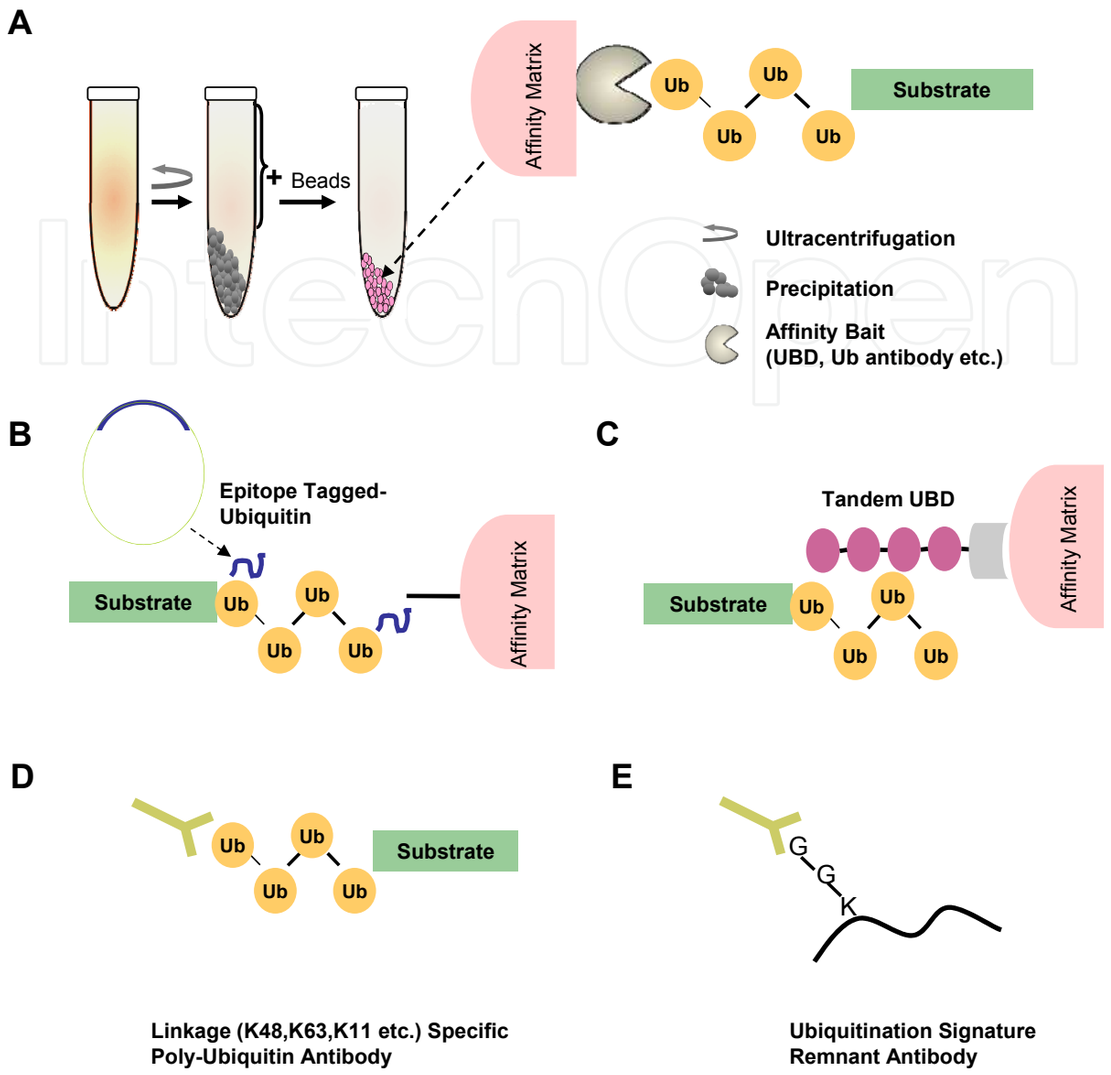

Fig. 4. A schematic presentation of ubiquitinated substrate purification strategies. A) Overall immunoprescipitation and purification procedure for ubiquitinated substrates. The affinity purification methods based on B) epitope-tagged ubiquitin, C) tandem ubiquitin-binding domain (UBD), D) linkage-specific poly-ubiquitin antibody or E) ubiquitination signature remnant antibody.

other methods that isolate the entire substrates. Using this reagent, hundreds of ubiquitination sites have been identified in human cells (*198, 201, 202*). Importantly, certain Ub-like modifications such as ISG15 or NEDD8 are also conjugated to their substrates through C-terminal Gly-Gly, as a result, the Gly-Gly remnant antibody does not distinguish ubiquitination from those modifications. It is necessary to couple with other purification method in order to obtain a homogenous population of ubiquitinated species for peptide IP (*211*) .

Ub binding domains (UBDs) are small structural entities that have affinity for Ub and can be used as natural affinity reagents to isolate endogenous poly-Ub proteins. Over 20 different UBDs have been discovered (*219-222*). The existence of a variety of UBDs implies their

functional diversities in the UPS signaling network. Although all UBDs bind Ub, the binding affinity varies greatly with the Kd value in the range of several hundred mM to the a few μM (*219*). The inherent low affinity of a natural UBD is generally not sufficient for large-scale purification of substrates. The development of engineered tandem UBD with appreciable affinity in the range of antibody makes it an attractive reagent for substrate purification. We have applied such strategy by engineering a GST fusion with four tandem UBA domains of ubiquilin 1 (GST-qUBA), which allowed us to detect close to 300 lysine ubiquitination sites from human cells that were not treated with proteasome inhibitors (*223*). A complication for this approach is that purification has to be performed under non-denaturing conditions; thus, "contaminant proteins" (especially true for UBD proteins that bind poly-Ub chains) are inevitably co-purified and sequenced by MS. These co-purified proteins may not only be misidentified as Ub substrates, but also masks the low abundant true substrates. Moreover, UBDs are more favorable for poly-Ub chains than mono-Ub and this approach could be biased towards polyubiquitininated substrates. Whether UBDs have preference towards specific type of poly-Ub or even the length of the chain remains unclear. A better understanding of UBD and Ub interaction require systematic investigation of more varieties of UBDs and may facilitate the design of poly-Ub linkage-specific reagents with higher affinity and selectivity (*211, 223*).

Precautions to be taken in affinity purifications

One of the major challenges for large-scale substrate purification is to distinguish the true ubiquitinated substrate from contaminant proteins. There are mainly three types of protein contaminants for Ub affinity purification: 1) non-specific binders to the solid matrix (e.g., agarose beads) for affinity reagents, 2) precipitated proteins that are accumulated during incubation, and 3) specific binders to poly-ubiquitinated proteins and/or Ub itself (*223*). Although it is very difficult to completely eliminate them all, the amount of contaminants can be significantly reduced by 1) carefully removing the insoluble precipitations after high-speed centrifugation, 2) shortening the affinity incubation time, 3) increasing protein solubility with stronger detergent such as 0.1% SDS, and 4) washing the beads with higher concentration of salt (*223, 224*).

The second challenge during purification is to preserve the ubiquitination by restricting the DUB activity. The use of DUB inhibitors of both Cys alkylation (such as iodoacetamide, choloroacetamide, or N-ethylmaleimide) and zinc chelating chemical (1, 10-σ-phenanthroline) can alleviate the loss of poly-Ub and preserve the intact modified substrates (*126*). In addition, avoiding freezing-thawing cycles by using freshly prepared cell lysate can help to preserve ubiquitinated sites.

Identification by mass spectrometry

The ubiquitinated species, once enriched and purified, can be analyzed by mass spectrometry (MS). While the ubiquitinated peptides purified from Gly-Gly antibody can be directly sequenced, ubiquitinated proteins are first subjected to digestion by proteases, such as trypsin, which cuts at carboxyl-terminus of lysine and arginine. The affinity purified proteins can either be first separated on SDS-PAGE and in-gel digested for peptides to be extracted, or directly digested on affinity matrix and extracted. Although on-beads digestion is fast, convenient, and suffers less sample loss, the SDS-PAGE separation provides a number of advantages. SDS-PAGE not only adds another purification step that eliminates the interference from abundant proteins that potentially mask the signals of less abundant

proteins, it also provides molecular size information for poly-ubiquitinted proteins that would migrate at higher molecular weight than their unmodified forms.
Ubiquitination can be identified by mass spectrometry with the detection of a mass shift of 114.043 Da- the ubiquitination signature that is derived from the di-glycine remnant of Ub after trypsin cleavage. Ubiquitination peptides (as well as sites) are identified by searching protein sequence database to match a tryptic peptide sequence with the addition of the ubiquitination signature mass on the particular amino acid, typically on Lys residues (*211, 223*).
The choice between peptide IP and protein IP has important implications. While peptide IP using Gly-Gly remnant antibody allows direct identification of Ub peptides and ubiquitination sites, these peptides may escape mass spectrometry detection if their sizes fall out of the mass range or they cannot be efficiently ionized. On the other hand, affinity-purified proteins that generate multiple peptides can be more readily detected, but cannot be distinguished from non-ubiquitinated proteins through association with ubiquitinated species. In this case, molecular weight information provided by SDS-PAGE can be used as a determinant.

6.2 Global Protein Stabilty (GPS) profiling of SCF Ub ligase substrates

Genetic screens are powerful tools for high throughput identification of biological targets through functional readouts. A strategy that combines global protein stability (GPS) profiling and genetic perturbation of E3 activity has been established to screen for substrates of the Skp1-cullin-F-box (SCF) ubiquitin ligase in mammalian cells (*225, 226*). In this approach, an HEK 293T reporter cell library expressing EGFP fused to ~8000 human open reading frames are established. In these cells, *Discosoma* sp. Red fluorescent protein (DsRed) and GFP fusion proteins are expressed under the control of the same promoter, serving as an internal control for protein expression. Thus, the GFP/DsRed ratio measured by FACS reflects the stability of the GFP-fusion protein, and the SCF targets can be detected in cells that show increased ratios in response to SCF inactivation. Since prolonged inhibition of SCF has the potential to alter cell cycle distribution, SCF is transiently reduced by lentiviral delivered dominant-negative Cul1. Among the >350 potential substrates identified, most known SCF targets are recovered and many previously unknown substrates involved in cell cycle, apoptosis, and signaling pathways are discovered (*225*). Although limited only to E3 ligases that regulate substrate stability, this approach has the potential for global discovery of any E3-substrate regulatory networks.

7. Concluding remarks

Protein modification by Ub and Ub-like molecules has emerged as a major regulatory mechanism for DNA damage signaling and repair. Accumulating evidence from last decade's research suggest that alteration of chromatin structure is a key step in the initiation and amplification of damage signals, as ubquitination of histones, such as H2A and H2AX, are substrates of several E3 ligases involved in DDR. Alternatively, it may also indicate that, while histones are previously known targets, other substrates remain unidentified due to technical difficulties. Likewise, two major E3 ligases (SCF and APC/C) appear to dominate the control of stability of most of proteins involved the cell cycle arrest. It is anticipated that technical advancement and availability of new affinity reagents will enable genome-wide, systematic characterization of UPS proteins in DDR.

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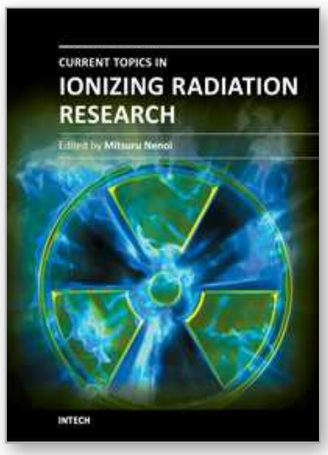

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Since the discovery of X rays by Roentgen in 1895, the ionizing radiation has been extensively utilized in a variety of medical and industrial applications. However people have shortly recognized its harmful aspects through inadvertent uses. Subsequently people experienced nuclear power plant accidents in Chernobyl and Fukushima, which taught us that the risk of ionizing radiation is closely and seriously involved in the modern society. In this circumstance, it becomes increasingly important that more scientists, engineers and students get familiar with ionizing radiation research regardless of the research field they are working. Based on this idea, the book "Current Topics in Ionizing Radiation Research" was designed to overview the recent achievements in ionizing radiation research including biological effects, medical uses and principles of radiation measurement.

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