Molecular Basis for K63-Linked Ubiquitination Processes in Double-Strand DNA Break Repair: A Focus on Kinetics and Dynamics

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Abstract

Cells are exposed to thousands of DNA damage events on a daily basis. This damage must be repaired to preserve genetic information and prevent development of disease. The most deleterious damage is a double-strand break (DSB), which is detected and repaired by mechanisms known as non-homologous end-joining (NHEJ) and homologous recombination (HR), which are components of the DNA damage response system. NHEJ is an error-prone first line of defense, whereas HR invokes error-free repair and is the focus of this review. The functions of the protein components of HR-driven DNA repair are regulated by the coordinated action of post-translational modifications including lysine acetylation, phosphorylation, ubiquitination, and SUMOylation. The latter two mechanisms are fundamental for recognition of DSBs and reorganizing chromatin to facilitate repair. We focus on the structures and molecular mechanisms for the protein components underlying synthesis, recognition, and cleavage of K63-linked ubiquitin chains, which are abundant at damage sites and obligatory for DSB repair. The forward flux of the K63-linked ubiquitination cascade is driven by the combined activity of E1 enzyme, the heterodimeric E2 Mms2-Ubc13, and its cognate E3 ligases RNF8 and RNF168, which is balanced through the binding and cleavage of chains by the deubiquitinase BRCC36, and the proteasome, and through the binding of chains by recognition modules on repair proteins such as RAP80. We highlight a number of aspects regarding our current understanding for the role of kinetics and dynamics in determining the function of the enzymes and chain recognition modules that drive K63 ubiquitination.

Introduction

DNA double-strand breaks (DSBs) are an insidious type of DNA damage that, left unaddressed, can lead to loss of genetic information, resulting in the onset of life threatening diseases including, for example, cancer, neurodegeneration, and metabolic disorders [1]. In response to such damaging DNA breaks, cells have evolved a sophisticated molecular defense system that leads to cell cycle checkpoint activation, cell cycle arrest, and ultimately repair of damaged DNA; the collective process is known as the DNA damage response [2]. Depending on cell cycle stage, DSBs are repaired either by non-homologous end-joining (NHEJ) or homologous recombination (HR) [3]. The NHEJ pathway carries out the joining of broken strands after end processing and is predominant throughout the cell cycle, whereas HR is active only in S and G2 phases, with the lost information in the broken strand regained through a homology search in the sister chromatid, thereby maintaining the fidelity of the repair process, as opposed to NHEJ, which is error-prone [4].

The repair of the broken strand by HR is enabled by timed recruitment and removal of different repair proteins [5]. These processes are guided by the action of different post-translational modifications such as phosphorylation, acetylation, methylation,
ubiquitination, and SUMOylation that orchestrate recruitment and removal of different DNA repair proteins at break sites, ultimately facilitating the repair of broken DNA strands [6]. This review will outline various aspects of our current understanding of the molecular basis for K63-linked ubiquitination in DSB repair by HR, with a specific focus on the role that kinetics and dynamics for the underlying protein components and protein interactions within this system play in the repair process.

**Ub and the Ub-like modifier SUMO**

Ubiquitin (Ub) is a 76-residue globular protein, conjugated to other proteins through an amide bond between its C-terminal glycine and an acceptor lysine on substrate. The Ub fold is composed of a five-stranded β-sheet, α-helix, and a 3_10 helix. Ub possesses seven surface lysines, which can be enzymatically linked to the C terminus of a sequential Ub; this represents a versatile platform for building chains composed of distinct links and topologies. Ub conjugation to protein substrates is carried out by the sequential action of three enzymes. Initially, the Ub C-terminal glycine is covalently attached to Cys within an E1 enzyme through a thioester bond, followed by interaction of the E1–Ub complex with an E2 Ub-conjugating (UBC) enzyme and subsequent Ub transfer to the E2 active site Cys. E3 Ub ligases bind E2–Ub complexes and substrate proteins, leading to the formation of an amide bond between the ε-amino group of a substrate lysine and the C terminus of Ub [7–9]. K48-linked Ub chains typically signal for proteasomal degradation of substrate, whereas K63-linked chains serve as a signaling platform for various pathways and play a major role in the DSB repair process. Ub chains composed of other linkages are involved in several biological processes. However, their functional roles have not been explored to the same extent as K48- and K63-linked chains [10,11].

Ub-like proteins possess a fold similar to that for Ub and include small Ub-like modifier (SUMO) and neural precursor cell expressed developmentally downregulated protein. These modifiers are involved in a number of signaling events in various cellular processes [12]. There are four different isoforms of SUMO, SUMO-1 shares ~45% sequence identity with SUMO-2 and 3 and lacks a SUMOylation motif, rendering it incapable of chain formation, whereas SUMO-2 and 3 share more than 90% sequence identity and form SUMO chains. SUMO is conjugated to substrates through the action of SUMO-specific E1, E2, and E3 enzymes in a manner similar to that for Ub. Initially, SUMO is transferred to its E1, the SAE1:SAE2 heterodimer, in an ATP-dependent manner. This is followed by conjugation to the only SUMO-specific E2, Ubc9, through a thioester bond, with the action of SUMO-specific E3 ligases ultimately attaching SUMO to substrates. SUMO-specific proteases, SENPs, remove SUMO modifications from substrates, regulating the signaling response. SUMO is recognized by proteins containing SUMO binding motifs such as the SUMO-interacting motif (SIM), a common and functionally important motif within a number of DSB repair proteins. It is composed of a stretch of hydrophobic residues flanked by acidic residues on one or both the sides of this hydrophobic module [13,14]. Upon binding with SUMO, the SIM forms an intermolecular β-sheet. The nature of acidic residues flanking the SIM hydrophobic module determines the orientation of the β-strand within the intermolecular complex. In addition, these regions potentially contain phosphorylation sites that impact the affinity of the SIM and the orientation of the SIM strand that contributes to the intermolecular β-sheet [14–18].

**Overview of HR-driven DSB repair**

The biological processes underlying the role of ubiquitination in the DNA damage response have been extensively reviewed [4]; in this section, we provide a brief overview of the HR repair pathway. Broken DNA strands induce chromatin relaxation, which is sensed by ataxia telangiectasia mutated (ATM) kinase, leading to its activation and interaction with the Mre11–Rad50–NBS1 complex (MRN complex), which binds broken DNA strands [6]. ATM phosphorylates histone H2AX at serine 139, and the resulting phosphorylated histone (γ-H2AX) acts as a recruitment scaffold for downstream repair proteins. Mediator of DNA damage checkpoint (MDC1) binds γ-H2AX, and as an ATM substrate, it is phosphorylated at damage sites to provide a binding platform for RNF8, an E3 Ub ligase [5]. A number of different Ub ligases, discussed in further detail below, lead to the conjugation of monoUb and Ub chains of different topologies at damage sites. These chains recruit repair proteins such as BRCA1, BRCA2, RAD51, and endonucleases such as EXO1 and CtIP and play important roles in repair pathway choice, chromatin reorganization, and repair pathway regulation [4]. Mechanistically, repair of DSBs by HR involves extensive resection of DNA ends by nucleases such as EXO1, DNA2, CtIP, and Mre11 of the MRN complex, resulting in the formation of single stranded DNA, which is then covered by replication protein A (RPA) [4]. In order to perform a homology search within the sister chromatid, RPA is exchanged with RAD51 by the assistance of BRCA2, and the resulting RAD51-ssDNA strand undergoes a homology search, followed by DNA synthesis, ligation, and conclusion of repair, thereby regaining the integrity of the chromosome [4,19].

**Overview of ubiquitination enzymes and co-factors involved in HR-driven DSB repair**

RNF20 and RNF40. Histone H2B monoubiquitination at K120 by this heterodimeric E3 Ub ligase
regulates chromatin compaction, a reorganization step that is required for transcription elongation [20–24]. Histone H2B modification also occurs in response to DNA DSBs, in an ATM-dependent manner with RNF20–RNF40, resulting in the accumulation of early repair proteins such as XRCC, RAD51, and BRCA1, for both NHEJ and HR repair pathways [20,23].

RNF2-BMI1: This heteromeric E3 Ub ligase belongs to the polycomb group proteins, an important group of epigenetic regulators. RNF2-BMI1-depleted cells show an impaired DNA damage response and increased sensitivity to radiation [25]. RNF2-BMI1 is responsible for the monoubiquitination of the core histone H2A at K119/K120 to enable gene silencing and to initiate DNA repair by facilitating the recruitment of the downstream repair proteins RAP80, 53BP1, and BRCA1 to damage sites [26,27].

RNF8 and RNF168: The E3 Ub ligase RNF8 is recruited to damage sites through the binding of phosphorylated MDC1 via its Forkhead associated (FHA) domain [28–30]. RNF8 in conjunction with the heterodimeric E2/UBC enzyme variant (UEV) complex Ubc13-Mms2 synthesizes K63 chains on linker histone H1 [31]. A second E3 ligase, RNF168, then binds these K63 chains through an N-terminal Ub-binding domain (UBD1), which leads to monoubiquitination of the core histone H2A at K13/K15, in conjunction with the E2 UbcH5 [32–36]. It is also possible that RNF168, with Ubc13, can synthesize K63 chains at damage sites. Subsequently, K63 chain recognition by RAP80 in complex with BRCA1 and BRCC36 is obligatory for HR-mediated DNA repair [37–39]. The monoubiquitination of histone H2A at K13/15 and dimethylation of K20 at histone H4 are recognized by the Ub-dependent recruitment motif and Tudor domain of 53BP1, respectively, and result in initiation of NHEJ [40–42]. In its constitutive state, histone H4 with dimethylated K20 is bound to the polycomb group protein L3MBTL1 and demethylases. These components are likely removed through attachment of K48 Ub chains by the E3/E2 pair RNF8/UbcH8, followed by removal of L3MBTL1 by the AAA-ATPase VCP/p97 segregase [43–49]. The ubiquitination activities of RNF8/RNF168 are thought to be involved in determining the balance between recruitment of the repair protein 53BP1 and the BRCA1-A complex to damage sites, which determines if repair will proceed by NHEJ or HR.

HERC2: HERC2 is a 500-kDa protein belonging to the homologous to E6-AP carboxyl terminus (HECT) family of E3 Ub ligases that is required for RNF8- and RNF168-dependent ubiquitination [50]. Upon DNA damage, it is phosphorylated by ATM and binds the FHA domain of RNF8 to facilitate the accumulation of 53BP1 and BRCA1 at damage sites by promoting stabilization, or formation, of the RNF8–Ubc13 complex. HERC2 is not directly involved in K63 chain formation through the Ub ligase activity of its HECT domain [51,52].

CHFR: The checkpoint with forkhead and RING finger domains (CHFR) E3 Ub ligase is recruited to sites of damage near the onset of repair, prior to recruitment of RNF8/RNF168 [53]. It contains FHA, really interesting new gene (RING), cysteine-rich domains, and a polyADP-ribose (PAR) binding zinc finger motif [54]. It interacts with the E2s Ubc13 and UbcH5C to synthesize K63- and K48-linked chains, respectively [54]. CHFR binds PAR modifications at sites of DNA damage through its PAR binding zinc finger motif and functions to ubiquitinate PAR polymerase 1, which leads to its early removal from the DSB repair process via proteasomal degradation [53].

RNF138: The E3 Ub ligase, RNF138, is recruited to damage sites through DNA binding mediated by its zinc finger domains; this promotes end resection and HR repair by ubiquitinating and displacing Ku80 from ssDNA and by binding Mre11 of the MRN complex through its zinc finger domains to recruit Ctp/EXO1 nucleosome to damage sites [55,56].

BRCA1-BARD1: BRCA1 is a tumor suppressor protein, found to be mutated in numerous malignancies; this protein is composed of an N-terminal RING and C-terminal BRCT domains, which facilitate interactions with multiple proteins to function in the maintenance of genome integrity [57]. BRCA1 interacts with BARD1, forming a heteromeric E3 Ub ligase that modifies substrates with different Ub linkages, including BRCA2, CtIP, and histone H2A [57–64]. While BRCA1 has been the subject of intense research efforts, the molecular basis for its involvement in HR-driven DSB repair remains mysterious.

BRCC36: BRCC36 is a Jab1/Mpn/Mov34 (JAMM)/MPN* deubiquitinase (DUB) that functions within a complex that includes RAP80, ABRAXAS, BRCA1, and MERIT40. This complex is recruited to sites of DNA damage through RAP80-mediated binding of K63 chains synthesized by RNF8/RNF168 [65]. BRCC36 selectively deubiquitinates K63 chains attached to γ-H2AX, thereby counteracting RNF8/RNF168-mediated ubiquitination at damage sites; these competing activities may be important for establishing the extent of the K63-linked polyubiquitination at DSBs [66].

Otubain 1 (OTUB1): OTUB1, a DUB belonging to the OTU class, acts as a negative regulator of RNF8 and RNF168-mediated K63 chain synthesis, with the inhibition separate from its catalytic activity as a K48-specific DUB [67]. Inhibition of K63 chain synthesis results through interaction with Ubc13, the cognate E2 for RNF8, which blocks the catalytic activity induced in Ubc13 by RNF8 [68,69].

Ub-specific protease (USP) 3: The Ub modifications on K119/120 of histone H2A and on K13/15 of histone H2B generated by BMI1/RNF2 and RNF20–RNF40 are attenuated by USP3, an H2A- and H2B-specific DUB. Thus, USP3 is involved in maintenance of
genomic integrity, potentially as a negative regulator of H2A/H2B-dependent recruitment of DNA repair proteins [70,71].

**USP11**: USP11 is DUB that has been implicated in HR-driven DSB repair through the finding that it associates with, and deubiquitinates BRCA2, and affects the recruitment of RAD51 and 53BP1 to damage foci, although the molecular mechanisms are currently unclear [72,73].

**POH1**: POH1/Rpn11 is a JAMM metalloprotease DUB that is associated with the 19S proteasome. POH1 activity associated with the proteasome results in cleavage of K63 chains at damage sites and limits the accumulation of 53BP1 at DSBs. In addition, it has also been shown to promote HR by facilitating the loading of RAD51 onto RPA-coated DNA strands for subsequent homology search and repair [74,75].

**BAP1**: The DUB BAP1 associates with BRCA1 and is mutated in a number of cancers, particularly mesothelioma and melanoma. BAP1 is phosphorylated by ATM kinase and promotes repair of DSBs through PAR-polymerase-dependent recruitment of the polycomb DUB complex to DNA damage sites [76].

**Ub carboxyl-terminal hydrolase (UCH)** L5: This DUB is a component of both the INO80 chromatin remodeling complex and the 19S proteasome and has been implicated in the positive regulation of resection of DNA ends at DSBs and HR repair [77]. UCHL5 exerts this positive regulation by protecting the NFRKB protein from proteasomal degradation. NFRKB is a component of the INO80 complex, which is involved in the remodeling of chromatin through DNA end resection, and appears to upregulate resection through recruitment of EXO1.

**SUMO modifications in DSB repair protein recruitment and removal**

Different isoforms of SUMO have been shown to gather at sites of DSBs along with the SUMO E3 ligases PIAS1 and PIAS4, whose activity is required for the proper formation of Ub chains at DSBs [78]. The polycomb group protein BMI1 has been shown to monoubiquitinate H2A, and its SUMOylation by CBX4 is a contributing factor for its accumulation at the DSB sites [79]. MDC1 is also SUMOylated upon DNA damage, a necessary step for its removal from sites of damage by the SUMO-targeted E3 Ub ligase RNF4 [80]. RAP80 binds K63 chains at damage sites through tandem Ub-interacting motifs (UIMs) and possesses a SIM adjacent to the UIMs that is important for its recruitment to the damage sites [81,82]. RAP80 also interacts with the SUMO E2 Ubc9, and the ensuing SUMOylation is important for its functional role at DSBs [83]; BRCA1 is also modified by SUMO and co-localizes with SUMO1, SUMO2/3, and Ubc9 at DSB sites. SUMO modification of BRCA1 has been shown to enhance its Ub ligase activity in cells, which in turn is important for DSB repair [84]. The Mre11 protein is a constituent of the Mre11-Rad50-Xrs2 (MRX) complex in yeast (equivalent to the MRN complex in humans) and possesses two conserved SIMs. SIM1 is essential for the assembly of Mre11-Rad50-Xrs2 (MRX) complexes at DSB sites, and SIM2 is thought to recruit SUMOylated conjugates to Mre11, facilitating the subsequent recruitment of SUMO E2 Ubc9 and the SUMO E3 Ub ligase Siz2 to enhance the general SUMOylation of DNA repair proteins, presumably to assist repair [85].

Exonuclease EXO1 resects DNA ends during HR-mediated DSB repair and is constitutively SUMOylated by PIAS1/4-Ubc9, and this is a requirement for its Ub-dependent EXO1 degradation at stalled replication forks to avoid excessive resection of free DNA ends. Moreover, it was found that the deSUMOylating enzyme SENP6 interacts with EXO1 to antagonize this process [86].

The SUMO-specific protease SENP7 assists DSB repair by cleaving SUMO chains on KAP1, a key transcriptional regulator; this releases CHD3, a chromatin remodeling complex, which then brings about chromatin decondensation, a physical requirement for repair proteins to gain access to the damage sites [87].

**Flux of the K63-linked ubiquitination cascade and DNA repair foci**

DSBs induce the formation of protein conglomerates, known as ionizing radiation-induced foci, that are considered to be affinity platforms for the recruitment of DDR proteins to damage sites [4,5,88,89]. A crucial early step in the development of ionizing radiation-induced foci, is the synthesis of K63-Ub chains on histone proteins, by the E3 ligases RNF8 and RNF168, in combination with the E2 heterodimer Mms2-Ubc13, which serve as recognition platforms for downstream repair proteins, as discussed previously. How the Ub landscape at DSBs is maintained by the flux of the ubiquitination cascade is not clearly understood. In this review, we focus largely on the structural, kinetic, and dynamic principles for the molecular mechanisms underlying the flux of K63-Ub signal, which is determined principally by the chain-building capacity of coupled E1, E2, and E3 enzyme activity and balanced by the counter-activity of DUBs, the p97 segregase, and the proteasome, including their respective chain recognition processes, and those of repair protein co-factors/receptors such as RAP80.

**E1 Activating Enzymes**

The first step in the Ub cascade involves the ATP-dependent activation of Ub by the E1 enzyme
UBA1 [90]. As all subsequent steps in the ubiquitination cascade are dependent on the function of UBA1, it is not surprising that inhibition of UBA1 activity has severe consequences in cells. The E1 enzyme has a vital role in the regulation of the cell cycle, as deletions of the gene in yeast [91] and temperature-sensitive mutations in CHO cells [92,93] show cell cycle arrest. Mutations in UBA1 or reductions in its activity within Drosophila affect apoptosis, cell cycle progression, tissue development [94,95], and motor impairment [96]. In humans, mutations in the UBA1 gene can cause X-linked spinal muscular atrophy, and reduced UBA1 activity has been found in Alzheimer’s, Huntington’s, and Parkinson’s disease [97]. In DNA damage repair, UBA1 is required for the formation of Ub chains and therefore the Ub-dependent recruitment of DNA repair proteins to sites of DNA damage, although UBA1 itself is not recruited to those sites [98].

Two isoforms of UBA1 are expressed [99,100], in which the shorter isoform is missing the first 40 residues of the complete 1058 residue sequence. The N-terminal sequence for the longer isoform may be involved in cell-cycle-dependent regulation of UBA1, as it contains a nuclear localization signal and phosphorylation sites, and the nuclear localization and phosphorylation of UBA1 are known to vary over the cell cycle [100,101]. However, the functional significance of this regulation has yet to be determined. Modification of UBA1 by the Ub-like protein FAT10 has been found to target UBA1 for degradation [102].

There are eight known proteins in humans that catalyze reactions analogous to UBA1, each of which is responsible for activating a particular Ub-like protein [103]. UBA6 can also activate Ub [104]; however, it is not involved in the DNA damage response [98]. As mentioned previously, modifications of proteins by the Ub-like protein SUMO has also been shown to play a role in the DNA damage response [78,84]. SUMO activation is catalyzed by the UBA2/SAE1 heterodimeric E1 enzyme [105].

In general, the E1 enzyme functions to maintain the charged E2–Ub pool, given that the thioester bond is chemically labile. Indeed, the apparent rate of E2–Ub thioester synthesis by E1 is $10^{11}$ fold greater than the rate of counterproductive hydrolysis [106].

**E2 Enzymes and E3 Ub Ligases: Ubc13, Mms2, RNF8, and RNF168, the K63 Chain Builders**

E2 enzymes bridge the barrier between activation of Ub by E1 enzyme and target selection by an E3 Ub ligase; given this central role, E2s have been extensively studied [107,108]. There are approximately 40 human E2 enzymes that share a structural domain known as a UBC domain of approximately 150 residues, with the family divided into 4 classes depending on the presence of N- or C-terminal functional extensions [109–110]. The gap between Ub activation and final attachment to a target is necessary to manage selective catalysis for the synthesis of stable, covalent amide bonds between Ub and a host of target proteins. In other words, the three-component ubiquitination enzyme cascade likely represents an overall accuracy rate trade-off [111] that allows efficient and selective ubiquitination of target proteins. In this regard, the full catalytic power of E2 enzymes, and ultimately their selection of target proteins, is reigned in until an E2 binds its cognate E3 Ub-ligase, a process that has been particularly well-characterized for the RING-type ligases [112], as typified by the RING E3-mediated, ~100-fold-rate enhancement for thioester hydrolysis for UbCH5b [113]. There are over 600 E3 ligases, divided into 3 classes, with the RING E3s as the largest class [114], the others being HECT E3s, and RING between RING E3s [115-118].

**Structures, interactions, and mechanism of Ubc13, the K63-chain builder**

Double-strand DNA breaks result in the formation of foci near the DSBs that are rich in DNA repair proteins. The repair process is fundamentally dependent on the synthesis of K63-linked polyUb chains on histones and their subsequent recognition by the protein RAP80 [4]. The E2 Ubc13 has been extensively studied given its function within numerous pathways and the fact that it is the primary E2 that synthesizes K63 Ub chains [119]. Within the nucleus, Ubc13 interacts with the UEV Mms2 to synthesize K63 chains [120,121]. This interaction allows the Mms2-Ubc13 heterodimer to position an “acceptor” Ub such that Lys63 is adjacent to the C-terminal thioester bond of the “donor” Ub bound to the active site Cys of Ubc13. Additionally, binding of acceptor Ub results in conformational changes to the active site gate of Ubc13, which are facilitated by hydrogen bonding interactions between residues Gln62 and Lys63 of the acceptor Ub, and Asn123 near the active site gate hinge for Ubc13. These structural studies provide a molecular basis for the ability of the heterodimer to synthesize K63 chains in the absence of an E3 ligase [122–126]. For the acceptor Ub, Lys63 is observed to be distant from the active site thioester (~10 Å) in the absence of E3. Interestingly, within structures of the heterodimer with the C terminus of donor Ub conjugated to an active site C to K mutant through a stable amide bond, and in the presence of the E3 ligase RNF4, which places the donor Ub in the closed state, acceptor Ub Lys63 remains distant (12 Å) from the active site (Fig. 1) [127]. These studies suggest that both acceptor Ub and Mms2 within the Mms2-Ubc13 heterodimer must undergo a wobbling motion of the entire domain to facilitate the attack of...
the acceptor Lys63 on the donor thioester. For acceptor Ub, this can be accomplished through a small amplitude rocking motion about Ile44, at the center of the Ub–Mms2 interface, to close the ~10 Å gap between the nucleophile and the thioester. Although the amplitudes and rates of such domain motions for this system have not been characterized, it is likely that they have a substantial impact on the catalytic activity of the heterodimer and the underlying biological processes.

Formation of K63 chains by the Mms2-Ubc13 heterodimer is driven by an array of protein–protein interaction kinetics that can be described by a set of coupled ordinary differential equations [128,129]. Various NMR spectroscopic methods have facilitated the measurement of the underlying on- and off-rates [128,129]. Mms2 and Ubc13 are tightly associated in the heterodimer, having a $K_D$ of 50 nM, with a generally fast association rate of $10^8$ M$^{-1}$ s$^{-1}$ and a slow off-rate of 4 s$^{-1}$; these values ensure that under physiological conditions, the heterodimer is intact at typically micromolar concentrations of E2 and UEV. Binding of substrate, or acceptor Ub, is substantially weaker, with a $K_D$ of ~30 μM, and a fast off-rate of ~1000 s$^{-1}$ [129]; this ensures that the release of a nascent Ub chain is not rate-limiting and that the invariable increase in binding affinity for K63 chains for the Ub-binding site on Mms2 is also not rate-limiting.

Molecular insights into the mechanism of Ubc13 have been derived from kinetic studies using small-molecule nucleophiles such as the free amino acid lysine, for which the neutral form of the side chain moiety (ε-NH$_2$) reacts with E2~Ub thioester, but not the α-NH$_2$ group [130]. Coupling such kinetic studies with measurement of the protein–protein interaction kinetics of Mms2-Ubc13 as described above, and the development of rate laws, facilitated the determination of a $k_{cat}$ value of ~0.002 s$^{-1}$ [128]. This represents an enormous ~10$^6$-fold increase in the rate of Ub attachment to substrate, even in the absence of E3 ligases, compared to non-catalyzed rates for small organic molecules [128,131]. To put this range in context, the catalytic proficiency for enzymes, or $(k_{cat}/K_M)/k_{noncat}$, ranges from $10^8$ to $10^{23}$ M$^{-1}$ [132], where $k_{noncat}$ indicates the second order rate constant for the non-catalyzed bimolecular reaction. For E2 enzymes without an E3 co-factor, their proficiency is at the lower end of the scale, with a value of ~$10^{11}$–$10^{14}$ M$^{-1}$ [128]. Thus, the Ubc13-Mms2 heterodimer is a proficient enzyme, capable of E3-independent K63 chain synthesis.

Early mechanistic insights regarding how E2 enzymes such as Ubc13 achieve large rate enhancements include the key discovery that the C terminus of Ub is positioned within the active site cleft of E2s, with Ub bound to the “underside” of the E2 within an extensive interface that includes the hydrophobic interaction surface of Ub composed of residues L8, I44, and V70, a conformation that is stabilized by E3 ligases [133]. Another seminal E2 study uncovered a substantial downshift for the $pK_a$ of the substrate lysine for Ubc9 [134], a SUMO conjugation enzyme, that was subsequently observed for Ubc13 [128]. The reaction of neutral lysine with a thioester bond, or aminolysis, is a nucleophilic acyl substitution, and such reactions have long been believed to proceed in a stepwise manner through a zwitterionic tetrahedral transition state bearing an oxyanion [135,136]. The oxyanion is likely to be electrostatically stabilized by the side
chain of an asparagine residue [137] within a structurally conserved HPN motif [138]. This view is consistent with theoretical views of electrostatic effects within enzyme active sites [139]; that is, solvation substitution within the active site of enzymes plays a major role in electrostatic stabilization of the transition state [140]. Furthermore, a hydrogen bond between the side-chain Asn within the HPN motif and the C-terminal Gly of Ub buried within the E2 active site has been observed for various crystallographic structures [141,142].

Further kinetic studies involving the reaction of Ubc13 with small-molecule nucleophiles have revealed that the loop, which buttresses the active site Cys and connects helices α2 and α3 within the UBC fold, has a fundamental role in catalysis, functioning as a stochastic or random gate to regulate catalysis [106]. These local dynamic fluctuations for the active site loop within Ubc13 represent precisely balanced gating rates that are critical for modulating reactivity, a view that is consistent with a number of theoretical studies for gated enzyme-catalyzed reactions [143–145]. Furthermore, the question for E2s, in general, is whether gating differs among family members, and if this contributes to biological and therapeutic specificity. In this regard, we demonstrated that K63 ubiquitylation by the E2 Ubc13 can be specifically inhibited with the small-molecule NSC697923 by exploiting sequence differences in the active site gate [146].

Accelerating K63 chain synthesis: RNF8 and RNF168, E3 ligases associated with Mms2-Ubc13

The interplay between the individual E3 ligase activities of RNF8 and RNF168 at DNA damage sites is critical for the formation of K63 chains and subsequent recruitment of repair proteins. However, our understanding of the underlying biochemical and mechanistic bases regarding chain synthesis by these E3 ligases remains largely incomplete. RNF8 consists of 485 residues, with the ligase activity confined to a large scaffold composed of the C-terminal 140 residues, which form a long ~50-residue N-terminal helix followed by a canonical C3HC4 RING domain, arranged in a dimer [35,147]. The dimerization is mediated through a coiled coil formed by the N-terminal helices, and by comparison to the GCN4 leucine zipper [148], the dimerization constant, while unknown, is likely in a range that allows RNF8 to exist predominantly as a dimer at concentrations above ~10 μM. The C-terminal region from RNF8 binds Ubc13 with a $K_D$ of ~2 μM, as determined by surface plasmon resonance (SPR), and enhances K63 chain synthesis by the Mms2-Ubc13 heterodimer [149]. From a structural standpoint, the rate enhancement arises in part from the RING domain of the RNF8 scaffold shifting Ubc13–Ub partly toward a closed conformation [149], as discussed above. Importantly, the biological relevance of the RNF8-induced shift toward a closed conformation for the Ubc13–Ub thioester was studied using a separation of function mutant for RNF8 (Leu451Asp) [149]. Biochemical and small angle X-ray scattering (SAXS) studies showed that this mutant is capable of binding Ubc13 with the same affinity as wild type; however, it populates a smaller fraction of the closed Ubc13–Ub conformation and results in a substantially diminished capacity for RNF8/Ubc13 to synthesize K63 chains [149]. Cell studies using the Leu451Asp separation of function mutant indicate that while recruitment of RNF8 to DNA damage sites is not impaired, synthesis of K63 Ub chains at these foci is markedly reduced [149].

Biochemical and in-cell studies have established that the RNF8/Ubc13/Mms2 complex builds K63 chains on H1 linker histones, which subsequently serve as a recognition platform for recruitment of RNF168 [31]. However, the nature of the recognition/ubiquitination site(s) for RNF8 on the linker histone is a mystery, as are the specific mechanisms underlying chain elongation, apart from the known mechanisms for K63 chain catalysis by the Mms2-Ubc13 heterodimer, as discussed above. Furthermore, H1 linker histones are transiently associated with nucleosomes, having mean residency times substantially shorter than those for the majority of core histones; this suggests that the K63 signal may not localize efficiently to nucleosomes [150,151].

RNF168 is composed of 571 residues, with an N-terminal RING domain, and 2 Ub-dependent DSB recruitment modules that are separated by ~200 residues [31]: UDM1, residues 110–188, and UDM2, residues 443–478. UDM1 contains an ligand recognition motif (LRM) [152], an motif interacting with Ub (MIU) [153], and a UIM- and MIU-related UBD [154,155]. UDM2 contains an MIU with an adjacent LRM. In contrast to RNF8, the RING domain from RNF168 is monomeric, binds Ubc13 weakly, and is deficient in accelerating K63 chain synthesis by Ubc13-Mms2 in vitro [147] but is capable of ubiquitinating K13 and K15 within histone H2A of the core nucleosome, in conjunction with the E2 UbcH5c [35]. UDM1 from RNF168 appears to bind K63 chains with high but unspecified affinity, whereas UDM2 appears to interact weakly with K63 chains and K33, K29, and K27 chains [31]. The kinetics and dynamics for the Ub-binding properties of UDM1 and UDM2 are not known; however, they can be expected to be similar to typical MIU and UIM domains that have $K_D$ values for Ub that range from tens to hundreds of μM [153,156,157]. Interestingly, the redundancy in chain recognition for UDM2 is consistent with biophysical studies of Ub$_2$ chains, which indicate that there is a high degree of flexibility between adjacent Ub molecules and that K27, K29, and K33–Ub$_2$ chains have similar interdomain orientations as K63–Ub$_2$ [158]. This suggests that generally, UBDs are likely to be promiscuous in recognizing the binding epitopes of extended chains.
such as K27, K29, K33, and K63 chains. In addition to ubiquitination of histone H1, RNF168 is believed to be involved in synthesizing K27 chains on histone H2A, which can be recognized by the tandem UIMs of RAP80 [159]. As in the case of RNF8, the biophysical parameters underlying the rate of synthesis for polyUb chains, the affinities of protein–protein interactions with E2s such as UbcH5c, UbcH8, and Ubc13, and polyUb chain recognition by the UBDs are not known. Furthermore, the roles of the LRMs in recognition and discrimination of Ub chains are not known and may be important for the recognition of hybrid SUMO–K63 chains for the regulation of promyelocytic leukaemia (PML) nuclear bodies within the nucleus [160].

Deubiquitinating Enzymes, Terminating the Proteasome-Independent K63 Chain Signal

Deubiquitinases are critical components for a number of DNA damage response pathways [161]. The five families of DUBs include four cysteine isopeptidases: USPs, the UCHs, OTUs, Machado–Joseph disease, and the JAMM metallopeptidases [162–164].

BRCC36 is a Zn²⁺-dependent protease that cleaves K63 chains

OTUB1 is specific for K48 chains, the canonical proteasome-dependent degradation signal [165]. However, as previously mentioned, OTUB1 also plays an important role in HR-regulated DSB repair, as it possesses the ability to bind conjugated Ubc13–Ub and inhibit K63 chain synthesis [67]. OTUB1 has been extensively reviewed [119] and will not be covered here. The JAMM (or MPN⁺), Zn²⁺-dependent protease BRCC36 is critical for the cleavage of K63 chains in the nucleus; it functions in the DSB repair process through its involvement in the Abraxas complex, which includes MERIT40 and BRCC45 [39,166], and is targeted to K63 chains at DSBs through interaction with RAP80 [39,166].

Mechanism, interactions, and structure of AMSH, a K63-chain specific, MPN⁺ protease

The structures, functions, and mechanisms of Zn²⁺-dependent proteases have been extensively characterized [167]. The first structural insights into the specific enzymatic cleavage of K63 chains were derived from studies of the Zn²⁺-dependent JAMM or MPN⁺ DUB associated molecule with the SH3 domain of STAM (AMSH)-LP [168]. AMSH-LP is composed of a core JAMM domain, with two insertions that are specific to the AMSH DUBs (Ins-1 and Ins-2). The K63 diUb substrate is bound in an extended conformation, with the distal (donor) Ub bound to the JAMM core domain and Ins-1 and with the proximal (acceptor) Ub bound to the JAMM core and Ins-2 (Fig. 2).

To enable crystallization, a key Zn²⁺ ligand (Glu) was mutated to Ala; thus, the active site Zn²⁺ and its associated catalytic water molecule were not observed in the structure. However, the arrangement of active site residues in AMSH suggests that the catalytic mechanism is likely to be similar to that for the zinc protease thermolysin [168,169]. For thermolysin, although the mechanism is still under debate [170], computational studies suggest that the zinc-bound catalytic water is deprotonated by an active site Glu and subsequently performs a nucleophilic attack on the substrate peptide carbonyl [171]. The transition state for nucleophilic attack has the largest energetic barrier in the reaction scheme and is therefore rate-limiting. The developing negative charge on the...
peptide carbonyl oxygen is stabilized by both the zinc ion and an active site His, and the developing positive charge on the amide nitrogen is stabilized by an active site glutamic acid. The reaction then proceeds through an oxyanion tetrahedral intermediate and a second transition state, which results in cleavage of the peptide N–C bond and proton transfer from the amide nitrogen to water that is mediated by the active site Glu, ultimately yielding a cleaved peptide bond. Interestingly, this reaction proceeds in a generally similar manner to the E2-catalyzed cleavage of the E2–Ub thioester bond, as discussed above.

In general, proteases are on the low end of the scale for catalytic proficiency with values of \( \approx 10^{13} \) to \( 10^{14} \) M\(^{-1}\) [172], similar to the proficiency for E2 enzymes, as discussed above. Thermolysin binds substrate peptides with lengths of three to four residues weakly, with \( K_M \) values ranging from 1 to 12 mM, and \( k_{cat} \) values ranging from 1 to 5000 s\(^{-1}\) [173]. In contrast, AMSH-LP and its yeast homolog Sst2 bind K63 diUb with \( K_M \) of \( \approx 50 \) μM and \( k_{cat} \) of \( \approx 1 \) s\(^{-1}\), substantially below the catalytic proficiency associated with cleavage of some small peptides by thermolysin. This enhanced proficiency may be related to the function of thermolysin as an extracellular protease. Its activity is highly inhibited as a preproenzyme in the host bacterium, but it is a highly proficient catalyst once outside the host, whereas AMSH activity must be tightly regulated, as it functions within the cellular milieu, to regulate endosomal sorting.

The Ins-2 region includes a loop whose apex buttresses the active site with a bulky hydrophobe (Phe). The Phe residue packs over the active site, resulting in a closed conformation, and interacts with K63 from the acceptor Ub substrate [168]. Interestingly, crystallographic B-factors combined with molecular dynamics simulations suggest that the loop acts as a flexible gate, whose fluctuations may modulate the catalytic activity of AMSH [174].

Mechanism, interactions, and structures of the BRCC36 super dimer, a K63-chain-specific MPN\(^+\) protease

BRCC36 is the main DUB for cleavage of K63 chains in DSB repair, and unlike AMSH-LP, which is a functional monomer, BRCC36 is minimally active only as a heterodimer with a catalytically inactive pseudo-DUB MPN\(^-\) domain (Abraxas in DSB repair, or KIAA0157 in cytokine receptor activation) [175]. Interactions with the Abraxas MPN\(^-\) domain mediate the association of BRCC36 with the additional subunits BRCC45 and MERIT40 of the Abraxas complex, and ultimately RAP80, which targets the DUB complex K63 chains to DSB sites [37,39,166,176–178]. Structural insights into the functionally relevant catalytic mechanism of BRCC36 have been obtained from crystallographic studies of the BRCC36-KIAA0157 (MPN\(^+\)–MPN\(^-\)) heterodimer and BRCC36 homodimer complexes [175]. Importantly, while the underlying protein–protein affinities are not known, it is clear that the structurally and functionally relevant MPN\(^+\)–MPN\(^-\) unit is a dimer of MPN\(^+\)–MPN\(^-\) heterodimers or a stable superdimer, using size exclusion chromatography with

![Fig. 3. Structure of the BRCC36-KIAA0157 MPN\(^+\)–MPN\(^-\) heterodimeric super dimer (5CW3), with BRCC36 (blue) and KIAA0157 (red) in the ribbon representation with Ins-1 and the catalytic Zn\(^2+\), and active site Ins-1 loop and Glu (green, stick representation) indicated.](image-url)
multi-angle light scattering (SEC-MALS), SAXS, biochemical, and in-cell studies. Indeed, an extensive coiled-coil helical bundle formed by two helices from each of the MPN+ and MPN- domains mediates the heterodimerization (Fig. 3). The MPN+ domain from BRCC36 is structurally similar to the JAMM/MPN+ domain from AMSH-LP, with the notable exception being a complete lack of Ins-2, or a structural alternative, which is surprising, considering that residues from the Ins-2 loop are involved in binding K 6 3 - U b 2 f o r A M S H - L P . W h i l e t h e BRCC36-KIAA0157 heterodimer structure was not determined in complex with K63-Ub2, the KD for binding is 2 μM, the Kcat for K63-Ub2 hydrolysis is 4 μM, and the substrate binding surfaces are generally highly conserved. Thus, it is likely that BRCC36 and AMSH-LP bind K63-Ub2 similarly.

Comparison of the BRCC36 homodimer and heterodimer structures suggests an explanation for the loss of catalytic activity in the homodimer [175]. There is a substantial 5-Å shift in the position of the catalytic Glu (positioned within the catalytic E-loop) that primes the active site water for nucleophilic attack and a major conformational shift for the Ins-1 loop. In addition, these structural changes in the homodimer are accompanied by an increase in disorder/dynamics. These observations raise a number of interesting points. How does the catalytic activity of BRCC36 compare to monomeric MPN+ DUBs, and how does this relate to its biological function in DSB repair? The Kcat value for the BRCC36 super dimer is ~10-fold smaller than Kcat for AMSH-LP [168] and its yeast counterpart Sst2 [174].

Fundamentally, the maximum attainable Kcat value for the BRCC36 super dimer will depend on the stability of the complex. If the super dimer lifetime is on the order of, or faster than, Kcat, the super dimer stability determines the upper limit on observed Kcat. For alternative scenarios, where stability is not an issue, the diminished Kcat appears to rule out positive cooperativity between the substrate binding sites, consistent with the measured KD and KM values, as mentioned above. Additionally, the small distance between putative binding sites for acceptor Ub on opposing heterodimers is expected to lead to steric clashes between opposing substrate acceptor Ub moieties. The structural data do not suggest an obvious mechanism for negative cooperativity in comparison to AMSH DUBs. One possible explanation for the diminished Kcat is that the Ins-2 loop plays a role in enhancing the catalytic activity and specificity of MPN+ DUBs, as suggested by mutational studies [174], but it is not obligatory for catalysis. In this regard, additional components of the BRCA1-A complex may substitute for the role of Ins-2, thereby increasing Kcat. Conceivably, the BRCC36 super dimer may be as proficient as it needs to be. Ultimately, the answers will most likely be found through studies of K63 chain cleavage within the intact BRCA1 complex.

K63 Chain Recognition by RAP80: A Mysterious Link to SUMO

RAP80 binds K63 chains using multivalency

Recruitment of the BRCA1-A complex to DNA damage sites is mediated through K63 chain recognition by the tandem UIM domains from RAP80, a process that is fundamental for DNA repair [4]. Individual UIMs consist of a short ~10-residue α-helix and bind Ub weakly, with affinities between 10 and 500 μM [156]. The UIMs from RAP80 bind mono-Ub with KD of ~700 μM [178,179]. The KD for K63-Ub2 chain binding by the tandem UIMs from RAP80 (~20 μM) is sevenfold greater than that for K48-Ub2 chains (~160 μM), and the affinity of the interaction is dependent on the length between the α-helical UIMs [179]. The X-ray structure of the tandem UIMs bound to K63-Ub2 indicates that each UIM binds one Ub within K63-Ub2 at the hydrophobic patch centered on Ile 44 (Fig. 4) [180]. Importantly, RAP80 does not make specific interactions with the covalent link between Ub moieties in K63-Ub2, NMR studies have shown that the increase in affinity for K63-Ub2 or tandem Ub2 compared to mono-Ub arises primarily from multivalent effects [178], that is, the flexible linker between Ub moieties in K63-Ub2 gives a large, effective concentration of one UIM with respect to the other UIM, at an average inter-UIM distance that matches the average inter-Ub distance in either K63-Ub2 or tandem Ub2. This mechanism leads to substantial increases in binding affinity, evident as a decrease in the macroscopic KD for binding of mono-Ub in comparison to Ub2 [178,179]. The minimal molecular units to achieve multivalent recognition are a K63-Ub2 chain and the tandem UIMs from RAP80. Interestingly, affinity continues to increase with increasing K63 chain length [178,179]. This increase is not due to multivalent effects; rather, the process can be viewed as entropically favorable, with the affinity increase arising from the availability of a larger number of different bound states for the tandem UIMs from RAP80 and chains longer than Ub2 [178]. Chain binding is also accompanied by fast kinetics, with on-rates of ~108 M−1 s−1 and off-rates of ~104 s−1. Such weak binding may be a defining feature of protein interaction networks in DNA repair, with DSB repair foci requiring plasticity to facilitate the reorganization of protein–DNA interactions at damage sites.

The functional importance of the RAP80–K63 chain interaction in DNA repair has been highlighted through screening studies for RAP80 mutations in BRCA1/BRCA2 mutation-negative families, which identified a deletion mutant (ΔE81) within the first UIM (UIM1) of RAP80 [181]. The deletion mutant demonstrates decreased capacity to bind
K63 chains, a diminished ability to localize to both ionization-induced foci and laser-induced DSBs, and a concomitant increase in chromosomal abnormalities in cells expressing ΔE81-RAP80 [181]. Subsequent NMR studies revealed that the α-helical structure of UIM1 remains essentially intact but undergoes a structural frameshift at the helix N-cap [182]. The frameshift leads to replacement of E81 with T80 in the mutant and results in a loss of favorable electrostatic interactions between E81 from RAP80 and residues Arg-42, Arg-72, and Arg-74 from Ub. The loss of these interactions leads to a 20-fold decrease in binding affinity of UIM1 for mono-Ub and abolishes multivalent recognition, providing a molecular basis for a role for ΔE81-RAP80 in disease progression.

RAP80 possesses an N-terminal SIM adjacent to the tandem UIMs that is necessary for optimal recruitment of the BRCA-A complex to DNA damage sites, in addition to the recognition of K63-linked chains [81,82]. The SIM consists of residues D38AFIVISDSD47, with the C terminus containing a CK2 phosphorylation site [S/T]-X-X-[D/E] [183]. The unphosphorylated RAP80 SIM binds SUMO-2 with a $K_D$ of ~200 μM [38]. Upon phosphorylation of the SIM by CK2, the affinity is substantially enhanced to a $K_D$ of 9 μM. The structure of the RAP80-phosphoSIM/SUMO-2 complex is similar to the antiparallel SIM/SUMO interaction (Fig. 4) [184] and suggests that the increase in affinity is a result of enhanced electrostatic interactions between Lys and His side chains on the surface of SUMO-2 and the phosphate groups on the SIM that elevate the on-rate from $\sim 10^6$ to $10^8$ M$^{-1}$ s$^{-1}$.

RNF4 is a crucial enzyme that functions within DSB repair and attaches Ub to SUMO chains with the E2 Ube2W, resulting in a mixed, or hybrid, Ub-SUMO chain, leading to the suggestion that RAP80 specifically binds hybrid Ub-SUMO chains through simultaneous binding of its N-terminal SIM and tandem UIMs [81,185]. The combined lengths of the C-terminal Ub/N-terminal SUMO-2 linkers in K63-Ub$_2$/SUMO-2 hybrid chains are reasonably matched to the length of the intervening region between the tandem UIMs and the SIM in RAP80. Theoretically, tethering these domains can give substantial multivalent effect [186], decreasing the individual dissociation constants from 150 μM (SUMO-2/SIM binding [38]) and 70 μM (Ub$_2$/RAP80-UIM binding [178]) to a multivalent binding constant of 1 μM and potentially as low as 50 nM, given that phosphorylation enhances SUMO-2/SIM affinity by ~20-fold [38]. In agreement with these calculations, the $K_D$ for RAP80 binding to K63-Ub$_2$/SUMO-2 hybrid chains was determined to be ~0.2 μM using analytical ultracentrifugation measurements [81].

Are hybrid chains bona fide signals in DNA repair and other processes? In the absence of RAP80 SIM phosphorylation, the affinity increase is not substantial compared to the affinity of the UIMs for K63-Ub$_4$. If DSB sites are awash in K63 chains longer than two Ub molecules, then it is difficult to imagine that the hybrid signal is biologically relevant. Phosphorylation of the
SIM may result in a high-affinity signal, but again, within a background of Ub chains, will the signal be buried in noise? A better understanding of the time-dependent molecular composition of DSB repair foci with respect to K63 ubiquitination, and further cell, biochemical, and structural studies, are necessary to provide a definitive answer.

**Proteasome**

The proteasome was initially characterized as a non-lysosomal, ATP-dependent enzyme that degraded abnormal and short-lived proteins [187, 188]. It was found to be composed of multiple subunits [189] and two major forms, an ATP- and Ub-independent 20S form and a Ub-dependent 26S form that contains the 20S particle [190–193]. It was eventually recognized as the primary enzyme for general turnover of proteins and protein degradation for antigen presentation [194]. The 26S proteasome recognizes K48-linked Ub chains attached to proteins, which target them for degradation [195]. It is responsible for the regulation of proteins involved in many cellular processes, including cell cycle and transcriptional regulation. Mutations within the genes that make up the proteasome have been implicated in cardiovascular, neurological, and auto-inflammatory diseases [196]. The proteasome is transcriptionally regulated by NRF1 and NRF2 [197] and contains many post-translational modifications [198].

Proteasomes are found in both the cytoplasm and nucleus in mammalian cells and are enriched in the nucleus in yeast [199]. In mammalian cells, both slow [200, 201] and fast [202] accumulation of 20S with γH2AX due to DNA damage has been observed. In DNA repair, the proteasome regulates the level of MDM2 and p53 in the cell, thereby indirectly regulating the expression of genes involved in DNA repair, cell cycle arrest, and apoptosis [203]. The proteasome is also involved in the removal and degradation of ubiquitinated proteins such as Ku80, L3MBTL1, and JMJD2A around sites of DNA damage, via the p97/Ub fold domain 1/NPL4 complex, to allow for the association of DNA repair proteins such as 53BP1 and BRCA1 [44, 45, 49, 204].

**Concluding Remarks and Future Directions**

The flux of the ubiquitination cascade is fundamental for reshaping the chromatin landscape and facilitating the DSB repair process. K63-linked Ub chains are central to HR-mediated DSB repair, and although other chain topologies are also involved (K6 and K27, for example), a mechanistic and biochemical understanding for the role of different chains is not well developed. Arguably, the most well-understood step in the ubiquitination cascade is activation of the C terminus of Ub by E1 enzyme. Structural, biochemical, and kinetic studies have painted a portrait of a proficient enzyme that effectively maintains the thioester charged E2 – Ub pool.

E2 enzymes primarily function in conjunction with E3 accessory proteins to attach the C terminus of Ub to substrate lysines. Mechanistically, E3 ligases are thought to stabilize the C terminus within the E2 active site cleft by favoring a closed conformation. In the case of the K63 chain builder, Ubc13, the active site cleft is narrow and likely requires a gating mechanism to facilitate the entry of Ub. Do E3s enhance the opening of the E2 active site gate, and if so, what are the implications for catalysis, and is such a mechanism common to all E2s? In this regard, targeting of specific E2s based on variability in mechanism represents an intriguing avenue for therapeutic intervention.

From a broad perspective, what can be gained from a quantitative understanding of the kinetics of catalysis and protein–protein interactions within the Ub cascade? An illustrative example concerns the large family of cullin-RING ligases (CRLs). The CRLs SCF<sub>β-TrCP</sub> have been extensively characterized using numerous biophysical studies and millisecond timescale enzyme kinetics assays [156]. Interestingly, k<sub>cat</sub> for the addition of the first Ub is 10-fold smaller for SCF<sub>β-TrCP</sub>. In addition, these two CRLs show different profiles for their Ub chain-length-dependent k<sub>cat</sub> values. The biological impact is significant: SCF<sub>β-TrCP</sub> more efficiently synthesizes chains competent for degradation by the proteasome. In contrast, the development of a mechanistic basis for the interplay between the E3 RING ligases RNF8 and RNF168 that maintain K63-Ub chains at sites of DNA damage is in its infancy. It is not known how RNF8 builds chains longer than two Ub, how quickly these chains are synthesized, and the role that DUBs play in chain maintenance and termination of the chain signal at DSBs. DNA damage foci are enriched in Ub chains; the nature of chain topology and chain length can be expected to have a significant impact on chain recognition processes. UIMs such as those in RAP80 generally bind Ub weakly; but within a region dense with long Ub chains, chain affinity will be large. In light of this, the biological relevance for hybrid SUMO–K63-Ub<sub>2</sub> chain recognition seems questionable. Furthermore, the role of LRMUs that are adjacent to UIMs in proteins such as RAP80 and RNF168 in chain binding and recognition is unclear.

The proteasome, much like E1 enzymes, has been intensively studied using a broad range of biophysical and structural approaches. However, a number of questions remain: how are Ub chains recognized by the
proteasome and its co-receptors, how are substrates positioned and translocated into the proteasome, and how is chain recognition coordinated with the timing of chain cleavage?

In closing, the grand challenge in the DNA repair field is to develop a detailed understanding of the structures, functions, and regulation of proteins that drive the DDR and ultimately use this knowledge for the treatment of disease. Blending quantitative biophysics with in-cell imaging techniques will be fundamental to illuminate how various post-translational modifications cooperatively regulate the dynamics of DDR proteins within the cell nucleus and its compartments in response to DNA damage.

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Abbreviations used:
DSB, double-strand break; NHEJ, non-homologous end-joining; HR, homologous recombination; Ub, ubiquitin; UBC, Ub-conjugating; SUMO, small Ub-like modifier; SIM, SUMO-interacting motif; ATM, ataxia telangiectasia mutated; MRN, Mre11–Rad50–NBS1 complex; MDC1, mediator of DNA damage checkpoint; RPA, replication protein A; FHA, forkhead associated; UEV, UBC enzyme variant; UBD, Ub-binding domain; HECT, homologous to E6-AP carboxyl terminus; CHFR, checkpoint with forkhead and RING finger domains; RING, really interesting new gene; PAR, polyADP-ribose; JAMM, Jab1/Mpn/Mov34; DUB, deubiquitinase; OTU, ovarian tumor protease; USP, Ub-specific proteases; UCH, Ub carboxyl-terminal hydrolase; UIM, Ub-interacting motif; MRX, Mre11–Rad50–Xrs2; SPR, surface plasmon resonance; SAXS, small angle X-ray scattering; PML, promyelocytic leukaemia; LRM, ligand recognition motif; MIU, motif interacting with ubiquitin; AMSH, associated molecule with the SH3 domain of STAM; SEC-MALS, size exclusion chromatography with multi-angle light scattering; CRL, cullin-RING ligase.

References


Review: K63-inked Ubiquitination Process


[144] A. Szabo, D. Shoup, S.H. Northrup, J.A. McCammon, Stochastically gated diffusion-influenced reactions,


