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REVIEW

Roles of histone ubiquitylation in DNA damage signaling

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Abstract **Histone ubiquitylation has emerged as an important chromatin modification associated with DNA damage signaling and repair pathways. These histone marks, laid down by E3 ubiquitin ligases that include RNF8 and RNF168, decorate chromatin domains surrounding DNA double-strand breaks (DSBs). Recent work implicated ubiquitylated histones in orchestrating cell cycle checkpoints, DNA repair and gene transcription. Here we summarize recent advances that contribute to our current knowledge of the highly dynamic nature of DSB-associated histone ubiquitylation, and discuss major challenges ahead in understanding the versatility of ubiquitin conjugation in maintaining genome stability.**

Keywords DNA damage, histone ubiquitylation, ubiquitin ligase, RNF8, RNF168

DNA damage, if left unrepaired, results in irreversible changes to nucleotide sequence and chromatin structure, which in turn contribute to tumorigenesis and compromise cell and organismal survival (Jackson and Bartek, 2009). DNA damage can arise endogenously during normal cell proliferation, including replication errors and attacks by free radicals that are generated during energy metabolism, and from exogenous mutagenic agents such as ultraviolet light and ionizing radiation (IR). Thus, the constant challenge of mutagenic assaults poses a serious threat to genome integrity.

In a life-long battle against these potentially deleterious entities, our cells have evolved protective mechanisms that safeguard our genetic material. These protective mechanisms encompass DNA damage detection and signal transduction, which culminate into cell cycle arrest and initiation of DNA repair (Ciccia and Elledge, 2010). These cellular responses, collectively termed "DNA Damage Responses" (DDR), are instrumental not only in ensuring faithful inheritance of genetic materials, but recent studies have functionally linked DDR to pleiotropic biological processes, including metabolic regulation, inflammatory responses as well as stem cell maintenance. Indeed, patient mutations and mouse models bearing genetic inactivation of DDR components not only predispose for cancer, but also display immunodeficiency, neurodevelopmental defects and progeria syndromes.

The key role of DDR in maintaining cell and tissue homeostasis implicates sophisticated signaling pathways to allow coordinated execution of cellular responses to cope with genotoxic stress. How are signals from DNA lesions detected and translated into biologic actions? In this regard, the ATM/ATR kinases orchestrate diverse DNA damage responses by regulating the phosphorylation of many DNA damage repair and signaling proteins (Bennetzen et al., 2010; Matsuoka et al., 2007; Mu et al., 2007; Smolka et al., 2007). It is now established that protein phosphorylation represents one of the major regulatory mechanisms to control damage-induced protein–protein interactions and protein activities (Bensimon et al., 2010). One of these include the ATM/ATR-dependent phosphorylation of the histone variant H2AX at Ser139 (γH2AX), which represents one of the earliest event upon DNA damage detection (Burma et al., 2001; Rogakou et al., 1998). γH2AX marks DNA breaks and is instrumental in productive assembly of a cohort of DNA damage signaling and repair proteins, including tumor suppressors BRCA1 and 53BP1, at the damage-modified chromatin (Paull et al., 2000). Consistent with pivotal roles of γH2AX in DNA damage signaling, mice engineered to carry null alleles of H2AX displayed elevated incidence of tumorigenesis, suggesting that H2AX plays a tumor suppressor role *in vivo* (Bassing et al., 2003; Celeste et al., 2003).

Apart from established roles of protein phosphorylation in DDR, recent studies have uncovered important functions of ubiquitin machineries in maintaining genome stability (Ulrich

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and Walden, 2010). In addition to targeting proteins for proteosomal degradation via Lys48-linked poly-ubiquitylation, protein ubiquitylation involving mono-ubiquitylation and non-degradative poly-ubiquitylation participates in many signal transduction events by promoting protein–protein interactions within the DDR network (Al-Hakim et al., 2010). Consistently, DDR components harboring modules that recognize ubiquitin conjugates and/or ubiquitin chains have been identified, implying that ubiquitin conjugates serve as recruiting factors to coordinate DNA damage-induced assembly of protein components for proper executing of DNA damage responses. In particular, DNA damage-associated ubiquitylation involving histone molecules has emerged as pivotal mechanisms for DNA damage signal transduction. Moreover, ubiquitylated histones have also been ascribed to functions in regulating various DNA transaction events on damage-modified chromatin. In this review, we will summarize how components of the ubiquitin machineries promote histone ubiquitylation, and how these histone marks are decoded and translated into biologic actions essential for protection of genome integrity.

Principle of protein ubiquitylation

Ubiquitin is a highly conserved 76 amino acid polypeptide that is covalently attached via its C-terminal glycine residue to ε-amino group of substrate lysine residues. Ubiquitin conjugation is accomplished by sequential actions of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s). E1 binds and transfers ubiquitin onto E2 molecules in an ATP-dependent manner. The E2-loaded ubiquitin is subsequently conjugated onto substrate proteins through substrate specificity-conferring E3 enzymes. To date, there exist two major E3 subfamilies, including E3 ligases that harbor the HECT (homologous to human papilloma virus E6 carboxyterminal domain) and RING (really interesting new gene) domain, respectively. While HECT-containing ubiquitin ligases unload E2 and form E3-ubiquitin thioester intermediate before they transfer the ubiquitin to target proteins, RING type E3s bring E2-bound ubiquitin to close proximity to the substrate protein such that ubiquitin can be directly transferred to substrate from E2.

Ubiquitin contains seven lysine residues situated at positions 6, 11, 27, 29, 33, 48 and 63. Hence, apart from mono-ubiquitylating substrate proteins, ubiquitin chains of different topology can form through extension via its own lysine residues. Together with the recent identification of linear ubiquitin chains (Iwai and Tokunaga, 2009), the diversity of ubiquitin conjugation greatly increases the complexity and versatility of this post-translational modification in temporal and spatial regulation of biologic processes. Protein ubiquitylation can be divided into 3 categories: mono-ubiquitination, multi-mono-ubiquitination and poly-ubiquitination (Komander, 2009). While K48-linked poly-ubiquitin chains play an established role in proteasome-mediated degradation, mono-ubiquitylation and non-degradative poly-ubiquitylation involving chains composed of K6- and K63-linkages have recently been implicated in cellular responses to genotoxic stress (Panier and Durocher, 2009).

Protein ubiquitylation at DNA double-strand breaks

Among the first hints that protein ubiquitylation is linked to DNA damage signaling and repair was the observation that ubiquitin conjugates (detected using the FK2 antibodies) form microscopically visible focal structures that overlap with those of the DNA damage marker γH2AX (Morris and Solomon, 2004; Polanowska et al., 2006). While the BRCA1-BARD1 heterodimer contributed partly to these ubiquitylated structures, exactly how these BRCA1-dependent ubiquitin conjugates are relevant to its functions in checkpoint control and DNA repair remain elusive. A major advance in understanding the possible roles of protein ubiquitylation at DSBs came when the ubiquitin-interacting motif (UIM)-containing protein RAP80 was identified as an upstream component that allows BRCA1 targeting to DSBs (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). Notably, RAP80 accumulates at DSBs in an UIM domain-dependent manner, indicating that non-degradative ubiquitin structures may serve to anchor the RAP80-BRCA1 complex at the vicinity of DNA breaks. In line with this possibility, the RAP80 UIM preferentially interacted with K63-linked ubiquitin chains (Sato et al., 2009; Sims and Cohen, 2009). Furthermore, UBC13, the only known E2 ubiquitin conjugating enzyme that catalyzes K63-based ubiquitylation was essential for FK2-labeled ubiquitin structure formation (hereafter referred to as FK2 foci) at DSBs (Zhao et al., 2007). Together, these data strongly suggest that certain K63-linked ubiquitin-modified proteins are present at DSBs.

Ubiquitylated histones in DNA damage signal transduction

Histone ubiquitylation has an established role in gene transcription (Weake and Workman, 2008). Interestingly, recent studies have functionally linked ubiquitylated histones in DNA damage signaling and repair processes. Like many other DDR components, use of antibodies against ubiquitylated H2A (uH2A) revealed foci structures at DSBs, suggesting that certain ubiquitin ligase(s) may mediate local histone ubiquitylation in response to DNA damage (Mailand et al., 2007). Roles of histone ubiquitylation in DNA damage signaling was corroborated with the identification of the E3 ligase RNF8 as an essential factor for damage-induced ubiquitin conjugation at DSBs (Huen et al., 2007a; Kolas

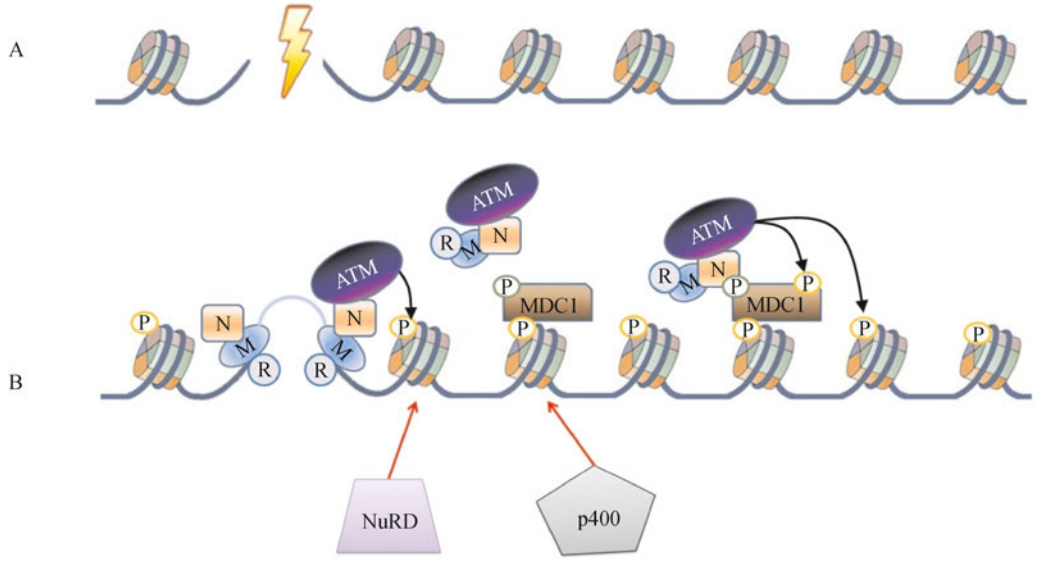
et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). RNF8 contains an N-terminal FHA domain, which supports its localization at DSBs through phosphorylation-dependent interaction with the upstream signaling component MDC1. Upon docking at the vicinity of DSBs, RNF8 promoted FK2 foci as well as ubiquitylation of H2A-type histones. Inactivation of RNF8 compromised not only DSB-associated ubiquitylation, but was also accompanied with defective recruitment of checkpoint and repair proteins 53BP1 and BRCA1, suggesting that RNF8 plays an early role in propagating DNA damage responses via ubiquitin signaling mechanisms. The observation that UBC13 interacted with RNF8 (Plans et al., 2006; Huen et al., 2008), and was similarly required for 53BP1 and BRCA1 foci formation led to the idea that RNF8-UBC13 represents an E3-E2 pair important for ubiquitin-dependent DNA damage signal transduction (Huen and Chen, 2010).

Interestingly, patient cells derived from the RIDDLE syndrome, a disease characterized by radiosensitivity and immunodeficiency, displayed similar phenotypic defects observed in RNF8-deficient cells, including abrogated damage-induced FK2 foci and defective recruitment of BRCA1 and 53BP1 (Stewart et al., 2007). Since ectopic expression of RNF8 or UBC13 in these cells did not restore these RIDDLE-associated defects, these observations hinted that additional DDR component(s) may play roles at levels between RNF8 and DSB-associated ubiquitylation events. Much like phosphorylation-dependent hierarchical signaling cascade that involves multiple kinases, using siRNA-based screens, subsequent studies identified RNF168 as an additional ubiquitin ligase that amplifies the RNF8-initiated ubiquitin signals on H2A-type histone molecules. RNF168 associated at damaged chromatin via its ubiquitin binding MIU domains, which interacted with the RNF8-primed mono- and di-ubiquitylated H2A and H2AX (Doil et al., 2009; Stewart et al., 2009). Similar to RNF8, RNF168 interacted with UBC13, and was required for productive assembly of K63-linked ubiquitin conjugates as well as 53BP1 and the RAP80-BRCA1 complex at DSBs. Taken together, these data are in favor of the idea that RNF8 and RNF168 function in a linear pathway to amplify the DNA damage signal via histone ubiquitylation (Fig. 1).

Congruent with the model that K63-linked ubiquitin chains at DSBs serve non-degradative roles to recruit checkpoint and repair proteins, RAD18 was subsequently identified as a DDR component that preferentially interacted with K63-linked poly-ubiquitin chains via its ZNF domain. Like RAP80, RAD18 concentration at DSBs required the RNF8-RNF168-UBC13 ubiquitin machinery, supporting that local ubiquitylation events at DSBs are prerequisite for loading of downstream effector molecules (Huang et al., 2009).

Regulators of RNF8-RNF168-UBC13-mediated ubiquitin signaling

The pivotal importance of ubiquitylated histones in propagating DNA damage signals is further underscored by recent identifications of DDR components which localize to DSBs and promote histone ubiquitylation through different strategies. One of these was the putative E3 ubiquitin ligase HERC2, which was identified as an RNF8-associated protein. DNA damage triggered HERC2 phosphorylation and promoted its interaction with RNF8 via the phospho-peptide binding FHA domain (Bekker-Jensen et al., 2010). Interestingly, HERC2 not only facilitated the RNF8-UBC13 complex formation but also independently stabilized RNF168 proteins. These observations suggest that HERC2 may function in at



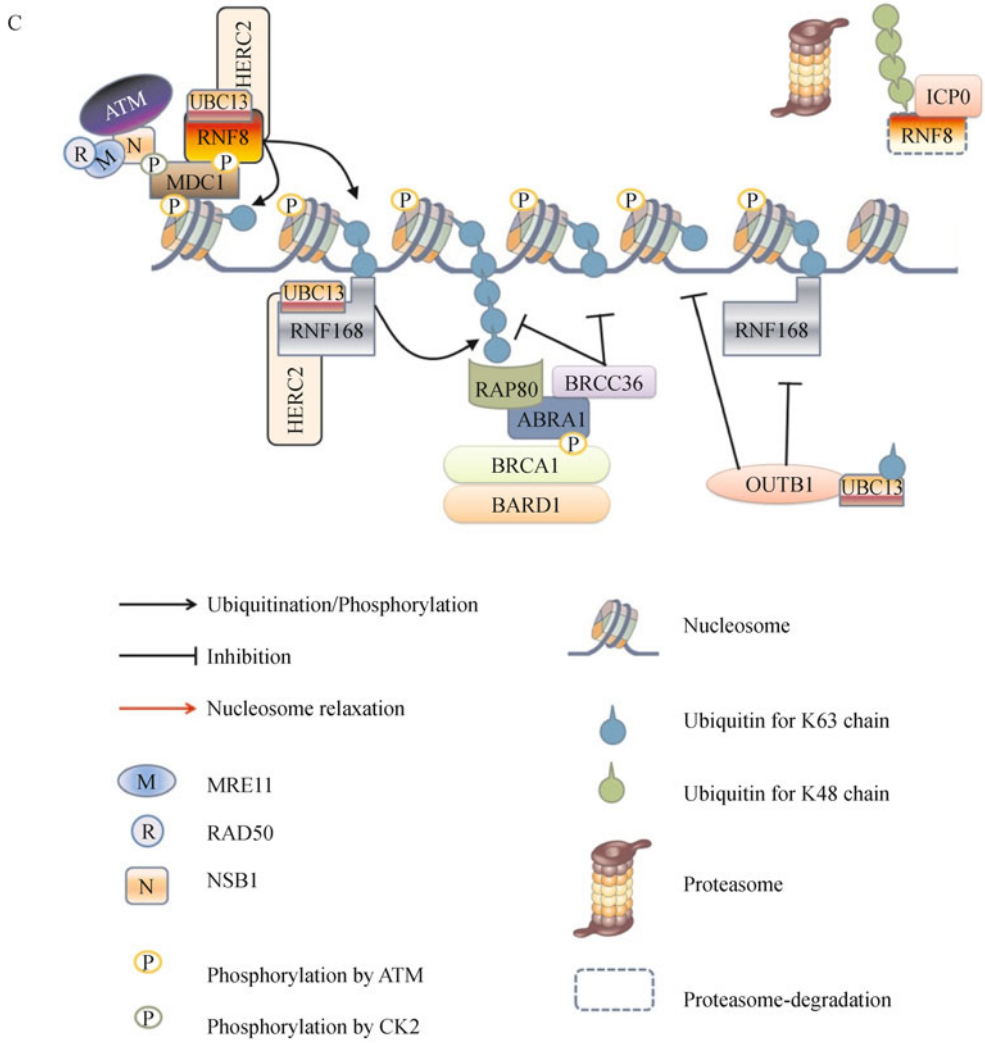


Figure 1 A schematic of RNF8-RNF168-UBC13-mediated ubiquitin signaling. (A) A DNA double-strand break (DSB) is induced on chromatin by e.g. ionizing radiation. (B) The putative DSB sensor MRE11/RAD50/NBS1 (MRN) complex recognizes DNA damage through the binding of RAD50 and MRE11 to DNA (Hopfner et al., 2001; Bhaskara et al., 2007) and the formation of the M2R2 head. NBS1 attaches to the M2R2 head and further recruits ataxia telangiectasia mutated (ATM) through its C terminus, leading to ATM-mediated phosphorylation of H2AX molecules (γH2AX) to provide binding sites for MDC1 (Stucki et al., 2005). CK2-phosphorylation of MDC1 allows further accumulation of MRN-ATM complexes to chromatin domains flanking DSBs (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). p400 and NuRD may contribute to chromatin remodeling to promote RNF8-dependent histone ubqiuitylation events at DSB. (C) DNA damage-induced ubiquitylation of H2A-type histones is catalyzed by the RNF8-RNF168-UBC13 axis. The ATM-dependent MDC1 phosphorylations anchor RNF8 to DSBs. In conjunction to UBC13, RNF8 mono- and di-ubiquitylates H2A and H2AX at DSBs, which recruit RNF168 to amplify the ubiquitin signals on histone molecules. HERC2 stabilizes the RNF8-UBC13 complex and RNF168 for full response of DSB-associated histone ubiquitylation. Ubiquitylated histones, including those involving K63-linked polyubiuqitin chains, allow recruitment of RAP80-BRCA1. OTUB1 binds UBC13 to suppress transfer of the UBC13-bound ubiquitin to RNF168 substrates. In addition, the viral protein ICP0 targets RNF8 and RNF168 for proteasomal degradation.

least two regulatory layers to fine-tune the ubiquitin-dependent DNA damage signaling cascade.

More recently, a number of chromatin remodeling complexes have been found to modulate the RNF8-RNF168-UBC13-dependent response at the damage-modified chromatin. One such complex is the nucleosome-remodeling and histone deacetylation (NuRD) complex. Consistent with direct roles of NuRD in DDR, several of its subunits, including CHD4, MTA1 and MTA2, were found to localize at DSBs (Chou et al., 2010a; Larsen et al., 2010; Polo et al., 2010; Smeenk et al., 2010). While inactivation of NuRD hypersensitized cells to DNA damaging agents, exactly how NuRD protects cells from DNA damage remains unknown. One possible route via which NuRD exerted its protective effects is through promoting the RNF8-dependent histone ubiquitylation at DSBs (Larsen et al., 2010; Smeenk et al.,

2010). Accordingly, ablating CHD4 function dampened DNA damage ubiquitin signaling (determined by reduction in FK2 foci and γH2AX ubiquitylation) and led to compromised recruitment of 53BP1 and BRCA1 to DSBs.

In line with the idea that histone ubiquitylation are intimately linked to DDR, the Polycomb repressive complex 1 containing BMI1 and RING2, an E3 ligase complex important for maintenance of steady-state levels of ubiquitylated H2A, was recently shown to localize at DSBs (Chou et al., 2010b; Ismail et al., 2010). Interestingly, concentration of BMI1 at DSBs was independent of the canonical DNA damage signaling pathway involving H2AX, suggesting possible crosstalks for optimal ubiquitin signaling in response to DNA damage (Ismail et al., 2010). Another chromatin remodeler that has been functionally linked to the DNA damage-induced ubiquitin signaling is the SWI/SNF DNA-dependent ATPase p400 (Xu et al., 2010). p400 is a component of NuA4 complex and is associated with the Tip60 acetyltransfase, the latter of which has been implicated in DNA repair (Murr et al., 2006; Ikura et al., 2007). Notably, p400 was required for nucleosome destabilization and efficient RNF8-dependent histone ubiquitylation in response to DNA damage. As RNF8 docking at damage-induced foci was not dependent on p400 status, it is possible that bipartite regulatory mechanisms exist to tightly govern the RNF8-RNF168-UBC13 axis in promoting DNA damage signaling. While MDC1 phosphorylation controls RNF8 localization at DSBs, nucleosomal destabilization mediated by p400 may temporally regulate RNF8-dependent histone ubiquitylation.

Antagonism of ubiquitin signaling at DNA double strand breaks

While we have gained considerable insights into how the RNF8-RNF168-UBC13 axis facilitates DDR by promoting DSB-associated histone ubiquitylation, functional relevance for negative regulators of these histone marks have largely been unexplored. To date, several deubiquitinases (DUB) have been implicated in DDR, including BRCC36 (BRCA1/BRCA2-containing complex subunit 36), USP3 and USP16. In line with roles of ubiquitylated histones in assembling checkpoint and repair factors to DSBs, both overexpression of BRCC36 and USP3 counteracted the RNF8-dependent histone ubiquitylation and led to defective recruitment of 53BP1 and BRCA1 (Mailand et al., 2007; Shao et al., 2009). In addition, USP16 was recently implicated in regulation of gene silencing in response to DNA damage by removing the RNF8-RNF168-dependent H2A mono-ubiquitylation at DSBs (Shanbhag et al., 2010).

More recently, OTUB1 was identified as another DUB that exerted negative effects toward damage-induced histone ubiquitylation (Nakada et al., 2010). OTUB1 was found to suppress histone poly-ubiquitination by inhibiting transfer of the UBC13-bound ubiquitin to protein substrates, thereby limiting the extent of the RNF168-dependent amplification of histone ubiquitylation.

While these studies point to exquisite and tunable control for DNA damage-induced ubiquitin signaling, prior to appreciating the cellular functions of these DSB-associated deubiquitinases, their mechanistic details will need to be dissected. Resembling the various protein phosphatases in DDR, one possible utility of these DUBs may be to terminate the ubiquitin signal to facilitate cell recovery upon completion of DNA repair. Alternatively, basal deubiquitinase activities may serve to limit the RNF8-RNF168-UBC13-mediated ubiquitin response until the level of DNA damage reaches a threshold. Regardless, the fact that multiple DUBs are involved in fine-tuning the magnitude of histone ubiquitylation underscores the importance of the coupling of DNA damage signaling and cell proliferation.

Viral strategy to counteract DSB-associated ubiquitin signaling

Apart from orchestrating DDR to protect genome integrity, the importance of the RNF8-RNF168-UBC13 axis in maintaining cell homeostasis is further exemplified by the identification of the HSV-1 viral factor ICP0 in regulating RNF8-RNF168 functions (Lilley et al., 2010). ICP0 encodes a RING domain-containing E3 ubiquitin ligase that was found to target RNF8 and RNF168 for proteasomal degradation. Accordingly, overexpression of ICP0 resulted in decreased protein levels of RNF8 and RNF168, leading to compromised ubiquitylation of H2A-type histones molecules and defective recruitment of 53BP1 to DSBs. Consistent with the idea that ICP0 limits RNF8-RNF168 functions at DSBs, overexpression of both RNF8 and RNF168 resulted in largely restored foci formation of 53BP1. Together with the observation that plaque-forming efficiency of an ICP0 null virus was partially restored in RNF8-deficient cells, these data suggests that the ubiquitin signaling machinery involving RNF8-RNF168-UBC13 may be important for cellular defense against viral infection.

Future prospective

Despite the rapid advance in our understanding of DNA damage-induced histone ubiquitylation and their utilities at DSBs, a number of important questions remain. Are histone molecules ubiquitylated in a uniform manner at chromatin domains flanking DSBs? Are there different topologies of ubiquitin chains on DSB-associated histone molecules? And if so, do they signal for activation of DDR sub-pathways? Since RNF8 promoted ubiquitylation of H2AX at sites other than Lys119/120 (Huen et al., 2007b), it is formally possible that H2A-type histones are post-translationally modified in response to DNA damage on various lysine residues, and by a

mixture of ubiquitin conjugates including multi-mono-ubiquitylation and K63-linked poly-ubiquitylation. Whether these different ubiquitin signals may lead to differential cellular responses will await more in-depth studies to unravel the nature of damage-induced RNF8-RNF168-UBC13-mediated histone ubiquitylation.

Moreover, exactly how ubiquitin conjugates allow tethering of DDR factors including 53BP1 and PTIP to DSBs remains to be determined. 53BP1 associates with chromatin via its tandem Tudor domain, which preferentially binds to methylated histones lysines including histone H4 Lys 20 (H4K20) and H3 Lys 79 (H3K79) (Huyen et al., 2004; Botuyan et al., 2006), whereas PTIP localizes to DSBs via its phospho-peptide BRCT domains (Manke et al., 2003; Munoz et al., 2007; Gong et al., 2009; Wu et al., 2009). Thus, whether the RNF8-RNF168-mediated ubiquitylation at DSBs may remodel nucleosomes to expose methylated histones or whether upstream components that recognize ubiquitin structures are required for DSB-association of 53BP1 and PTIP will require further work.

Since the RNF8-RNF168-UBC13 axis in promoting DNA damage signaling via ubiquitylation-dependent mechanisms has emerged, studies have uncovered important roles of protein sumoylation in propagating DDR (Galanty et al., 2009; Morris et al., 2009). To date, a number of DDR components, including BRCA1 and 53BP1, have been shown to be subjected to sumoylation (Galanty et al., 2009; Morris et al., 2009). Possible roles of protein sumoylation in DNA damage signaling are illustrated not only from observations where dysregulation of sumoylation machineries compromised DSB-association of checkpoint and repair proteins, but sumoylation of the tumor suppressor BRCA1 also potently increased its E3 ubiquitin ligase activities. Thus, it will be of significant interest to examine in details how protein sumoylation may provide yet another layer of control for full response of the RNF8-RNF168-UBC13-dependent ubiquitin signaling.

Conclusion

Since the original inception of possible involvement of ubiquitylation at DSBs, fueled by the identification of novel DDR components, we have now gathered an intricate ubiquitylation-dependent DNA damage signaling cascade. Notably, the versatility of protein ubiquitylation affords temporal and spatial controls for diverse cellular events that, in concert, promote genome stability and cell survival. As our understanding for DDR expands, it is likely that we will see more of protein ubiquitylation as regulatory mechanisms for proper cellular responses to DNA damage.

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