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Review

RNF8-dependent histone ubiquitination during DNA damage response and spermatogenesis

Teng Ma†, Jennifer A. Keller†, and Xiaochun Yu*

Division of Molecular Medicine and Genetics, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor 48109, USA
†These authors contributed equally to this work.
*Correspondence address. Tel: +1-734-615-4945; Fax: +1-734-936-6684; Email: xiayu@umich.edu

Histone ubiquitination regulates the chromatin structure that is important for many biological processes. Recently, ubiquitination of histones was observed during the DNA damage response (DDR), and this modification is controlled by really interesting new gene (RING) domain E3 ligase, RNF8. Together with the E2 conjugating enzyme UBC13, RNF8 catalyzes ubiquitination of the histones H2A and H2AX during the DDR, thus facilitating downstream recruitment of DDR factors, such as p53 binding protein 1 (53BP1) and breast cancer type 1 susceptibility protein (BRCA1), to the damage site. Accordingly, the RNF8 knockout mice display phenotypes associated with failed DDR, including hypersensitivity to ionizing radiation, V(D)J recombination deficiency, and a predisposition to cancer. In addition to the DDR phenotypes, RNF8 knockout mice fail to generate mature sperm during spermatogenesis, resulting in male sterility. The RNF8 knockout mice also have a drastic reduction in histone ubiquitination in the testes. These findings indicate that the role of histone ubiquitination during chromatin remodeling in two different biological events could be linked by an RNF8-dependent mechanism. Here, we review the molecular mechanism of RNF8-dependent histone ubiquitination both in DDR and spermatogenesis.

Keywords acetylation; RNF8; UBC13; chromatin remodeling

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Introduction

Chromatin fibers are composed of nucleosomes, in which DNA is wrapped around a histone octamer core. The core histones include H2A, H2B, H3, and H4 [1,2]. These four canonical histone proteins are composed of a structured central (globular) domain that is in close contact with the DNA and much more flexible N-terminal and C-terminal tails [3]. Both the globular domain and histone tails undergo post-translational modifications, which can either directly change the chromatin structure by affecting the accessibility of DNA to other proteins or provide docking sites to recruit downstream chromatin remodeling factors [4,5]. These modifications, such as phosphorylation, acetylation, methylation, and ubiquitination, combine to form the 'histone code' that is associated with diverse cellular processes such as chromosome condensation, gene expression, and DNA damage repair [6–14]. Histone modifications are not insulated from each other. Instead, these modifications display cross-talks and function together in biological events [15,16]. One recently identified example is the RING domain E3 ligase RNF8-dependent histone ubiquitination, which mediates histone acetylation to promote histone eviction during both spermatogenesis and DNA damage response (DDR) [17].

Histone ubiquitination

Protein ubiquitination is a chemical reaction with three sequential steps in which ubiquitin, a 76 amino acid polypeptide, is covalently conjugated to the substrate in the presence of ubiquitin E1, E2, and E3 enzymes [18]. The first-reported ubiquitination substrate was histone H2A, identified *in vivo* by Goldknopf and Busch in 1977 [19]. Subsequently, histone H2B was found to be ubiquitinated as well by West and Bonner [20]. Like other protein ubiquitinations, histone ubiquitination is catalyzed by the formation of an isopeptide bond between the carboxy-terminal glycine of ubiquitin and lysine resides on H2A and H2B [18]. The ubiquitination sites have been mapped to lysines 119 and 120 on the tails of H2A and H2B in mammals, respectively [21]. Considering that H2A and H2B contain only 131 and 125 residues, respectively, the large molecular size of ubiquitin relative to the histones makes histone ubiquitination unique among protein

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modifications. Although structural analysis indicates that ubiquitin protrudes to the outside of the nucleosome, this bulky modification existing in the nucleosome potentially changes the chromatin structure. Thus, it is not surprising that both H2A and H2B ubiquitination regulate chromatin remodeling during gene transcription. Interestingly, the roles of ubH2A and ubH2B are different in transcription. It has been shown that ubH2A is enriched in gene loci with low transcription activity and participate in gene silencing with Polycomb repressive complex 1 [22–24]. In addition, during the pachytene stage of meiotic prophase I, ubH2A is highly enriched in the XY body where X and Y chromosomes are transcriptionally silenced [25]. In contrast, ubH2B marks highly transcribed gene loci and facilitates transcription elongation [26–30]. Recently, both ubH2A and ubH2B have been shown to be involved in DDR [31–36].

RNF8 regulates histone ubiquitination during DNA damage response

Genomic DNA that stores genetic information can easily be damaged by numerous environmental and internal hazards. The most deleterious damage is DNA double-strand breaks (DSBs). In response to DSBs, a group of PI3-like kinases, including Ataxia Telangiectasia Mutated (ATM), Ataxia Telangiectasia and RAD3 related (ATR), and DNA-dependent protein kinase catalytic subunit (DNAPKc), are activated and transmit signals through various mediators to arrest cell cycle progression and facilitate DNA damage repair [37,38]. One of those important mediators during DDR is histone H2AX, a variant of H2A with a C-terminal tail that can be phosphorylated by ATM at DNA lesions [37]. When DSBs occur, ATM-phosphorylated H2AX recruits mediator of DNA damage checkpoint 1 (MDC1), which can also be phosphorylated by ATM at DNA damage sites. The H2AX and MDC1 complex stabilizes a large group of DNA damage repair factors, such as p53 binding protein 1 (53BP1) and breast cancer type 1 susceptibility protein (BRCA1), at DNA damage sites, which mediates cell cycle arrest and DNA damage repair [39,40]. In addition to this, protein phosphorylation cascade, phosphorylated H2AX, and MDC1 also regulate a unique ubiquitination cascade at DNA damage sites through the E3 ligase RNF8 [31,33,34].

First reported in 1998, RNF8 is a 485-amino acid nuclear polypeptide ubiquitously expressed in human tissues [41]. The RNF8 protein contains an N-terminal forkhead-associated (FHA) domain and a C-terminal RING domain [42]. The FHA domain is a phospho-threonine binding domain [43]. Peptide library screening indicates that the RNF8 FHA domain recognizes a pTXXF motif [33]. Following DNA damage, we and others have found that the RNF8 FHA domain recognizes three different pTXXF motifs in MDC1, and MDC1 targets RNF8 to DNA damage sites through this phospho-dependent interaction [31,33,34]. The RING domain of RNF8 is an E3 ubiquitin ligase. It can interact with Ubc13 to catalyze lysine-63 polyubiquitin chain formation as well as with class III E2s (UBE2E2, UbcH6, and UBE2E3) for lysine-48-based polyubiquitin chains [42,44]. Ubiquitination of H2A, H2AX, and H2B are known to be regulated by RNF8 at DNA damage sites. H2A and H2AX can be both mono- and poly-ubiquitinated, while H2B is only mono-ubiquitinated. Although RNF8 is an E3 ligase and does ubiquitinate histones *in vitro* [34], it is not clear as to whether RNF8 or other E3 ligases directly ubiquitinate histones *in vivo*.

Accumulating evidence suggests that histone ubiquitination could be recognized by ubiquitin-binding proteins [45]. For example, the ubiquitin interacting motif (UIM) domain of receptor-associated protein 80 (RAP80) recognizes ubH2A and ubH2B at DNA damage sites [36]. RAP80 forms a complex with CCDC98 and BRCA1 [46–50]. The UIM domain of RAP80 targets the whole complex to DNA damage sites, which facilitates the DNA repair function of BRCA1. Recently, we found that MRG15, a subunit of both the histone acetyltransferase complex and deacetylase complex [51–54], might also recognize ubH2B and induce histone acetylation by two acetyltransferases, males-absent on the first protein (MOF) and tat-interactive protein 60 kDa (TIP60) (T. M., J. A. K., X. Y.). Since histone acetylation brings negative charges onto the chromatin, it may potentially change the topology of chromatin into a more relaxed status, thus allowing other DNA damage repair factors to access DNA damage sites.

Although RNF8 can trigger DSB-associated ubiquitinations, it might not be sufficient to sustain conjugated ubiquitin at DNA damage sites due to the weak E3 ligase activity of RNF8 *in vitro* [34] and competition with strong deubiquitinase activity *in vivo* [55–57]. The persistence of ubiquitinated histones at DNA lesions was unexplained until the discovery of another E3 ligase, RNF168. Performing a meticulous monitoring of the DSB-associated ubiquitinations during the first 10 min after DNA damage, researchers found that the temporal accumulation of conjugated ubiquitin at DSBs tightly correlated with the retention of RNF168 in this compartment, and that no increase in local ubiquitin concentration was observed in cells with depleted RNF168, even at the earliest time points. RNF168 contains ubiquitin-binding domains (MIU1 and MIU2) that allow interaction with ubH2A [32,35]. Like RNF8, RNF168 interacts with UBC13 to ubiquitinate histones adjacent to DSBs [32,35]. RNF168 ubiquitination is RNF8 dependent, and, by targeting H2A and H2AX, amplifies the local concentration of ubiquitin conjugates to the

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threshold required for retention of 53BP1 and BRCA1 [32,35]. These data indicate that the ubiquitin conjugates generated by RNF8 are transient and/or unstable and require amplification and/or stabilization by RNF168 to achieve the threshold needed for the completion of the DSB-induced chromatin response. Interestingly, it was found that overexpression of RNF8 rescues cellular phenotypes in cells with moderate, but not strong, down-regulation of RNF168 [32], indicating that high activity of RNF8 can maintain unstable ubiquitin conjugates to compensate for a weaker, but not absent, RNF168 response. Additionally, recent work has shown that the silencing of genes near sites of DNA damage (DISC, Double-strand break-Induced Silencing in Cis) is dependent on H2A ubiquitination, and that DISC is only lost when both RNF8 and RNF168 are inactivated [58]. Thus, the functional interaction between RNF8 and RNF168 needs to be further elucidated.

In addition to RNF168, it was also reported that another factor, HERC2, forms a complex with RNF8 in response to ionizing radiation and is involved in the DDR [59]. HERC2 is an HECT-type E3 ubiquitin ligase. The HERC2-RNF8 interaction requires ionizing radiation-inducible phosphorylation of HERC2 at Thr 4827, which is recognized by the FHA domain of RNF8. HERC2 facilitates assembly of the ubiquitin-conjugating enzyme Ubc13 with RNF8, thereby promoting DNA damage-induced formation of poly-ubiquitin chains. It has also been shown that HERC2 interacts with and maintains the levels of RNF168, implicating HERC2 in maintenance of both components of the histone ubiquitination pathway.

Taken together, RNF8 plays a central role in DDR. RNF8 recognizes phosphorylated MDC1 in order to relocate to DSBs and ubiquitinate histones at DNA lesions. RNF8 acts upstream of a number of repair factors including RNF168, HECT domain and RCC-like domain-containing protein 2 (Herc2), 53BP1 and BRCA1, and its activity tethers these proteins to the damaged chromatin to transduce the repair signal for DNA damage in the cell. To examine the function of RNF8 *in vivo*, we and others have generated RNF8-deficient mice [17,60,61]. To our surprise, the phenotype of RNF8 null mice is very mild. Although RNF8 null mice are sensitive to ionizing radiation and have subtle defects in V(D)J recombination during T-cell development and immunoglobulin class-switching during B-cell differentiation, the mice are viable and seldom develop T-cell or B-cell lymphomas [17,60,61]. These mild phenotypes lead us to search for other similar proteins that could play a redundant functional role with RNF8.

From a similar domain architecture search, Chfr could be a paralog of RNF8 [62]. RNF8 and Chfr are the only two human E3s that contain both the FHA domain and RING domain. Like RNF8, the RING domain of Chfr is also an E3 ligase and can interact with Ubc13, the key E2 enzyme to catalyze histone ubiquitination at DNA damage sites [63]. More interestingly, Chfr is down-regulated in 20%–40% of primary tumors and tumor cell lines, mainly due to promoter hypermethylation-induced *Chfr* gene silencing, suggesting that Chfr may play a role in tumor suppression [64–71]. Since RNF8 and Chfr share similar functional domains and interact with the same E2 ubiquitin conjugase, we generated Chfr-deficient mice. Like RNF8-deficient mice, Chfr-deficient mice are also viable and have a mild phenotype. However, after we crossed RNF8-deficient mice and Chfr-deficient mice to generate double-knock-out mice (DKO), we found that DKO mice were not only hypersensitive to ionizing radiation, but also have significant V(D)J recombination defects during T-cell development and develop T-cell lymphomas (unpublished data). These phenotypes of DKO mice are very similar to ATM-deficient mice [72]. In the mouse embryonic fibroblasts (MEFs) extracted from DKO mice, the basal level of histone ubiquitination is significantly abrogated, indicating that RNF8 and Chfr may regulate not only DNA damage-induced histone ubiquitination but also the basal level of histone ubiquitination. As acetylation and destabilization of the nucleosome have been linked to histone ubiquitination [17,73], RNF8 and Chfr-dependent histone ubiquitination indirectly modulate chromatin structure and condensation. In response to DNA damage, RNF8 could be recruited to DNA damage site [31,33,34]. Its ubiquitination activity could relax the chromatin adjacent to DNA lesions and allow DDR factors to access DNA damage sites for proper repair. In the absence of RNF8 and Chfr, DSBs, particularly generated during V(D)J recombination, could not be correctly repaired, inducing genomic instability and ultimately causing T-cell lymphoma.

RNF8 in spermatogenesis

In addition to playing important roles in DDR, histone ubiquitination is critical for spermatogenesis. Correspondingly, loss of RNF8-dependent histone ubiquitination suppresses spermatogenesis [17,61]. During spermatogenesis, progenitor cells undergo successive mitotic and meiotic divisions (spermatocytogenesis) and a metamorphic change (spermiogenesis) to produce spermatozoa. During the pachytene stage of meiotic prophase I, ubH2A is highly enriched in the XY body [25], where X and Y chromosomes become partially synapsed through pseudo-autosomal regions and are transcriptionally silenced. This phenomenon is known as meiotic sex chromosome inactivation (MSCI) [74]. Consistent with its transcriptionally silenced status, the XY body contains a unique combination of histone modification marks associated with gene silencing including dimethylation of histone H3 on lysine 9 (H3K9) and

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deacetylation of histone H3 and H4 [75]. MSCI is important for proper meiosis, and is controlled by H2AX. Disruption of MSCI leads to the arrest of spermatocytes at the pachytene stage of meiotic prophase in the H2AX-deficient mice [76]. The role of ubH2A in the XY body is not clear, but it is thought that these modifications may mediate MSCI [77]. In RNF8 knockout spermatocytes, ubiquitinated conjugates on the XY body in pachytene-stage cells are strikingly lost, which is correlated with RNF8's role as the E3 for histone ubiquitination [31,33,34]. However, although ubH2A enrichment at the XY body is abolished, both XY body formation and meiosis are unaffected in RNF8-deficient testes as marked by normal γH2AX [17]. The transcription and replication machinery are inactivated in the RNF8-deficient mice as in the wild-type mice, as shown by the exclusion of RNA polymerase II from the XY body and the low-expression pattern of X chromosome genes [17]. Thus, these findings indicate that RNF8-dependent histone ubiquitination is not required for MSCI and meiosis [17].

Ubiquitinated histones occur in other stages of spermatogenesis beyond meiosis. For example, ubiquitinated H2A and H2B are also enriched in elongating spermatids [23,78]. During spermiogenesis, sperm DNA is highly condensed and tightly wrapped around histone-like protamines instead of histone octamers [79]. The transition from nucleosomes to protamines occurs in round haploid spermatids that elongate and transform into mature sperm. During this process, most nucleosomal histones are initially replaced by two transition proteins, transition protein 1 and 2, and subsequently by two protamines, protamine 1 and 2 [80,81]. Both histone ubiquitination and hyper-acetylation are implicated in nucleosome removal at post-meiotic stages [81]. Although the biological function of these massive chromatin remodeling events is not clear, it is hypothesized that the protamines promote increased DNA condensation to facilitate the packaging of DNA into the sperm heads. Failure to accomplish this global chromatin restructuring causes male sterility [82–84]. In fact, the male infertility in RNF8-deficient mice occurs during this post-meiotic stage. Histological analysis of the testes revealed that RNF8-deficient testes contained fewer condensing spermatids and drastically fewer condensed mature spermatids. Further investigation indicated that chromatin-bound transition proteins and protamines were reduced in the testes of RNF8-deficient mice. During histone replacement, it has been suggested that the N-terminal tail of histone H4 is highly acetylated [85]. Since acetylation adds negative charges to nucleosomes, it has been hypothesized that acetylation of H4 could loosen chromatin fibers to enhance histone replacement [86–88]. Interestingly, the H4 acetylation level is also significantly reduced in testes from RNF8-deficient mice, whereas other histone markers like H3 methylation showed no change [17]. Similarly, the chromatin-associated histone acetyltransferase MOF, which accounts for the majority of H4K16Ac, is also decreased in the RNF8-deficient testes [17].

Collectively, these findings pose a trans-histone modification model in which RNF8-dependent histone H2A/H2B ubiquitination induces the H4 acetylation by MOF. In support of this model, the N-terminal tail of H4 has been shown to make an inter-particle contact with the H2A/H2B heterodimer of adjacent nucleosomes [3]. H4 acetylation could be an essential step for histone removal in elongating spermatids. A defect in H4 acetylation could significantly suppress histone removal and histone-like protein incorporation during spermiogenesis. Thus, RNF8-dependent ubH2A/ubH2B induces H4 acetylation in adjacent nucleosomes and promotes removal of histones from the chromosomes of elongating spermatids.

Concluding remarks and perspectives

The role of RNF8 in DDR and in spermatogenesis underscores the similarities between these diverse cellular events. RNF8-dependent histone ubiquitination is required for both biological processes, which are linked by the necessity for loosening histone–DNA interactions (Fig. 1). The only difference is that during DDR, RNF8-dependent histone ubiquitination regulates histone acetylation at DNA damage sites to induce local chromatin relaxation and potential local histone eviction; whereas during spermiogenesis, it is

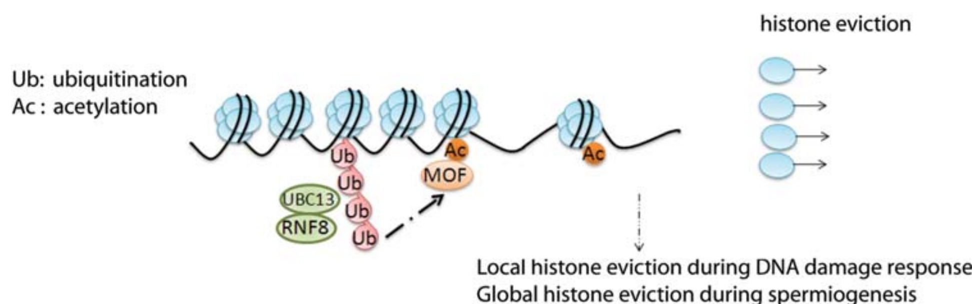

Figure 1 A model of RNF8-dependent histone eviction During DNA damage response or spermiogenesis, RNF8-dependent histone ubiquitination regulates histone acetylation and facilitates histone eviction.

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RNF8-dependent histone ubiquitination that mediates global histone acetylation, global chromatin relaxation, and global histone eviction. However, the molecular mechanisms underlying these two biological events are almost identical. It is possible that other histone ubiquitination-dependent biological processes, such as gene transcription, adopt a similar mechanism for chromatin remodeling. Certainly the importance of RNF8 *in vivo* is broader than originally expected, and suggests that many other factors may have more extensive roles than are currently known.

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