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Review

Histone Ubiquitination: An Integrative Signaling Platform in Genome Stability

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Complex mechanisms are in place to maintain genome stability. Ubiquitination of chromatin plays a central role in these mechanisms. The ever-growing complexity of the ubiquitin (Ub) code and of chromatin modifications and dynamics challenges our ability to fully understand how histone ubiquitination regulates genome stability. Here we review the current knowledge on specific, low-abundant histone ubiquitination events that are highly regulated within the cellular DNA damage response (DDR), with particular emphasis on the latest discovery of Ub phosphorylation as a novel regulator of the DDR signaling pathway. We discuss players involved and potential implications of histone (phospho)ubiquitination on chromatin structure, and we highlight exciting open questions for future research.

Highlights

Histones are heavily post-translationally modified by ubiquitin. Lately, many more ubiquitin-based modifications have been mapped on histones, which are far less abundant than the canonical ones but essential to safeguard genome stability.

Histones at damaged DNA can be modified by phospho-ubiquitin (i.e., pUb at Thr12), resulting in chromatin regions with new properties that impact DNA repair pathways.

The discovery of new ubiquitination sites on histones and new ubiquitin modifications expands dramatically the landscape of chromatin-based signaling and crosstalk, paving the way for future studies on novel mechanisms in genome stability programs (i.e., new writers, readers, and erasers).

The Complexity of Histone Ubiquitination Events

Histone ubiquitination differs substantially from the other histone post-translational modifications (PTMs) by small chemical groups because it entails the covalent binding of a 76–amino acid protein; that is, ubiquitination is the result of sequential actions of E1 activating, E2 conjugating, and E3 ligase enzymes, yielding the covalent conjugation of ubiquitin (Ub) to a lysine (Lys) residue on proteins, or on Ub itself to form different flavors of polyUb chains [1–3]. In parallel, deubiquitinating enzymes (DUBs) are responsible for the removal of these Ub marks [4]. The complexity of the Ub system was further increased by the discovery that Ub-like modifiers (UbLs) and PTMs target Ub to create an exponentially complex Ub code (Figure 1 and Box 1).

Histones are among the most abundant monoubiquitinated proteins, with 5–15% of H2A and 1% of H2B, leading to H2AK118/119ub and H2BK120ub, respectively. In recent years, it appeared that histones undergo many other Ub modifications, which are far less abundant than the canonical H2AK118/119ub or H2BK120ub, but recognized as essential players in orchestrating fundamental processes on chromatin [5] (Figure 2A). In this review, we examine the most recent data on histone ubiquitination, highlighting the impact of the recently identified noncanonical modifications in the context of genome stability. In the last part, we present and discuss the new concept of Ub phosphorylation and its effect on ubiquitinated chromatin, which promises to bring intriguing new directions for future research in chromatin and Ub biology.

Histone Ubiquitination in Response to Genotoxic Stress

Unscheduled alterations to the DNA structure, as a consequence of DNA damage, elicit a rapid chromatin response, promoting a variety of histone PTMs that are in place to react to and resolve the potentially harmful situation. These events are part of the DDR, a signaling cascade involving many factors able to sense the lesion, to halt cellular pathways (e.g., cell cycle, transcription), to repair the damage, and to resume cellular processes thereafter. Similar events occur in response to stress, such as during perturbed DNA replication or at dysfunctional telomeres, when DNA structure intermediates may become exposed and recognized as harmful DNA damage [6,7].

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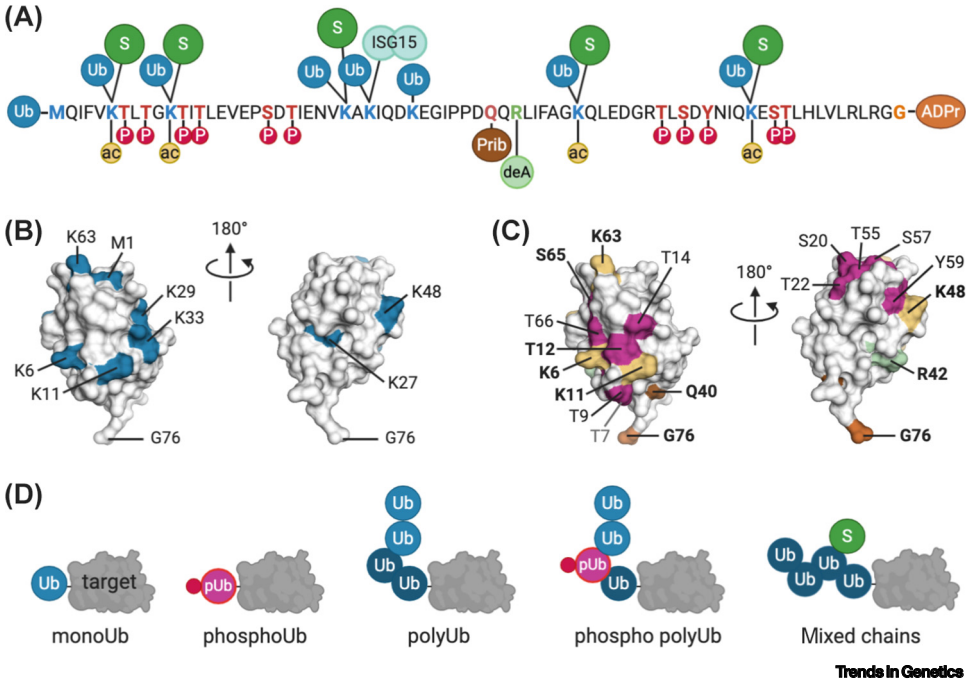

Figure 1. The Complexity of the Ubiquitin (Ub) System and Histone Ubiquitination. (A–C) Many residues within the Ub sequence are targeted by a variety of post-translational modifications, which include modifications by Ub (blue); by the UbL interferon-stimulated gene 15 (ISG15; light blue) and SUMO (S; green); and by small chemical groups, such as phosphorylation (P; pink), acetylation (ac; yellow), deamidation (deA; light green), ADP ribosylation (ADPr; orange), and phospho-ribosylation (Prib; brown). (D) Ub targets can be modified by one Ub moiety (monoubiquitination or mono-Ub) or by a chain of Ub molecules (polyubiquitination or polyUb). Ub can also form polyUb chains by mixing different linkages (dark and light blue circles) and UbL modifiers (mixed chains). An additional level of complexity is achieved by the conjugation of Ub in its modified forms (indicated as phosphoUb), adopting many possible combinations.

Histone ubiquitination is at the heart of the DDR, functioning as a signaling platform that integrates multiple inputs from inside and outside the cell. While the impact of histone H3 and H4 ubiquitination in the DDR is far from clear, the effect of DNA damage-induced ubiquitination of histones H2A, H2B, and H1 has been addressed in recent studies. Table 1 summarizes the currently known histone ubiquitination events and the involved players. Here we discuss the most validated mechanisms.

Ubiquitination of H2B

In mammals, histone H2B monoubiquitination (H2Bub) occurs at Lys120 (H2BK120ub; Figure 2) by the heterodimeric Ub ligases RNF20/RNF40. H2BK120ub is associated with transcription elongation, and it is also promoted by genotoxic agents and required for DNA double-strand break (DSB) repair [8–10]. Ub conjugation at this H2B site on the nucleosome was shown to interfere with the compaction of chromatin fibers *in vitro* [11]; therefore, it may promote chromatin opening at the sites of DNA lesions. This results in timely accumulation of DNA repair proteins at DSBs, suggesting a role for H2BK120ub early in the DDR before cells commit to the repair pathways [8–10]. Interestingly, this modification and its function in chromatin structure and in the DDR is conserved in yeast, where the E3 ligase Bre1 ubiquitinates Lys123 on H2B (H2BK123ub) [12].

In yeast, H2BK123ub has recently been linked to signaling during **DNA replication stress** (see Glossary) [13–16]. Cells expressing H2BK123R are sensitive to **hydroxyurea (HU)** treatment and show delays in **S phase** progression, suggesting a role of H2Bub in DNA replication

Glossary

Acidic patch: surface on the H2A-H2B dimer rich in acidic residues, used by many nucleosome-binding proteins to interact with the nucleosome.
ATP-dependent chromatin remodeler: ATP-dependent protein complexes responsible for nucleosome sliding on DNA and histone exchange or eviction.
DNA damage bypass and postreplication repair: DNA repair pathway acting in conjunction with DNA replication processes.
DNA end resection: nucleolytic processing of a DNA end to produce a long 3′ single-stranded DNA tail that can invade the homologous DNA strand, required for proper DNA repair via homologous recombination.
DNA replication stress: conditions that alter the DNA replication process, causing reduced or accelerated DNA replication fork progression.
DNA satellite repeat: large arrays of tandemly repeating noncoding DNA sequences, usually located in proximity of centromeres.
Fork reversal: process where replication fork regress in their progress and form intermediate structures where the two newly synthesized DNA strands anneal to each other.
Histone chaperone: histone-binding protein that shields histones from nonspecific interactions with DNA or other proteins.
Homologous recombination (HR): DNA repair pathway relying on DNA recombination between two homologous DNA templates and active mainly in S and G_2 phases of the cell cycle.
Hydroxyurea (HU): inhibitor of the ribonucleotide reductase enzyme, inducing decreased levels of deoxyribonucleotides (dNTPs), ultimately leading to replication fork stalling.
Liquid-liquid phase separation (LLPS): reversible process of a homogeneous fluid demixing into two distinct liquid phases: one condensed phase and one dilute phase. LLPS drives important biological processes, ranging from the formation of liquid condensates with no membrane enveloped (e.g., nucleolus and stress granules) to regulation of chromatin remodeling and signal transduction.
Nonhomologous end joining (NHEJ): DNA repair pathway relying on

Box 1. Histone Modifications by UbLs

UbLs closely resemble the Ub fold (Figure I), but due to their divergent sequence (indicated as percentage of homology compared with Ub), they exert specific functions when conjugated to substrate proteins. UbL conjugation requires specialized E1-E2-E3 enzymes, which generally function through mechanisms similar to the Ub enzymes [111]. Histone modification with UbLs has recently been described. SUMO [112–115], NEDD8 [116,117], and UFM1 [118,119] are conjugated to histones within the DDR and within other pathways. Histone SUMOylation has been linked to transcriptional repression and to genome stability. This modification is not site specific; it is possible that histones are targeted by SUMO during the DDR, as part of the 'protein group modification' mechanism that is characteristic of SUMO signaling in response to DNA damage [120].

Histone H4 and H2A NEDDylation has been linked to the DDR, with RNF111 and RNF168 as the responsible E3 ligases [116,117]. Finally, UFMylation of histone H4K31 acts as an amplification step for ATM signaling via the STK38 protein [119], thus playing an important role in genome stability [118]. Interestingly, this modification crosstalks to H3K9me3, again stressing the complexity of the Ub and its ramifications for other cellular signaling.

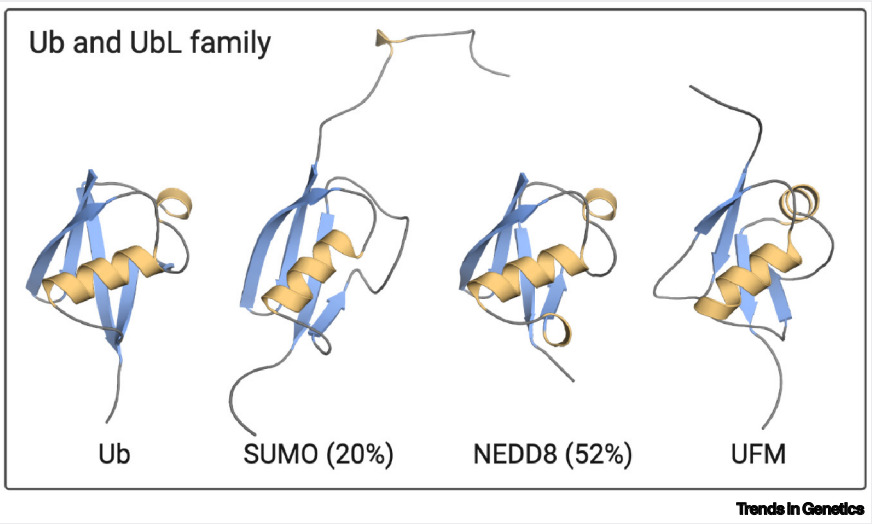

Figure I. Ub-like Proteins Involved in Histone Modification.

the direct ligation of two DNA ends with no or minor processing of the ends and active throughout the cell cycle (except during mitosis).

PARKIN: Ub ligase that targets proteins on mitochondria that have lost membrane potential, promoting the elimination of damaged mitochondria. Localization and activity of PARKIN are regulated through interaction with pUbS65.

Polycomb repressive complex 1 (PRC1): multisubunit vertebrate complex responsible for ubiquitination of H2AK118/119 and leading to repression of gene transcription.

Proliferating cell nuclear antigen (PCNA): the processivity factor for DNA polymerases at the leading and lagging strands of the fork.

S phase: phase of the cell cycle where DNA is replicated in preparation for cell division.

Single-strand annealing (SSA) repair: is initiated when a DSB is made between two repeated sequences. Single-stranded regions are created around the lesion such that the complementary strands can anneal to each other.

Stalled replication forks: DNA replication forks that are not progressing due to encounter of damage or the presence of a stress factor.

[17]. Importantly, genetic mutations of the E3 ligase Bre1 further support a role for H2Bub in genome stability [18]. Both in yeast and in humans, H2Bub recruits FACT (FAcilitates Chromatin Transaction) [19–21], a **histone chaperone** that governs chromatin dynamics during DNA replication. It is therefore possible that the reported phenotypes supporting the involvement of H2Bub in DNA replication mechanisms are directly or indirectly linked to FACT function in this process or in transcriptional mechanisms [22].

Interestingly, in contrast to a role for H2BK120ub in the DDR, data in human cells have recently shown that H2BK120 undergoes a switch from ubiquitination to acetylation when DSBs are induced by expression of a site-specific restriction enzyme [23]. This discrepancy may be explained by different sources of DSB induction, which may cause DNA damage in different genomic contexts, leading to different chromatin modification dependencies.

Ubiquitination at the C-Terminal Tail of H2A

The canonical H2AK118/119ub (Figure 2), catalyzed by the Ub ligase RING1A/B, is a highly abundant histone mark with central roles in transcriptional silencing by the **Polycomb repressive complex 1 (PRC1)**. Initially, this mark was thought to directly participate in the DDR signaling.

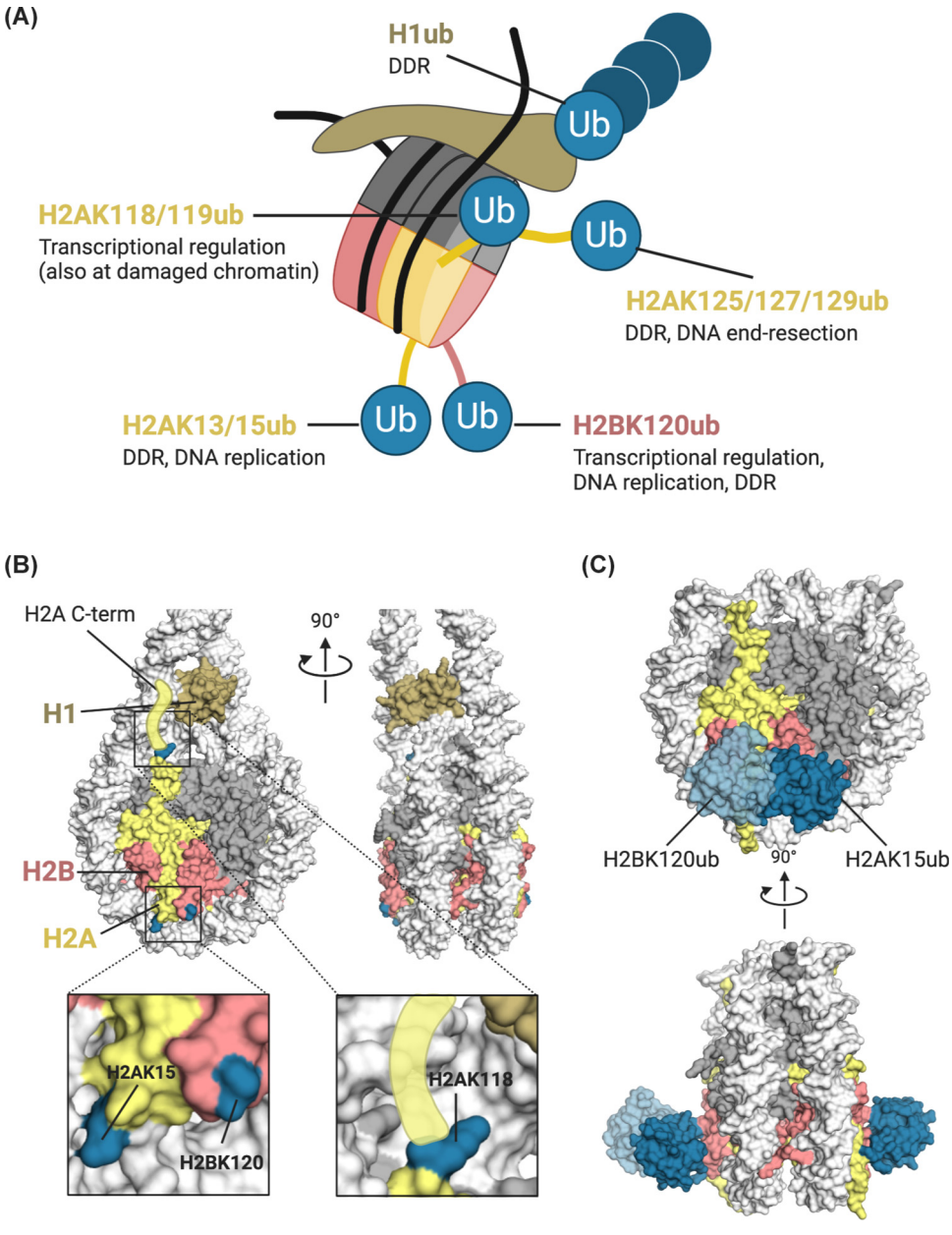

Figure 2. Roles of Different Histone Ubiquitination in DNA Damage Response (DDR). (A) Schematic representation of the H1-bound nucleosome carrying ubiquitinated histones H2A, H2B, and H1 linker. The specific sites at H2A and H2B and the functional effects are indicated. Histone H1 is modified by K63-linked polyUb chains (blue circles). (B) The nucleosome is composed of an $(H3\text{-}H4)_2$ tetramer (gray) and two H2A-H2B dimers (H2A in yellow, H2B in red), wrapped by 150 bp of DNA (white). H1 (in sand) interacts with the nucleosome at the dyad. H2A-H2B ubiquitin (Ub) conjugation sites are shown in dark blue. Canonical H2AK118/119 lie nearby the nucleosome dyad, preceding H2AK125/127/129 on the flexible C-terminal tail (not visible in the crystal structure, shown here with a schematic line). On the opposite sides of the nucleosome, H2AK13/15 are in close proximity to H2BK120. (PDB 5NL0). (C) Flexibility of the Ub moiety allows both H2AK13/15ub (dark blue) and H2BK120ub (light blue) to be present. However, how they can simultaneously signal in the DDR remains an open question. Superposition of H2AK15ub-NCP: PDB 5KGF, 53BP1 fragment not shown. H2BK120ub: PDB 6NJ9, DOT1L, and nucleosome not shown.

Table 1. Summary of Ubiquitination Events on Histones and Players Involved[a]

Histone	Target lysine	Ub(L)	E3	DUB	Reader	Crosstalk	Type	Process	PTMs	Refs
H2A	K13/15	Ub	RNF168	USP51, USP44, USP11, USP3	53BP1, RNF169, RAD18, BARD1	H2AK125/127/129ub	monoUb	DDR, DNA replication	pUbT12[b]	[37–44,75–77,83–86, 92,95,105–107]
	K118/119	Ub	RING1A/B (Polycomb repressive complex 1)	BAP1, USP16	RYBP, JARID2, ZRF1	H3K27me3	monoUb	Transcriptional regulation		Reviewed in [5]
	K125/127/129	Ub	BRCA1/BARD1	USP48	SMARCAD1	H2AK13/15ub	monoUb	DDR		[24–28,79–83]
	Not specified	NEDD8	RNF168					DDR (negative regulator)		[116]
H2B	K120 (K123 in yeast)	Ub	RNF20/40 (Bre1 in yeast)	USP22 (SAGA complex) (Ubp8 in yeast), USP51	RAD18, RNF169, DOT1L, COMPASS	H3K79me3, H3K4me3	monoUb	Transcriptional regulation, DNA replication, DDR		[8–18,59–74,106, 107,110]
H3	K14/18/23	Ub	UHRF1		DNMT1	DNA methylation	multi monoUb	Maintenance of DNA methylation during DNA replication		[47–49]
	K14	Ub	Cul4 (CRLC) in *Schizosaccharomyces pombe*		Clr4	H3K9me3	monoUb	Heterochromatin regulation		[50]
	K23/36/37	Ub	NEDD4		GCN5	H3K9ac	monoUb	Transcriptional regulation		[51]
	K121/122/125	Ub	Rtt101^{Mms1} in yeast and Cul4A^{DDB1} in human					Histone dynamics during DNA replication		[52]
	Not specified	Ub	Cul4-DDB-ROC1					DDR		[57]
H4	K91	Ub	BBAP (also known as Dtx3L)					DDR	ADPr[c]	[58]
	K31	UFM1	UFL1		STK38	H3K9me3		DDR		[118,119]
	Not specified	NEDD8	RNF111					DDR		[117]
	Not specified	Ub	Cul4-DDB-ROC1					DDR		[57]
H1	Not specified	Ub	RNF8				polyUb	DDR		[45]
	Not specified	Ub	HUWE1				monoUb	DDR		[46]

[a]DDR, DNA damage response; DUB, deubiquitinating enzyme; Ub, ubiquitin.
[b]Phosphorylation.
[c]ADP ribosylation.

This notion is now changing, where H2AK118/119ub may only appear after initiation of DDR signaling to silence transcription of the affected genomic region (reviewed in [5]).

More recent was the discovery that additional lysines on the C-terminal tail of H2A are targeted by the critical E3 ligase breast cancer–associated protein 1 (BRCA1), tumor suppressor and key mediator of DNA repair via **homologous recombination (HR)**. BRCA1 is an active E3 ligase only when in complex with BRCA1-associated RING domain 1 (BARD1) [24]. The activity of BRCA1 toward H2A was first demonstrated in the context of heterochromatin [25], where BRCA1 binds and silences **DNA satellite repeat** regions. An elegant rescue experiment using a chimeric H2A-Ub fusion determined a direct role for BRCA1-mediated H2A ubiquitination [25]. To match those findings, it has been demonstrated that BRCA1/BARD1 targets Lys125/127/129 on H2A [26–28]. This mark promotes **DNA end resection** as part of the HR repair pathway (discussed in the following text).

Ubiquitination at the N-terminal Tail of H2A

A milestone in understanding the role of histone ubiquitination in the DDR cascade was the identification of the two Ub ligases RNF8 and RNF168 as essential factors for reshaping chromatin around the genotoxic lesion (Figure 3, Key Figure). These ligases generate highly specific Ub-based docking sites at DNA lesions that trigger two distinct and counteracting DNA repair pathways to resolve the damage: HR and **nonhomologous end joining (NHEJ**; [29–34]; reviewed in [35]).

Although spreading of a DNA damage-induced phosphorylated form of the histone variant H2A.X (named γH2A.X) extends over megabases surrounding the lesion [36], histone ubiquitination appears confined in space. Initially, it was inferred that both RNF8 and RNF168, in association with the E2-conjugating enzyme UBC13, promote Lys63-linked polyubiquitination of H2A at the canonical site (i.e., H2AK118/119), respectively working as initiator (RNF8) and amplifier (RNF168) of the DDR-dependent chromatin remodeling. This assumption made it difficult to reconcile the high abundance of H2AK118/119ub with the need to locally and specifically regulate the DDR. The conundrum was solved with the discovery that these Ub ligases do not target the C-terminal tail of H2A. Rather, RNF168 specifically and efficiently targets Lys13 and Lys15 at the N-terminal tail of H2A (yielding H2AK13ub and H2AK15ub, also referred to as H2AK13/15ub; Figure 2) [37,38]. Differently from RNF8/UBC13, which were convincingly validated to promote Lys63 polyubiquitination of various proteins, RNF168 mediates primarily the monoubiquitination of H2A [38]. Interestingly, RNF168 activity depends on Lys27 of Ub (UbK27). Indeed, mutations of UbK27 impair the accumulation of DDR factors at lesion sites [39]. Whether UbK27 is required for the RNF168 activity or for ubiquitinating unknown targets is still unclear.

As master regulator of genomic stability, the DDR cascade is activated during telomere uncapping and at challenged DNA replication forks [7,40]. Importantly, RNF168 colocalizes with **proliferating cell nuclear antigen (PCNA)** in unperturbed S phase, and its activity on H2AK13/15 is required to prevent accumulation of **stalled replication forks** and **fork reversal** at difficult-to-replicate sequences. In support of a direct role for H2AK13/15ub in replication fork stability, electron microscopy experiments showed that nucleosomes regularly assemble at reversed forks [40].

RNF168 modifies histone H2A, as well as its variants H2A.X [38], H2A.Z, and macroH2A1/2 at the corresponding N-terminal lysines [41]. RNF168 activity depends largely on the **acidic patch** residues on the H2A-H2B dimer, which functions as a docking site for the its RING domain [42–44]. Lysine specificity for H2AK13/15 is achieved by an additional negative residue on H2B in proximity to the acidic patch that directs RNF168 to the N-terminal lysines of H2A [44].

Key Figure

The Regulation of DNA Damage Response (DDR) by Ubiquitin (Ub) and pUbT12

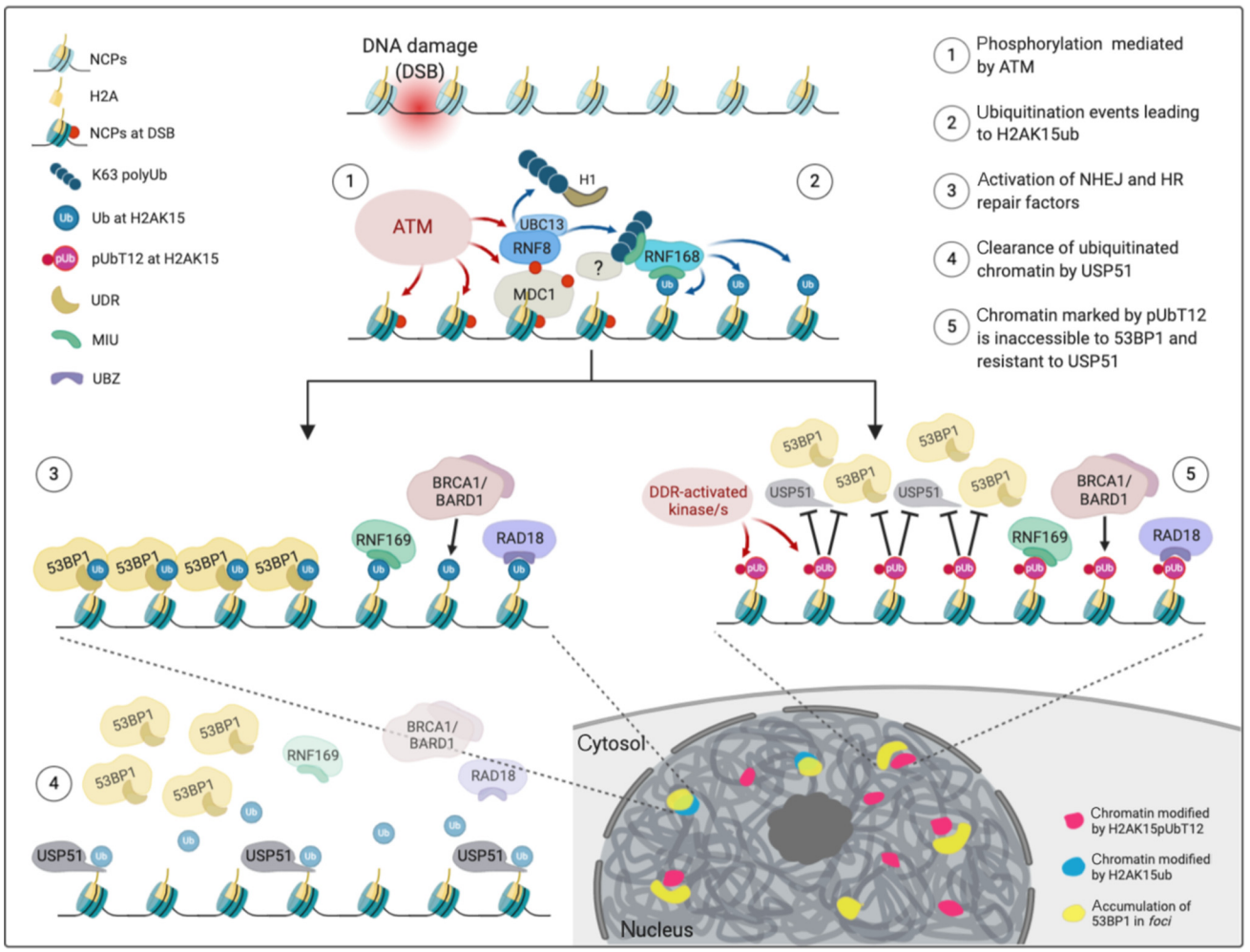

Figure 3. Formation of DNA double-strand breaks (DSBs) leads to the recruitment of the MRN (MRE11/RAD50/NBS1) complex (not shown) and activation of the ataxia-telangiectasia mutated (ATM) kinase (1), which induces the phosphorylation of several factors on chromatin (red circles), including histone H2A.X (named γH2A.X). γH2A.X directly recruits MDC1 (mediator of DNA damage checkpoint protein 1), which in turns allows recruitment of the ubiquitinating complex RNF8/UBC13 and the subsequent formation (or elongation) of Lys63 Ub chains around damage. Targets of RNF8/UBC13 are the linker histone H1 and an as yet unidentified factor on chromatin, which allows the recruitment of the second Ub ligase RNF168, by means of its three Ub-binding domains (UBDs) UMI (UIM- and MIU-related UBD; not shown) and MIUs (motif interacting with Ub). RNF168 ubiquitinates the N-terminal tail of histones belonging to the H2A family at Lys13/15 (indicated only as H2AK15 for simplicity) (2), leading to the activation of both NHEJ (53BP1) and HR (BRCA1/BARD1, RNF169, and RAD18) pathways (3). H2AK15ub is directly recognized by specific UBDs, 53BP1 via UDR, RNF169 via MIU and RAD18 via UBZ, while the mechanism for BRCA1/BARD1 is still unclear. Clearance of Ub from chromatin and termination of the DDR is promoted by USP51 (4). Phosphorylation of Ub at Thr12 (pUbT12) conjugated to H2AK15 (H2AK15pUbT12) depends on the apical DDR kinases and occurs in a subset of chromatin regions, from which 53BP1 is specifically excluded while accumulation of other DDR factors is allowed (5). pUbT12 inhibits the activity of USP51, leading to prolonged chromatin ubiquitination.

In contrast with H2Bub and its associated factors, the E3 ligases and the DDR-related Ub marks on H2A are found only in vertebrates, raising the question whether H2AK13/15ub or H2AK125/127/129ub occurs in, for example, yeast or fly cells. This places the H2Aub-dependent

mechanisms as a recent evolutionary addition to the DDR, opening interesting questions on the origin of these signals in vertebrates.

Ubiquitination of the H1 Linker Histone

Different from RNF168 and BRCA1, the lysine targets of RNF8 on chromatin are still unresolved. The RNF8/UBC13 complex was reported to extensively ubiquitinate the H1-type linker histone upon DSBs, proposing polyubiquitinated H1 as a platform on chromatin for RNF168 recruitment [45]. Later, this concept was extended by showing that, upon UV-induced DNA damage, the Ub ligase HUWE1 primed ubiquitination of histone H1 at multiple lysines to allow further chain elongation by the RNF8/UBC13 [46]. Therefore, it remains unclear whether RNF8 acts on histones directly or functions primarily in extending Lys63 Ub chains on ubiquitinated substrates. This would be in line with early observations that RNF8/UBC13 is a highly effective ubiquitinating pair with little target specificity [38].

While the ubiquitination of H1 upon DNA damage has been clearly demonstrated by these studies [45,46], the hypothesized role for RNF168 as a docking site remains controversial. Interestingly, a recent ChIP-based genome-wide analysis of chromatin features at DSBs shows that H1 is evicted from chromatin [23]. A time-resolved and mechanistic analysis of RNF8-dependent ubiquitination in relation to the recruitment of RNF168 at DSBs would greatly clarify the involvement of RNF8 and the role of H1 in the DDR.

Ubiquitination of H3 and H4

Histones H3 and H4 are also targets for Ub conjugation; yet, their direct involvement in the DDR cascade remains unclear. One well-characterized H3 ubiquitination event occurs on the N-terminal tail at Lys14/18/23, catalyzed by the E3 ligase UHRF1, and then recognized by the DNA methyltransferase DNMT1. The Ub-DNMT1 interaction stimulates the methyltransferase activity of DNMT1, a key step in the maintenance of DNA methylation patterns after DNA replication [47–49].

Work in *Schizosaccharomyces pombe* has found that H3K14ub also stimulates the activity of the methyltransferase Clr4, responsible for the silencing histone mark H3K9me2/3. Interestingly, the Cul4-dependent E3 ligase responsible for H3K14ub is part of the Clr4 methyltransferase complex (CLRC) [50]. Similarly, in human cells, Lys23/36/37 on the H3 tail are ubiquitinated by the E3 ligase NEDD4 in response to glucose stimulation; this mark further recruits GCN5 to stimulate acetylation of H3 Lys9 and Lys14 [51]. These works provide multiple examples of Ub-dependent crosstalk on chromatin between histone PTMs.

Ubiquitination of the C-terminal Lys121/122/125 on H3 or its centromeric variant centromere protein A (CENP-A) have also been reported [52–55]. This modification occurs outside of the chromatin context (i.e., on histones that are not yet incorporated into nucleosomes), consistent with the H3K121/122/125 residues being located in a critical interface for nucleosome formation [56]. These marks regulate **histone chaperone**–mediated pathways that are essential for genomic stability through the cell cycle.

In addition to these modifications, promiscuous ubiquitination of H3 and H4 [57] and the ubiquitination of H4K91 [58] have been reported to occur in response to DNA damage. The mechanistic details of these modifications and their downstream effects still require further investigation.

Readers of Histone Ubiquitination and Crosstalk with Other Histone Modifications

Ub signaling relies on reader molecules that mediate downstream events to the Ub modification. Not surprisingly, Ub-binding domains (UBDs) are present on many DDR factors, and their

involvement in the pathway is being investigated. Interestingly, many of the readers of ubiquitinated histones also contain binding domains for other histone modifications, or they contain catalytic domains that promote additional PTMs on histones (reviewed in [20]). This creates a network of signaling moieties on chromatin that interplay to ensure proper responses to genotoxic stresses.

Crosstalk of H2Bub and H3 Methylation

H2Bub acts as a binding platform for many factors associated with transcription, post-transcriptional RNA modifications, and DNA replication and repair [59]. As such, this mark crosstalks to several other histone PTMs.

Perhaps the most well-known crosstalk involving histone ubiquitination is between H2Bub and H3 methylation. H2Bub stimulates histone methyltransferase activity of DOT1L on H3K79 and SET1 on H3K4 [60–64], leading to increased H3K79me3 and H3K4me3 [65]. This crosstalk is conserved from yeast to humans. Recent studies of the DOT1L enzyme bound to a nucleosome carrying H2BK120ub have elucidated the structural and mechanistic features of this activation [65–69]. DOT1L binds Ub at the Ile36 hydrophobic patch, and it binds the nucleosome on the acidic patch. The histone H4 tail is further involved in positioning DOT1L and remodeling the H3K79 residue for catalysis, explaining the mechanism by which DOT1L accesses H3K79, which is located in the histone core rather than on the flexible tails.

Structural details for the mechanism of SET1 recognition of nucleosomes containing H2Bub are emerging [70,71]; however, the basis for activation of the methyltransferase activity are still unclear. The multisubunit organization of the SET1 complexes and the location of H3K4 on the flexible H3 tail suggest that distinct mechanisms may be in place for this ubiquitination–methylation crosstalk involving H2Bub. How this crosstalk influences the DDR locally is still a subject of investigation.

Interestingly, H2Bub levels are affected by the presence of other histone PTMs on the nucleosome or by the presence of histone-modifying enzymes [65,72–74]. These observations underscore the bidirectionality of histone PTMs crosstalk. The local balance between these interplays will determine local chromatin signatures that control the DDR and many other signaling cascades.

Readers of H2A Ubiquitination at K125/127/129

The BRCA1/BARD1-dependent ubiquitination at K125/127/129 on H2A recruits the **ATP-dependent chromatin remodeler** SMARCAD1 [27]. Indeed, SMARCAD1 accumulation at damaged sites is reduced upon mutation of its UBD or upon BARD1 depletion. Upon recruitment, SMARCAD1 promotes sliding or eviction of H2AK125/127/129ub-containing nucleosomes. This results in the mobilization of 53BP1 and the completion of DNA end resection as part of the HR repair pathway [27]. Importantly, the DUB that hydrolyzes H2AK125/127/129ub, USP48, may also function as a reader of the same mark. Indeed, USP48 activity is stimulated when a Ub moiety is present on the nucleosome (i.e., in the presence of multiple monoUb marks on H2A) [28]. This indicates that BRCA1-dependent histone ubiquitination promotes DNA repair by promoting changes in chromatin architecture, and, surprisingly, it may control its own dynamics by activating the deconjugation machinery.

Readers and Consequences of H2A Ubiquitination at K13/15

The first identified reader of the H2AK15ub mark is the Ub-dependent recruitment (UDR) motif of p53 Binding Protein 1 (53BP1) [75–77], key factor of the NHEJ repair pathway, providing the

molecular basis of RNF168-dependent recruitment of 53BP1 to damaged chromatin [32–34]. Accumulation of 53BP1 on chromatin depends not only on H2AK15ub but also on the dimethylation of Lys20 on H4 (H4K20me2) [78], a mark that is broadly distributed across the genome, enriched in prereplicative chromatin, and diluted after DNA replication [79,80]. This bivalent interaction allows highly specific recruitment of 53BP1 at DNA damage sites (via H2AK15ub) on nonreplicated genomic regions (via H4K20me2), explaining how NHEJ is preferred in the G_1 phase of the cell cycle. In turn, this raised the question about the readers of H2AK13/15ub in regions depleted of H4K20me2. This was recently solved by the finding that BARD1, the protein partner of BRCA1 in HR, interacts with H4K20me0, explaining why HR repair mechanisms are strongly promoted in S and G_2 phases of the cell cycle in human cells [79,81,82]. Very recently, BARD1 has also been suggested to recognize H2AK15ub in human cells [83], highlighting a possible Ub reader–writer crosstalk between N- and C-terminal H2A Ub marks, which should be further explored and characterized.

H2AK13/15ub is also directly bound by other DDR factors: RAD18, RNF169, and RNF168 itself [77,84–86]. RAD18 has a well-established role in promoting PCNA ubiquitination at DNA replication stalled forks, in **DNA damage bypass and postreplication repair** [87–89], but it is also recruited at the DSBs in an RNF8-dependent manner to facilitate HR by directly binding the recombinase RAD51C [90]. Similarly, RNF169 has been involved in HR by promoting DNA end resection and **single-strand annealing (SSA) repair** [91]. Interestingly, RNF169 and RAD18 bind H2AK13/15ub with much higher affinity than 53BP1, explaining why 53BP1 relocation at the DNA damage site is terminated by RNF169 and RAD18 [77,85,92]. Conversely, RNF168 affinity for H2AK13/15ub is low and similar to that of 53BP1, thereby amplifying the spread of H2AK13/15ub across damaged chromatin and allowing the recruitment of 53BP1 [77].

Notably, both histone marks – H2AK13ub and H2AK15ub – are recognized by RNF168, RNF169, and RAD18, and it was shown that the two modifications can, in principle, coexist on the same molecule (i.e., H2AK13/15ub2) [37,77], opening new possibilities in terms of recognition of this 'bidentate' Ub site. Similar questions remain open about the coexistence of H2AK13/15ub with H2BK120ub, due to their close proximity on the nucleosome (Figure 2B,C).

Overall, H2AK13/15ub is central to the DDR pathway ubiquitination by triggering the activation of opposing DNA repair pathways, HR (i.e., BRCA1, RAD18, RNF169) and NHEJ (i.e., 53BP1). The molecular events occurring at the chromatin level and responsible for shifting the balance in favor of one or the other DNA repair pathway have recently emerged. A suggested mechanism involves the acetylation of H2AK15 by the NuA4/TIP60 acetyltransferase complex, which may prevent the ubiquitination of H2AK15 by RNF168 [93]. An additional determinant for the regulation of HR over NHEJ is represented by the very recent discovery of Ub phosphorylation as a fine regulator of signaling at damaged chromatin [94] (Figure 3).

Ub Phosphorylation as Novel Determinant of DNA Repair Pathways

In recent years, several mass spectrometry studies revealed that Ub can be modified by small chemical groups, representing a major breakthrough in many fields (Figure 1; reviewed in [2,3]). Despite the huge interest, to date, only very few PTMs on Ub have been functionally characterized [95–98]. The first detailed characterization is represented by Ub phosphorylation at Ser65 (pUbS65), a key event in the activation of the Ub ligase **PARKIN** and the regulation of mitochondrial metabolism [97,99,100]. Several reports showed that pUbS65 results in a wide range of effects for substrate conjugation/deconjugation and downstream signaling [101–103].

By exploring the effect of different phosphoinhibitory Ub mutants on chromatin ubiquitination, Walser *et al.* identified and characterized a novel phosphorylation site on Ub at Thr12 (pUbT12) as a regulator of the DDR [94]. Following DNA damage, pUbT12-positive nuclear foci are induced in a manner dependent on DDR kinases (i.e., ATM/ATR/DNAPK) and RNF168 (Figure 3). pUbT12 marks H2AK13/15 (i.e., H2AK15pUbT12) and is functionally relevant because chromatin regions decorated by pUbT12 specifically prevent 53BP1 recruitment but are permissive to BRCA1/BARD1, RAD51, and RNF169. Mechanistically, pUbT12 impedes 53BP1's UDR to recognize ubiquitinated nucleosomes at H2AK15, while RNF169's MIU retains the binding. These findings position H2AK15pUbT12 as a new modification in the landscape of damaged chromatin and open the way for investigations into the unexpected further complexity of Ub PTMs in the DDR pathway.

Histone Deubiquitination in the DDR: The Case of USP51

A balance between Ub conjugation and deconjugation defines the timing and specificity of Ub signaling in the DDR and in all other Ub-dependent pathways [4]. Several DUBs, the enzymes that remove Ub from its substrates, have generally been associated with the DDR pathway, based on the phenotypes observed upon depletion or mutation of these proteins. However, the specificity of DUBs (listed in Table 1) is generally less clear than for E3 ligases. Indeed, most 'histone' DUBs have been shown to act on a variety of substrates, and where and how these DUBs directly function in the DDR cascade remain largely unclear. The current challenge is to disentangle indirect cellular effects from direct activities on substrates. Here, we discuss the case of USP51, in view of its latest and exciting findings, and refer elsewhere to the description of DUBs' activity in the DDR [5,104].

Many DUBs have been implicated in the removal of H2AK13/15ub from damaged chromatin to terminate the DDR signaling. However, only USP51 has clearly been shown to have direct activity *in vitro* toward H2AK13/15ub. Despite initially being considered selective for the N-terminal sites of H2A [105], recent studies demonstrated that USP51 does not show preference for H2AK13/15ub over H2AK119ub and that it can also target nucleosomes carrying H2BK120ub [106,107], making USP51 a central DUB in the dynamics of Ub events on chromatin and together raising questions about its (histones and sites) specificity.

Intriguingly, USP51 activity toward histones is severely impaired by modifications of Ub at Thr12 [94] (Figure 3). The possible molecular explanation resides on the USP51/Ub interaction surface. DUBs belonging to the USP (Ub-specific protease) family contact Ub via a surface comprising Thr12 of Ub [108]. Therefore, modifications on this site, such as pUbT12, likely interfere with the direct contact of USP51 with Ub, impairing its deubiquitinating activity. This may result in the persistence of H2AK15pUb on chromatin and a consequent prolonged local inhibition of 53BP1, with implications in the balance between HR and NHEJ. Also, the expression of noncleavable UbT12 mutants in cells revealed that the H2AK15ub mark is far more frequent than previously inferred, even in the absence of exogenous genotoxic stress [94]. These cleavage-resistant UbT12 mutants could represent a powerful tool to address specific questions on the dynamics of Ub-based histone marks.

Concluding Remarks

Over the past decade, considerable progress has been made in understanding the mechanisms by which the combination and complexity of histone modifications determine cell behavior and fate. Ubiquitination is at the center of these mechanisms, occurring on all histone types and regulating most nuclear signaling pathways. Among others, ubiquitination events on histone H2A are leading the way for their specificity and known involvement in response to genotoxic

Outstanding Questions

How do ubiquitinated histones directly affect nucleosome and chromatin structure? How do they affect histone chaperones and ATP-dependent chromatin remodelers?

What are the functional implications linked to the evolution of H2Aub signaling in the DDR for vertebrates?

Are there additional PTMs on Ub/UbLs involved in the regulation of genome stability? How is the crosstalk regulated and what is its functions?

What is the effect of Ub phosphorylation (or other PTMs) on nucleosome stability and on the biophysical properties of chromatin?

Are there additional targets of pUbT12 on chromatin (other sites and/or histones) or on chromatin-associated proteins, which are functionally relevant?

What are the specific chromatin regions marked by H2AK15pUbT12, as opposed to H2AK13/15Ub? How is this distinction controlled?

What are the writers, readers, erasers of pUbT12, and how are they regulated in the DDR?

stress. Further studies are needed to pinpoint when and where H2Bub acts in the DDR, considering its widespread presence on chromatin. Moreover, due to its close localization to H2AK13/15 on the nucleosomes (Figure 2), it will be important to unravel how specificity is ensured both for the Ub enzymes E2, E3s, and DUBs and in the downstream signaling.

The recent identification of Ub phosphorylation on H2A significantly expands the histone code, representing the first phosphoUb–based epigenetic mark that reveals unforeseen complexity in the chromatin landscape and promises to offer novel mechanisms regulating the highly ordered structure of chromatin (Box 2). A fascinating new concept in chromatin biology is that **liquid-**

Box 2. Dynamics, Recognition, and Clearance of (H2AK15)pUbT12

Ub is a small globular protein highly conserved throughout evolution, and modification of Ub by small chemical groups affects surface charge and structure properties, often resulting in functional alterations of its conjugation, deconjugation, and interaction activities. The discovery of pUbT12 as regulator of the DDR opens different possible scenarios regarding the writers, readers, and erasers of phospho-ubiquitinated chromatin (Figure I).

Writers: pUbT12 is induced by DNA damage, but whether the phosphorylation occurs on free Ub or rather on chromatin at regions already marked by H2AK15ub is still unknown. RNF168 is able to conjugate pUbT12 *in vitro* [94], making it a possible phospho-Ub writer. However, it is likely that the chromatin context is important to ensure spatial and temporal specificity of the signal, pointing toward the existence of Ub kinases that selectively operate on damaged chromatin.

Readers: pUbT12 prevents the recognition of specific types of UBD, such as 53BP1's UDR, but is permissive to the RNF169 (and likely to RNF168) because it can bind H2AK15ub, regardless of the phosphorylation of Ub [94]. The next compelling challenge will be to assess the existence of a new class of binding motifs – referred to as pUb binding domains, or PUBs – that may specifically recognize pUb.

Erasers: One of the major histone DUBs, USP51, is unable to remove Ub from chromatin when modified (i.e., phosphorylated or mutated) at Thr12 [94], suggesting an additional regulatory step in the DDR cascade. Which are the mechanisms/players and dynamics of pUbT12 removal from chromatin? This may depend on the local activity of phosphatases (PPase) acting on Ub (referred as Ub PPase) and/or on the presence of Ub proteases that are either insensitive to pUbT12 modification (such as the viral protease LB^{pro}) or even specific for pUbT12.

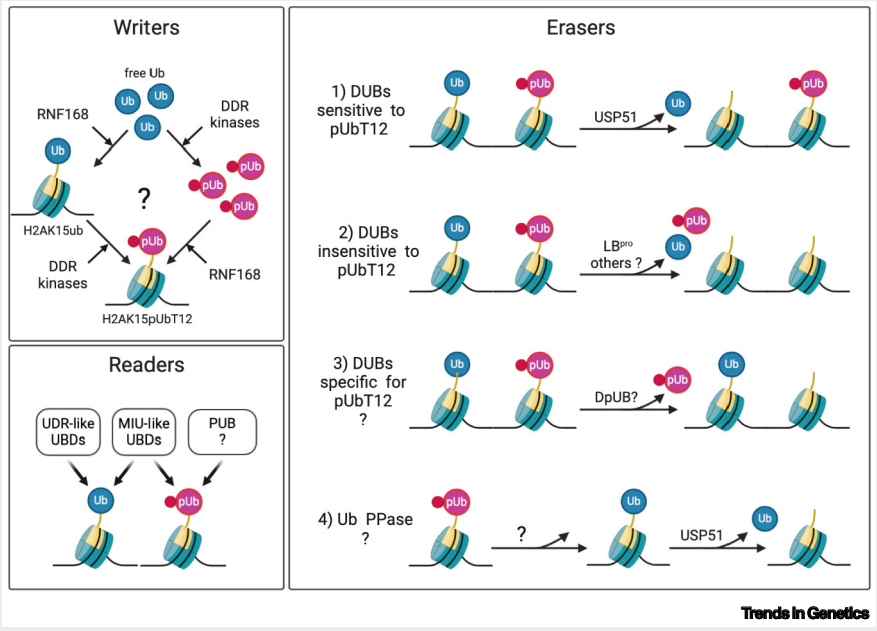

Figure I. Possible Modes of Action of Writers, Readers, and Erasers of pUbT12.

Box 3. Challenges in Studying Noncanonical Ub-Based Histone Modifications

Despite the relevance of noncanonical histone ubiquitination in key biological processes, the understanding of their complexity, abundance, and crosstalk with other modifications are still limited. One of the reasons for this elusiveness is common to both systems and relies on their complex gene regulation and high conservation throughout evolution. Ub is encoded by different genes in mammalian cells: two genes, *UBA52* and *RPS27A*, encode Ub fused to ribosomal subunits, whereas *UbB* and *UbC* code for three and nine tandem-repeat, head-to-tail Ub units, respectively. The sole family of histone H2A (not including the histone variants) is organized in 15 gene copies, and histone H2B is coded by 23 different genes, all organized into clusters that are transcribed and translated via noncanonical mechanisms. Such a complexity makes the genetic manipulation of Ub and histones H2A/H2B very arduous and, even when modulation of their expression and simultaneous replacement with mutants are possible, as in the case of Ub [94], it often results in extreme toxicity that severely limits the number of possible functional phenotypes that can be followed in cells.

Another important issue relies on the limited detectability of these modifications in cells, attributable to multiple reasons. First, they are relatively rare compared with canonical histone ubiquitination events (occurring at the C-terminal tails of H2A and H2B), which regulate gene expression and are diffused across the genome. Indeed, noncanonical histone ubiquitination usually occurs in defined chromatin regions and entails highly specific functions (in DNA replication- and DDR-related events) with tightly controlled kinetics. Exemplary of this is the activity of RNF168 on damaged chromatin at H2AK15, which is essential for promoting DNA repair, though it is far less abundant than other histone PTMs.

Experimental observation of these modifications also suffers from the lack of specific and validated tools to monitor these marks in physiological conditions. The complication in generating and successfully using these reagents resides in (i) the high degree of conservation of the epitopes (Ub and histones), which renders particularly difficult the generation of specific antibodies; (ii) the crowded landscape of PTMs found on these small proteins; and (iii) the high number of factors accumulating at these sites, which may hinder epitope recognition. Inadequacy of detection tools can result in simplistic interpretation of the complexity of the system; hence, efforts in developing high-quality reagents should be encouraged to reach unambiguous conclusions on important biological phenomena.

liquid phase separation (LLPS) enables the formation and maintenance of distinct chromatin compartments [109] and is implicated in stimulating histone (i.e., H2B) ubiquitination [110]. What would be the impact of Ub phosphorylation on chromatin organization and whether occurrence of pUbT12 (and/or other Ub PTMs) on ubiquitinated histones may locally alter chromatin properties, interfering with the compartmentalization of specific nuclear processes, are compelling questions that need to be addressed.

Predictably, many more PTMs on Ub, and possibly on other UbLs, can affect the multitude of sophisticated events tuning chromatin dynamics. Future investigations and the development of new specific tools and methods (Box 3) are urgently required to monitor and understand the growing complexity of the integration/crosstalk of these Ub/UbLs PTMs (see Outstanding Questions).

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