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Histone ubiquitylation and chromatin dynamics

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1. ABSTRACT

Histones are subject to several post-translational modifications, which act to regulate gene expression and other processes on the DNA template. One such modification is the addition of a single ubiquitin moiety, which has been reported to influence chromatin dynamics and exhibit cross-talk with other histone modifications. Mono-ubiquitylation of H2B has been reported in eukaryotes as divergent as budding yeast, flies and humans, and is linked to transcriptional activation and gene silencing. Furthermore, ubiquitylation of H2A is also important for transcriptional repression in higher eukaryotes, and both histones play key roles in DNA repair. In this review, we will give an overview of the enzymes important for ubiquitylation and deubiquitylation of the various histone species, before examining the role of ubiquitylated histones in shaping the chromatin landscape and thus controlling the accessibility of DNA to effector proteins, through putative roles in promoting histone-histone interactions and stabilizing the structure of nucleosomes. We will finally discuss other processes reported to involve ubiquitylation of histones, including DNA repair, recombination and mRNA processing, underlining the diverse actions of these modifications.

2. INTRODUCTION

Eukaryotic DNA exists in complex with histone proteins, forming nucleosomes, the fundamental units of chromatin. The basic nucleosome structure consists of a histone octamer (a tetramer of H3-H4 histones, flanked by two dimers of H2A-H2B) around which is wrapped 147 base pairs of DNA. In addition, a linker histone, H1, associates with the nucleosome and protects inter-nucleosomal DNA located at the nucleosome entry point (see Figure 1) (for recent reviews see references (1, 2)). Histones are the targets of extensive post-translational covalent modifications; acetylation, lysine and arginine methylation, phosphorylation, SUMOylation, ADP ribosylation, proline isomerization, biotinylation, citrullination and ubiquitylation (3, 4). While many of these modifications are targeted to the N terminus or to the histone fold domain (4), ubiquitylation is targeted to the C terminus (5) (sites of mono-ubiquitylation in histones are summarized in Figure 1). These modifications act to regulate gene expression and other processes, and function both through recruiting additional proteins and by altering nucleosome stability and structure (2, 6-9). For example, acetylation of lysine residues on various histones weakens their association with DNA, and acetylation of K16 of H4 inhibits formation of the chromatin 30 nm fiber (3). This is

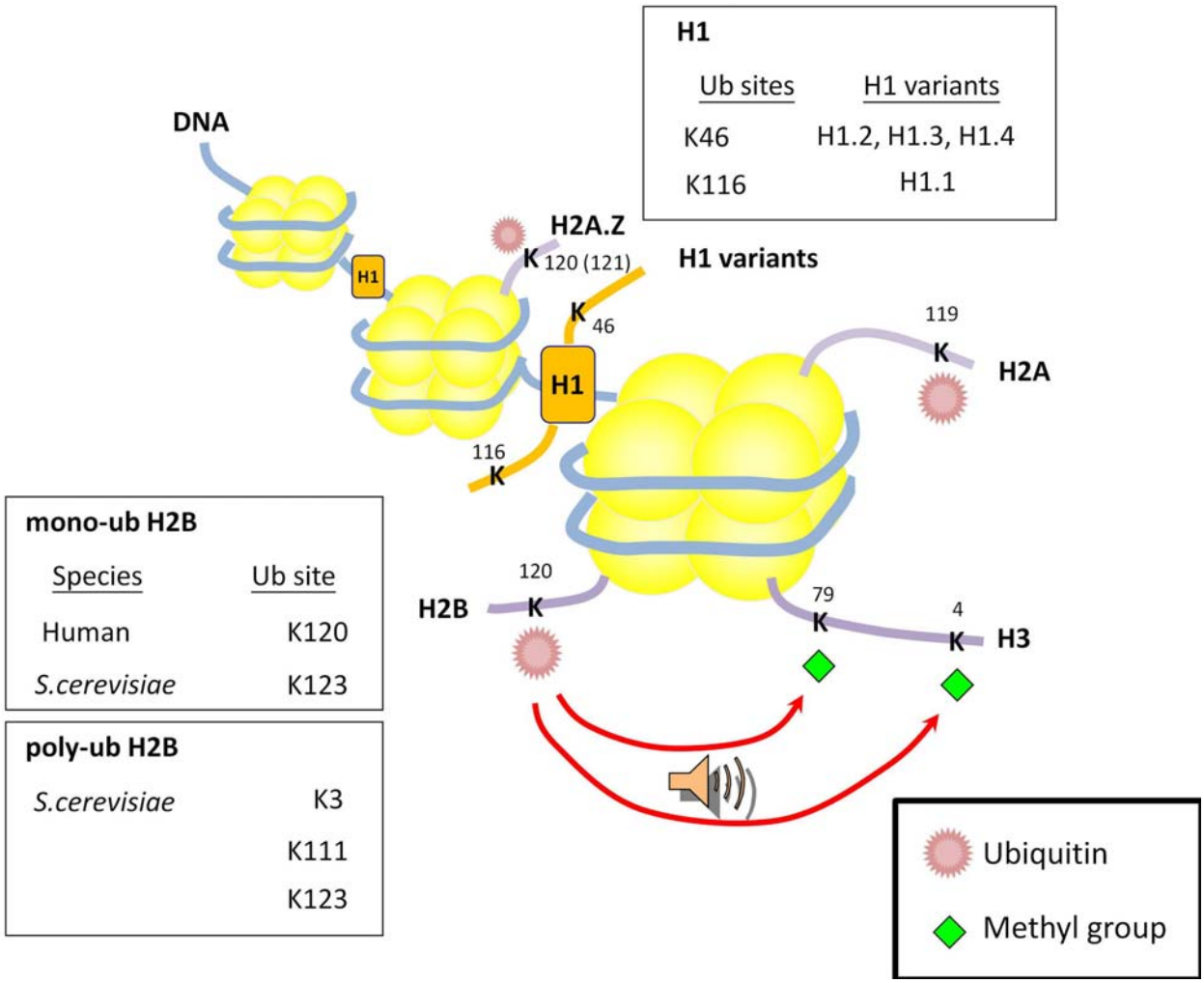

Figure 1. The nucleosome consists of 147 base pairs of DNA (represented by the blue line) wrapped around a histone octamer - two dimers of H2A-H2B flank a tetramer of H3-H4 (the yellow circles each represent a single histone). The histones bind one another through their histone fold domains, forming the nucleosome's globular core. Carboxy and amino terminal tails protrude from the globular domains (indicated by the purple lines), and these are subject to post-translational modifications, including mono-ubiquitylation and lysine methylation, at the amino acid residues indicated (numbers above lysine (K) residues). H2B is mono-ubiquitylated in both budding yeast and humans, and the position of the ubiquitylated lysine is indicated in the upper left-hand box. Lysine residues revealed to be poly-ubiquitylated are listed in the lower left-hand box. Ubiquitylation of H2BK120/yeast H2BK123 has downstream effects on the methylation of H3K4 and H3K79 (indicated by the red arrows). The linker histone H1 (shown in orange) also associates with the nucleosome, and is important for the regulation of higher order chromatin structure. Certain variants of H1 have been observed to be mono-ubiquitylated at specific lysine residues, as indicated in the right-hand box. The histone H2A variant, H2A.Z, is also mono-ubiquitylated, the majority of which is reported to occur at K120, but K121 also appears to be an alternative, minor target.

hypothesized to destabilize the nucleosome, predisposing it to targeting by chromatin remodeling factors, thereby enabling transcriptional initiation (3).

Ubiquitin is a 76 amino acid polypeptide of about 8.5kDa, and its C terminal glycine can be ligated to the epsilon amino group of certain lysine residues of proteins via an isopeptide linkage (10, 11). The ability of ubiquitin to form polymers enables the poly-ubiquitylation of proteins, and this is associated with protein degradation via the 26S proteasome (11). The addition of a single ubiquitin moiety attached at one or multiple lysine residues is critical for the priming of poly-ubiquitylation. However, mono-ubiquitylation itself is known to be biologically relevant (1, 11, 12). Approximately 5-15% of total H2A, and 1-10% of total H2B have been reported to be ubiquitylated in a number of higher eukaryotes, whilst other ubiquitylated histones are less prevalent (13, 14). Histone ubiquitylation is a dynamic process, through its reversal by ubiquitin proteases, and this is critical for the regulation of a number of processes (10, 13, 15, 16). Roles for mono-ubiquitylated histones are diverse, ranging from an implied involvement

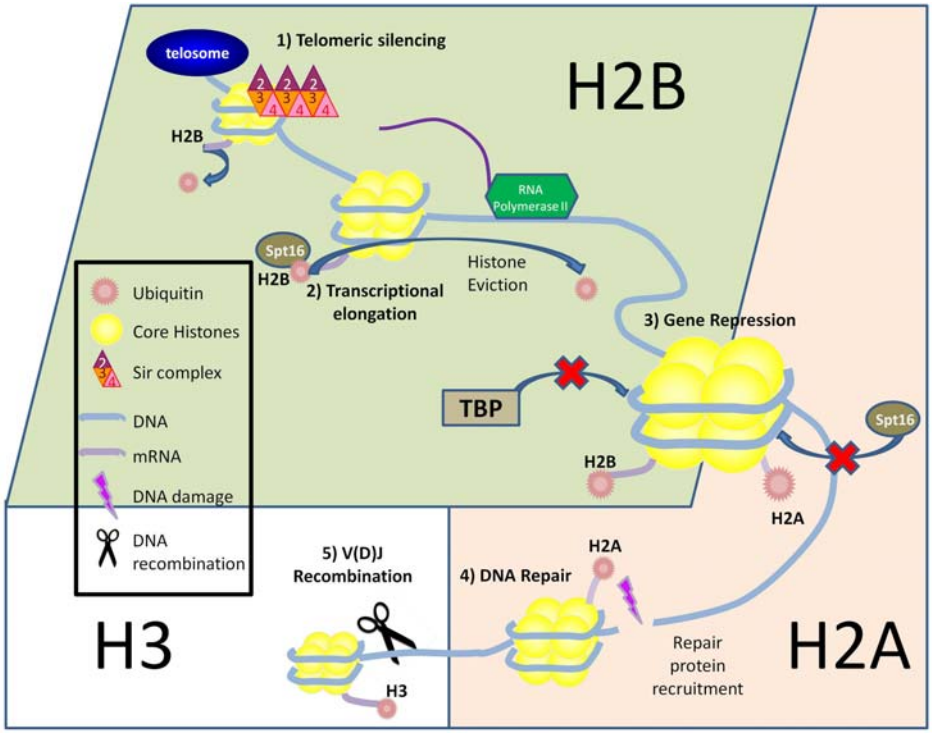

Figure 2. Summary of the known biological roles of ubiquitylation of histones (yellow circles) H2B, H2A and H3 on the DNA template (shown in blue). Deubiquitylation of H2B.Ub1 is required for the establishment of silencing at the telomere (1), thereby allowing the binding of silencing factors (triangles), and both ubiquitylation and deubiquitylation are important for transcriptional elongation (2) (mRNA shown in purple), as they are believed to be required for Spt16 binding and nucleosome eviction respectively. H2B.Ub1 and H2A.Ub1 are both implicated in the repression of specific genes (3), through preventing the recruitment of TATA-binding protein (TBP) and Spt16 respectively. H2A is also involved in DNA repair (4), as its ubiquitylation is important for the recruitment of repair proteins. The ubiquitylation of H3 is reported to be important for V(D)J recombination (5), through an as yet undelineated mechanism.

for the linker protein H1B in the HIV-1 resistance phenotype exhibited by HRF(+) cells (17), to the control of flowering time by ubiquitylated H2B in *Arabidopsis thaliana* (18-20). Regulation of histone ubiquitylation is also critical for the differential regulation of gene expression, DNA repair and other processes on the DNA template (10, 16, 21), which will be considered within this review, and are summarized in Figure 2. The mechanisms by which histone ubiquitylation exerts its effects are also diverse; H2B ubiquitylation influences chromatin remodeling, and exhibits cross-talk with other histone modifications, including regulating methylation of histone H3. The focus of this review however will be on the effects of histone ubiquitylation on nucleosome structure that appear to be independent of H3 methylation; the reader is referred to reviews on histone cross-talk by Shukla *et al.* (2009) (1) and on the regulation of transcription and silencing by Emre and Berger (2006) (22) for further information on the relevance of downstream lysine methylation. We will first give an overview of the enzymes involved in histone ubiquitylation and deubiquitylation, and then explore the ways in which these processes regulate gene expression, and DNA repair, recombination and replication, through downstream effects on chromatin structure.

3. MECHANISMS OF HISTONE UBIQUITYLATION

Histone ubiquitylation consists of three steps, for which an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase are required (reviewed by reference (23)). In brief, the E1 enzyme activates the C terminus of ubiquitin, forming a thiol ester of E1 and ubiquitin. The ubiquitin moiety is then transferred from the E1 to the E2 enzyme, before being transferred to an appropriate substrate through the action of an E3 ligase (23). The E3 ligases themselves are important for the specificity of substrate ubiquitylation, and thus the vast repertoire of E3 ligases underlies the diversity of targets (see Table 1 for a list of E3 ligases reported to target H2A and H2B in budding yeast, flies and humans) (23). Histone H2B has been demonstrated to be mono-ubiquitylated at lysine 123 (K123) in *Saccharomyces cerevisiae* (budding yeast) by the E2 ubiquitin-conjugating enzyme Rad6, and the E3 ubiquitin-ligase Bre1 with its associated protein Lge1, the latter complex being required for substrate recognition (24-27). Rad6 exhibits *in vitro* H2B-ubiquitin conjugating activity in the absence of Bre1 (28), but the latter is required for ubiquitylation of K123 *in vivo* (25, 26) and for restriction of ubiquitylation to K120 in human H2B (corresponding to K123 in yeast H2B) *in vitro*

Table 1. Known E3 ubiquitin ligases targeting histone H2B in *Saccharomyces cerevisiae*, and histones H2A, H2B, H3 and H4 in *Drosophila melanogaster* and humans

Known Histone E3 Ubiquitin Ligases			
S.cerevisiae	***D.melanogaster***	***H.sapiens***	**Function**
Bre1	dBre1	RNF20/40	Ubiquitylates H2B; involved in transcriptional initiation and elongation, and gene silencing.
		BAF250b/ARID1	Ubiquitylates H2B; physiological relevance unknown.
		UBR1	Involved in N-end rule proteolytic pathway; ubiquitylates H2A *in vitro*
	CG9086	UBR2	Involved in N-end rule proteolytic pathway; mediates transcriptional silencing of mammalian sex chromosomes during meiosis by ubiquitylating H2A
	dRING (Sce)	Ring1B (RING2/RNF2)	Ubiquitylates H2A; linked to Polycomb silencing.
		Ring1A (RING1)	Ubiquitylates H2A; linked to Polycomb silencing.
		2A-HUB/hRUL138 (DZIP3)	Ubiquitylates H2A; prevents FACT recruitment to promoters, blocking transcriptional elongation.
		RNF8	Required for initial ubiquitylation of H2A and H2AX during DNA damage repair.
		RNF168	Amplifies the amount of H2A.Ub1 and H2AX.Ub1 during DNA damage repair, necessary for the retention of repair factors at DNA double-stranded breaks
		CUL4-DDB-ROC1	Mono-ubiquitylates all core histones *in vitro*; targeting of H3 and H4 may lead to its eviction, allowing DNA repair proteins to access damage sites. Targeting of H2A also reported in lymphoblastoid cell lines
		BBAP	Mono-ubiquitylates H4, necessary for the recruitment of the PR-SET7/SET8 methylase and subsequent methylation of H4, enabling recruitment of 53BP1 during DNA damage repair

Homologous proteins that show the greatest similarity in terms of amino acid sequence and/or function between the three species are collated in the same row.

29). It was subsequently discovered that mono-ubiquitylation of H2B is required for the expression of certain inducible genes (see Figure 3A), and that Rad6 and ubiquitylated H2B (henceforth referred to as H2B.Ub1) show a transient association with the core promoter of the *GAL1* gene, prior to the emergence of *GAL1* transcripts, implicating histone ubiquitylation in the control of transcriptional initiation (14, 30). The functional relevance of H2B.Ub1 at this stage appears to be mediated at least in part through its role in the regulation of tri-methylation of H3 at lysines 4 and 79 by the histone methyltransferases Set1 and Dot1 respectively (31-34) via an interaction with Swd2 (Cps35) and proteasomal ATPases (as reviewed in (1)), as these methylated histones mark actively transcribed regions (14). The Paf1 transcription elongation complex, along with Bre1 and the kinase Kin28 (which phosphorylates Pol II at serine 5 of its C terminal domain (CTD), required for the transition from transcriptional initiation to elongation), have been demonstrated to be required for the association of Rad6 with the elongating (phosphorylated) form of RNA polymerase II (Pol II), implicating H2B.Ub1 in the regulation of transcriptional elongation, as well as initiation (33, 35). Bre1 is required for the initial association of Rad6 with gene promoters (26), whilst components of the Paf1 complex are required for the subsequent association of Rad6 with elongating Pol II, and release into the coding regions of genes (35). Moreover, despite Rad6 accumulation at promoter regions being unaffected in mutants bearing deletions of components of the Paf complex, H2B.Ub1 fails to accumulate, indicating an absolute requirement for the Paf1 complex for Rad6 activity (35). More recently, a physical interaction between yeast Bre1 and Paf1 has also been observed, providing further evidence for coupling between the ubiquitylation machinery and transcription (29). Rad6 in turn is activated by phosphorylation at serine 120 by the Cdc28-related cyclin-dependent protein kinase Bur1 and its associated cyclin Bur2 (36, 37). Interestingly, Bur1/Bur2 appear to play roles in the localization of the Paf1 complex to certain regions of chromatin and in augmenting serine 2 phosphorylation of Pol II CTD, as well as in the activation of Rad6 (37, 38). It has also been demonstrated that glycolysis is necessary for mono-ubiquitylation of H2B, with the ubiquitylated form being undetectable on glucose depletion (39).

Geng and Tansey (2008) recently re-examined the ubiquitylation state of yeast H2B, and found that in addition to proteins mono-ubiquitylated at K123, poly-ubiquitylated forms are also present *in vivo* (40). K123 itself may be poly-ubiquitylated (mediated by Rad6), and other lysine residues within H2B are also targeted, independently of Rad6 (40). Although the observed stability of H2B led the authors to speculate that its poly-ubiquitylation is not linked to its destruction (40), the biological relevance of this process remains unknown.

In mammalian systems, both H2A and H2B are ubiquitylated (unlike in yeast, in which mono-ubiquitylation of H2A has not been reported (10)), at K119 and K120 respectively (reviewed by Zhang 2003 (13)). The two human homologues of yeast Rad6, hRad6A and B (encoded by *HR6A* and *HR6B* respectively) have been unequivocally identified as the cognate E2 ubiquitin-conjugating enzymes for H2B in human cells (41) and the RNF20/40 complex (hBRE1/hBRE2) as the interacting E3 ligase (41-44), Analogous to the situation observed in yeast, the Rad6 enzymes were observed to associate with the human PAF complex *in vitro*, and this interaction is mediated through binding of PAF1 to RNF20/40 (41).

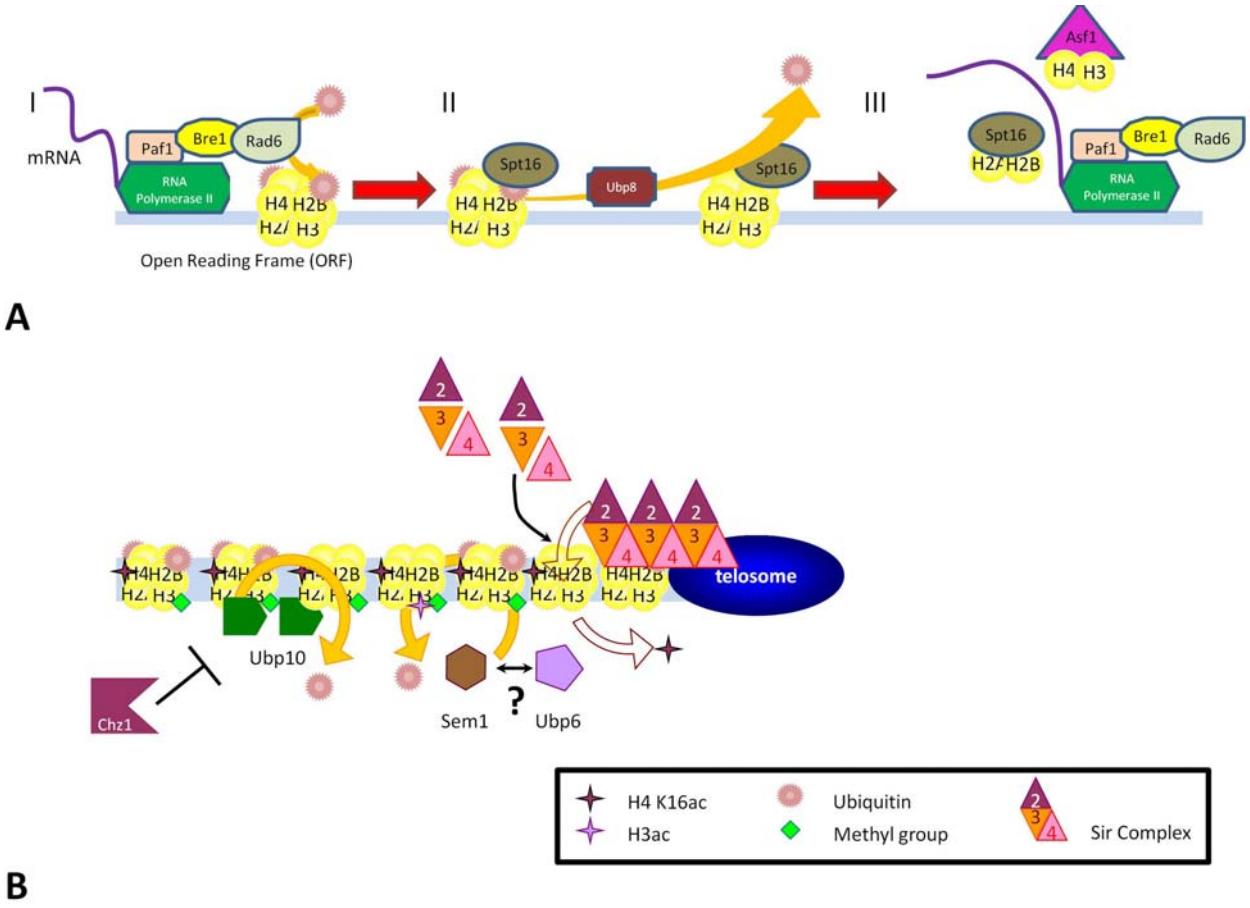

Figure 3. Proposed mechanisms for the control of transcription and heterochromatin formation by H2B ubiquitylation. (A) Ubiquitylation and subsequent deubiquitylation of H2B are important for transcriptional elongation at the *GAL1* gene. (I) The E2 ubiquitin-conjugating enzyme Rad6, in association with Bre1 and Paf1, enters the open reading frame and travels along the gene with RNA Polymerase II (Pol II). Rad6 and Bre1 ubiquitylate H2B, which has been hypothesized to stabilize the nucleosome, enabling recruitment of the histone chaperone FACT (required for eviction of H2A-H2B dimers) and its Spt16 subunit (required for subsequent histone deposition) (II). Deubiquitylation of H2B by Ubp8 may destabilize the nucleosome, thereby allowing histone eviction by FACT and Asf1, which in turn enables passage of Pol II (III). (B) Telomeric heterochromatin is compacted through the interaction of Sir2–Sir4 with the telosome (a complex of telomere-associated proteins), which results in the deacetylation of histone tails (H4K16) by Sir2, leading to the recruitment of additional Sir proteins. Ubiquitylation of H2B, and the resultant increase in methylated H3 prevents spreading of the Sir complex, and as such, the telomere-associated ubiquitin protease, Ubp10, enables heterochromatin formation by maintaining low H2B.Ub1 at the telomere. Two subunits of 26S proteasome, Sem1 and Ubp6, are also reported to regulate telomeric silencing by modulating levels of H2B.Ub1 and H3 acetylation through a proteasome-independent pathway, although the interaction between these two proteins in the context of silencing remains unknown. The histone chaperone Chz1 is proposed to help enable H2B ubiquitylation by preventing Ubp10 association with subtelomeric DNA.

There are also reports that the E2 enzyme hUbcH6 is able to mono-ubiquitylate H2B *in vitro* (42, 43), and a fascinating recent study by Li *et al.* (2010) demonstrated that the BAF250b/ARID1b subunit of the human chromatin-remodeling complex SWI/SNF-A ubiquitylates H2B *in vitro* and plays a role *in vivo*, although the respective roles for RNF20/40 and BAF250, and any relevance of this to chromatin remodeling, remain unexplored (45). A mutant form of the homologue of BAF250 in *Drosophila melanogaster*, Osa, reduces levels of global H2B.Ub1, suggesting conservation of function in higher eukaryotes (45). The Rad6 homologue hRad6B appears to be sufficient for ubiquitylation of human H2A *in vitro*, and this is further enhanced by addition of the E3 ligases UBR1 and UBR2 (46). Ubiquitin ligase activity specific for H2A has been identified in HeLa cells, mediated through hPRC1L (human Polycomb repressive complex 1-like) (47, 48). Ring1B (RING2/RNF2) (dRING being the *Drosophila* orthologous protein) is the only E3 ligase within hPRC1L that specifically mono-ubiquitylates H2A (47, 49, 50) although another Polycomb-group protein, Ring1A (RING1) contributes to H2A ubiquitylation *in vivo* (51). A third ubiquitin ligase, 2A-HUB/hRUL138, is required for gene repression through prevention of FACT-

Table 2. Known ubiquitin proteases targeting histone H2B in *Saccharomyces cerevisiae*, and histones H2A and H2B in *Drosophila melanogaster* and humans

Known Histone Ubiquitin Proteases			
S.cerevisiae	***D.melanogaster***	***H.sapiens***	**Putative Function**
Ubp8	Nonstop	USP22	Associates with SAGA/STAGA and associated complexes, regulates transcriptional initiation and elongation, mRNA export; targets H2B (and H2A in higher eukaryotes).
Ubp10	dUSP36 (Scrawny)	USP36	Localizes to silenced regions of chromatin, maintains low levels of H2B.Ub1, and H3K4me3 and H3K79me2, thereby enabling binding of Sir proteins required for silencing.
Ubp6	[CG5384 – Uncharacterized function]	USP14	Role in the proteasome; reported role in telomeric silencing in yeast through targeting of H2B.
Ubp15	USP7	USP7 (HAUSP)	Stabilizes p53 in human cells; role in silencing of homeotic genes and co-repression of ecdysone-targeted genes through deubiquitylation of H2B in flies.
		2A-DUB (MYSM1)	Deubiquitylates H2A, reported to cause H1 dissociation from the nucleosome; required for full activation of androgen receptor-regulated genes.
		USP3	Required for S phase progression following DNA damage repair; targets H2A and H2B
		USP16 (Ubp-M)	Required for expression of *Hox* genes and mitotic progression; targets H2A
		USP21	Relieves H2A.Ub1 mediated-repression of H3K4 di-/tri-methylation, enabling transcriptional initiation

Homologous proteins that show the greatest similarity in terms of amino acid sequence and/or function between the three species are collated in the same row.

mediated eviction of histones at the promoters of targeted genes (52). Finally, it was recently shown that BRCA1/BARD1 can ubiquitylate both H2A and H2B in the context of the nucleosomal particle (53).

Besides H2A and H2B, ubiquitylation of other histone proteins has also been reported (13). Mono- and poly-ub H3 were identified in rat spermatids (54), and H1 is mono-ubiquitylated by the coactivator $TAF_{II}250$ in *Drosophila*, as detected *in vitro* and *in vivo* (55). This is an especially relevant finding, as diminished H1.Ub1 in *Drosophila* embryos is correlated with reduced expression of Dorsal-target genes, hinting at a possible role for H1.Ub1 in gene activation (55). Additionally, analysis of H1 proteins in human cell lines and murine tissues by mass spectrometry was recently used to map sites of ubiquitylation (56), and additional sites of ubiquitylation were found on H1 and H4 isolated from rat brain (57). Analysis of protein fractions using similar techniques in *Arabidopsis thaliana* has also detected mono-ubiquitylated H1 (58). Human H3 and H4 were shown to be targeted for mono-ubiquitylation by the CUL4-DDB-ROC1 complex, and the activation of this complex is linked to UV irradiation (59). More recently, the E3 ligase BBAP was found to mono-ubiquitylate human H4. This has been observed *in vitro* and *in vivo*, and is linked to the cellular response to DNA damaging-agents (60).

4. MECHANISMS OF HISTONE DEUBIQUITYLATION

Mono-ubiquitylation of histones is a highly dynamic process (61-66). Deubiquitylation is catalyzed by ubiquitin proteases, also known as deubiquitylating enzymes, or DUBs (those with reported activity on H2A.Ub1 and H2B.Ub1 are shown in Table 2) (67). In *S. cerevisiae*, deubiquitylation of H2B at the promoters of certain stress-induced genes is catalyzed by the ubiquitin protease Ubp8 (14) (30, 61, 65). Ubp8 has been found to function in yeast as a component of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) (which comprises several functional domains, including modules required for histone acetylation (reviewed by Daniel and Grant 2007; Rodríguez-Navarro 2009 (68, 69))) or SAGA-like (SLIK) complex (also known as SALSA (SAGA altered, Spt8 absent) (61, 68, 70). SAGA-associated Ubp8 accumulates at the *GAL1* TATA region following gene induction, and was shown to deubiquitylate promoter-bound H2B.Ub1 (30) (see Figure 3A). Recruitment of Rad6 precedes that of Ubp8, and the two enzymes exhibit different patterns of promoter association, with Rad6 binding transiently, whilst binding of SAGA is more persistent, presumably indicative of its multiple roles during transcription (61). Transcriptional initiation and elongation requires not only H2B ubiquitylation, but also deubiquitylation; *UBP8* delta mutants show defective accumulation of *GAL1* and *ADH2* mRNA, comparable to that observed in *htb1-K123R* mutants (30). This has been suggested to be due to a disruption in the methylation patterns of H3K4 and K36; H2B.Ub1 is required for di- and tri-methylation of H3K4, whilst H3K36 methylation by Set2 requires deubiquitylation – H2B.Ub1 is proposed to block interaction of the kinase Ctk1 with H2A and H2B at SAGA-dependent genes, thereby preventing phosphorylation of Pol II CTD at serine 2 and hindering Set2 recruitment (30, 71). As for Rad6 and Bre1, Ubp8 enters into the coding region of genes through interacting with Pol II phosphorylated at serine 2 or serine 5 (71). It is subsequently able to deubiquitylate H2B.Ub1 during elongation (71). However, Ubp8 detected within the coding region is not associated with SAGA, and as such it is hypothesized that it may instead be part of the SLIK or some other related Ubp8 containing-complex (71). Song and Ahn (2009) observed that recruitment of Ubp8 to promoter regions of the *PMA1* gene in yeast was significantly increased in the absence of the Bre1-associated protein Lge1, coincident with a decrease in the association of Bre1 at the same location (27). This suggests

that Lge1 may control both ubiquitylation and deubiquitylation of H2B, through facilitation and attenuation of the recruitment of Bre1 and Ubp8 respectively, and as such could play an important role in coordinating initiation and elongation (27). Mammalian USP22 and *Drosophila* Nonstop are homologous to yeast Ubp8, and are associated with the SAGA-orthologous complexes hSAGA and dSAGA respectively (previously TFTC/STAGA), that, together with ATXN7L3 and ENY2 (dSgf11 and E(y)2 in flies), possess deubiquitylating activity towards H2A.Ub1 and H2B.Ub1 *in vivo* and *in vitro* (72, 73). It was shown that the deubiquitylation activity of Nonstop and USP22 relieves transcriptional repression and is required for androgen receptor-mediated transactivation (72), and that USP22 is required for transcriptional activation of Myc-targeted genes (73). It is interesting to note that whilst yeast Ubp8 functions in the activation of stress-inducible genes, its mammalian homologue is required for de-repression.

In addition to Ubp8, the yeast ubiquitin protease Ubp10 (Dot4) has also been found to deubiquitylate H2B *in vivo* and *in vitro* (63, 74, 75). Ubp10, but not Ubp8, has been demonstrated to target H2B at silenced regions of the genome (Figure 3B), and mutant analysis has also indicated additional targets at non-silenced loci (63, 74, 75). Conversely, Ubp8, but not Ubp10, is required for regulation of SAGA-dependent genes (63). There also appears to be some overlap in the targets of Ubp8 and Ubp10, based on a synergistic increase in H2B ubiquitylation in the double mutant, and the observation that, as for wild type, most ubiquitylated H2B is lost in either single mutant on entry to diauxie but in the double mutant H2B.Ub1 is retained (indicating that in the absence of Ubp8 or Ubp10, the remaining protease will remove the ubiquitin mark from H2B during diauxie) (75). The fruit fly orthologue of Ubp10, dUSP36 (Scrawny; Scny), has also been reported to target H2B, and stem cell maintenance is impaired in mutants (76). This is correlated with an increase in the levels of acetylated H3 (indicative of increased global transcription, suggesting dUSP36 represses gene expression) and expression of *bam*, a gene required for stem cell differentiation in certain germline stem cells, although it is unclear whether this is causative or reactive to stem cell loss (76). The involvement of H2B.Ub1 (if any) in these processes remains unclear. Human USP36 has not as yet been reported to ubiquitylate H2B *in vitro* or *in vivo*, but recent studies have shown that it is important for the development of nucleolar morphology and rRNA processing, through its role in deubiquitylating proteins associated with the nucleous (e.g. fibrillarin) (77).

A third ubiquitin protease in yeast, Ubp6, is associated with the 26S proteasome (78). Ubp6 interacts with Sem1p, and together, are believed to be involved in proteasome-dependent proteolysis, but an independent function in telomeric silencing via H2B deubiquitylation has also been reported (79) (see Figure 3B). It should be mentioned that a fourth ubiquitin ligase in yeast, Ubp15, has been shown to deubiquitylate H2B *in vitro*, but its biological significance remains unreported (see supplementary Figure 3 of Weake *et al.* 2008 (80)). Its homologue in *Drosophila*, USP7, deubiquitylates H2B in complex with guanosine 5'-monophosphate synthetase (GMPS) *in vitro* and *in vivo*, and is required for silencing of homeotic gene clusters and down-regulation of target genes of ecdysone (a steroid hormone required for several developmental processes in arthropods, including moulting and morphogenesis (81)) through co-repression of the ecdysone receptor *in vivo* (82, 83).

The human ubiquitin protease USP3 has also been shown to deubiquitylate histones (H2A and H2B) *in vivo* (84). A number of other ubiquitin proteases, including USP16 (Ubp-M), USP21, 2A-DUB and USP22 have been shown to deubiquitylate H2A *in vitro* (67, 85-87). USP16 is believed to be important for chromatin condensation during mitosis through the removal of ubiquitin from H2A and downstream phosphorylation of H3, and is itself activated by phosphorylation at the start of mitosis (87, 88). Finally, 2A-DUB is involved in eviction of H1 from the nucleosome (89), and USP21 impacts on transcriptional initiation through crosstalk with di- and tri-methylation of H3K4 (90).

5. UBIQUITYLATION, HISTONE DYNAMICS AND GENE EXPRESSION

Ubiquitylation of H2B influences both transcriptional initiation and elongation, as reviewed in reference (16). At least part of the effect of H2B on the initiation and elongation of certain genes appears to be through the promotion of H3K4 and H3K36 tri-methylation (required for the early and late stages of elongation) following H2B ubiquitylation and deubiquitylation respectively (30). Additionally, a recent paper by Mohan *et al.* (2010) reports that deletion of the Bre1 homologue in *Drosophila melanogaster* (dBre1) reduces global levels of H2B.Ub1 and H3K79me3 in embryos, and RNA_i knock down of *dBre1* in wing imaginal discs decreases expression of gene targets of the Wingless signaling pathway, suggesting an interaction between this pathway, H2B.Ub1 and H3K79me3 (91). However, elongation is also believed to be partly regulated by its effects on nucleosome dynamics, independently of H3 methylation (14, 35). It should also be noted that H2B.Ub1 is additionally involved in the recruitment of the 19S proteasome to genes (92), which itself participates in transcription elongation (93, 94). As such, findings from *in vivo* studies need to be considered in the light of the potential influence of 19S.

Nucleosome occupancy at genes has been shown to act as a barrier to transcriptional elongation by RNA polymerase II (Pol II), and nucleosomes at the promoters of actively transcribed genes exhibit reduced occupancy and rapid turnover (62, 95-99). During transcriptional elongation, all four histones are evicted from, and then re-deposited on the transcribed gene in the wake of the passage of Pol II (96, 100, 101). In yeast, the chromatin remodeling complex FACT (facilitates chromatin transcription) is required for removal of a H2A-H2B dimer (102), whilst the histone chaperone Asf1 is required for eviction of the H3-H4 tetramer (103). Eviction of histones

removes a barrier to Pol II transcription, and reassembly is important for preventing subsequent transcription from cryptic initiation sites (8, 64, 104, 105). Post-translational modification of histones is known to be important for both nucleosome eviction and reassembly; H3 acetylation by the Gcn5 histone acetyltransferase (HAT) results in histone eviction, enabling transcription (64), and methylation of H3K36 by Set2 affects histone deposition through signaling for deacetylation of H3 and H4 by the Rpd3S histone deacetylase complex (104-107).

H2B.Ub1 is proposed to play a role in transcription elongation, in addition to its reported roles in initiation (14, 35, 108, 109). The implication that H2B.Ub1 may be involved in elongation initially came from the finding that H2B.Ub1 and the E2 Rad6 locate throughout the galactose-induced *GAL1* gene, suggesting a role beyond initiation of transcription (14). Moreover, in a recent study, Minsky *et al.* (2008) utilized a chromatin immunoprecipitation sequencing (ChIP-Seq) approach to demonstrate that human H2B.Ub1 is preferentially associated with the open-reading frame of highly-expressed genes, adding credence to its role in elongation (110). Based on the findings that H2B.Ub1 is required for H3K4 di-methylation by Set1 (32), and that Set1 is recruited to the hyperphosphorylated (elongating) form of Pol II (111), Xiao *et al.* (2005) searched for a similar interaction of Rad6 with elongating Pol II (35). As predicted, Rad6 was found to co-purify with Pol II phosphorylated at both serines 2 and 5 of the CTD. Recruitment of Set 1 to Pol II is dependent on serine 5 phosphorylation by Kin28 (111), and is mediated through the Paf1 elongation complex (112); again, a requirement for components of the Paf1 complex was also found for Rad6 recruitment to elongating Pol II (and subsequent entry into the coding region; this is in contrast to a requirement for Bre1 for the initial association of Rad6 with gene promoters), and global ubiquitylation of H2B was found to be absolutely dependent on Kin28 (35). Reduced levels of Rad6 at the *GAL1* coding region in deletion strains of *bre1* and *rtf1* (the latter encoding a component of the Paf1 complex) were also correlated with a decrease in Pol II occupancy. Additionally, recruitment of Pol II to coding regions following induced activation of p53-target genes was found to coincide with an increase in H2B.Ub1 at these genes in human cells, indicating that the interaction between Pol II and H2B.Ub1 is conserved between budding yeast and humans (110). A more recent study by Kim and Buratowski (2009) revealed that mutations in *RAD6* or *BRE1*, and the resultant downstream decrease in di- and tri-methylation of H3K4, caused an increase in H4 and H3 acetylation at the 5' region of genes, due to a failure to recruit the Set3 histone deacetylase complex (the latter being demonstrated to promote transcription) (113). However, H2B.Ub1 also appears to perform a role in elongation distinct from its effects on H3 methylation, based on the observation that *rad6* delta, *bre1* delta and *htb*1-*K123R* mutant strains (all defective in H2B ubiquitylation) are sensitive to treatment with 6-azauracil (which reduces the nucleotide pool for Pol II elongation), whereas strains harboring mutations of H3K4 and K79 (preventing methylation) are unaffected (35, 64). Studies in *Schizosaccharomyces pombe* (fission yeast) have also shown that H2B.Ub1 affects gene transcription in a histone methylation-independent manner (109). Shukla and Bhaumik (2007) subsequently confirmed that loss of ubiquitylation, but not H3 methylation alone, resulted in decreased kinetics of Pol II recruitment to gene coding regions, whilst having no effect on recruitment to the promoter (108). Decreased elongation rate in mutants of H2B ubiquitylation is also associated with a skewed H3 distribution within certain gene coding regions in *S. pombe*, suggesting that H2B.Ub1 affects chromatin assembly, and genetic studies suggested an interaction between H2B.Ub1 and regulators of nucleosome structure (109). Interestingly, the *hem2+* gene, which exhibits a decrease in Pol II occupancy at the 3' end in the *htb1-K123R* (but not *set2* delta) mutant, shows no such skewed H3 distribution, suggesting that the effect on elongation by H2B.Ub1 may have some component beyond both effects on nucleosome occupancy and methylation, in a gene-specific manner (109). As predicted, ubiquitylation, but not methylation, also impairs transcriptional elongation of the *GAL1* gene, by enhancing the rate of Pol II recruitment to the coding region (108, 114). This control of elongation is gene differential, as no such effect of H2B.Ub1 on recruitment of Pol II to coding regions was observed for *PHO84*, *ADH1* or *PYK1* (114). Human H2B.Ub1 has been shown to cooperate with FACT (that displaces and deposits H2A-H2B (100, 102)) to promote efficient Pol II elongation in a reconstituted chromatin transcription system; this indicates that the ubiquitylated histone may exert its effects on elongation through regulating histone association with chromatin (43). Neither FACT nor the factors required for mono-ubiquitylation of H2B could stimulate transcription in the system alone, and FACT was found to be necessary for mono-ubiquitylation of histones bound to the promoter, in a transcription-dependent manner (43). Adding FACT, PAF or the E3 ligase RNF20 to the *in vitro* system before or after formation of the preinitiation complex showed no difference in reaction efficiency, indicating that their role is effected post-initiation. It was found that whilst H2B.Ub1 affects the levels of the H2A/H2B dimer on chromatin, it has no effect on levels of the H3/H4 tetramer (6). Although transcripts were detected in both the presence and absence of H2B.Ub1, the size of the transcripts were significantly increased in the system with H2B.Ub1, implicating it in Pol II processivity (43). Again, this effect was independent of H3K4 methylation (43). However, a recent investigation by Kim *et al.* (2009) found no differences in the level of transcription between unmodified chromatin and that containing a semi-synthetic H2B with pre-ubiquitylated K123, in either the presence or absence of FACT, suggesting that H2B.Ub1 *per se* has no effect on transcription (41). It was thus concluded that any effects on transcription observed in other experimental setups must be mediated by downstream effectors, although it was noted that the unexpected result may in part be due to the use of NAP1 rather than FACT as a histone chaperone in the assay used, perhaps resulting in a different chromatin conformation (41).

More recent evidence from work in budding yeast has further elucidated the relationship between H2B.Ub1 and histone dynamics, by alluding to a role for

H2B.Ub1 in histone deposition in coding regions following passage of Pol II (43, 64). It was reported that both H2B.Ub1 and the Spt16 subunit of FACT were required for nucleosome reassembly during elongation, and as *in vitro* for human H2B (43), they exhibited a reciprocal relationship, with Spt16 being required for H2B.Ub1 recruitment at the ORF of constitutively transcribed genes (suggested to be either mediated through PAF-binding, or indirectly, through reduced Pol II accumulation at coding regions in the absence of Spt16), and H2B being necessary for stable accumulation of Spt16 at the ORF (perhaps through its presumed role in stabilizing nucleosome structure) (6, 64, 115). Incubation of a strain with a thermosensitive mutation of *spt16* at its restrictive temperature, or a *htb-K123R* mutation, was found to disrupt the inverse relationship between Pol II and H2B and H3 levels; low levels of histones become associated with low levels of the polymerase. Without efficient reassembly of nucleosomes (for example, in yeast mutants of *spt16* or *asf1*), initiation of transcription from cryptic sites within coding regions is observed (103, 116); this is also seen in a subset of genes in the *htb-K123R* mutant, and intragenic transcription is elevated further on inactivation of Spt16 (64). *spt16*/*htb-K123R* double mutants exhibit heightened sensitivity to MNase digestion (suggesting nucleosome disruption), whilst histone deposition was shown to be intermediate between the two single mutants; this suggests that prevention of H2B ubiquitylation in the *spt16* mutant can somewhat rescue histone deposition, but that the chromatin is inherently unstable (64). It has been hypothesized that H2B ubiquitylation may promote histone deposition by both stabilizing the interaction of Spt16 with chromatin, and repression of other, competing reassembly factors. In the absence of Spt16, these alternate factors deposit histones, but are unable to recapitulate the stable nucleosome structure, accounting for the double mutant phenotype (64). In addition, the elongation rate of the double mutant as measured at the *GAL1* gene is faster than that of the wild-type, as opposed to both single mutants, which have an elongation rate slower than wild-type, perhaps due to a more open chromatin conformation in the double mutant (64). However, despite the increased elongation rate of Pol II, production of full-length *GAL1* mRNA was greatly diminished compared to wild type, highlighting the requirement for maintenance of appropriate histone distributions at coding regions (see supplemental Figure 4 of Fleming *et al.* 2008 (64)).

Further evidence for the interaction between FACT and H2B.Ub1 comes from genetic studies in *Arabidopsis thaliana*; double mutants of *ssr1*/*spt16* (FACT subunits) and *HUB1*/*2* (histone monoubiquitination 1 and 2; E3 ligases) revealed a synergistic interaction for leaf growth, independent effects on flowering induction, and that the FACT subunits are epistatic to H2B ubiquitylation with regard to certain other developmental processes, such as branching (117). Clearly the interactions between ubiquitylation of H2B and histone chaperones are complex and intimate.

It was found that chromatin from yeast possessing a mutant H2B lacking the consensus ubiquitylation site exhibited greater sensitivity to MNase DNA digestion, suggesting a more open chromatin conformation. This was found to be due to decreased histone occupancy within gene coding regions (6). Ubiquitylation of H2B was also found to decrease its solubility in salt, further suggesting that H2B.Ub1 affects nucleosome stability, and the presence of H2B.Ub1 led to increased H2B binding to the coding regions of genes, implying that ubiquitylation affects histone deposition (6). Chandrasekharan *et al.* (2009) propose that rather than H2B.Ub1 mediating Spt16 binding through disrupting chromatin structure, the increase in stability observed may provide a suitable binding site for Spt16. As such, ubiquitylation of H2B may initially stabilize nucleosomes ahead of the elongating Pol II in order to enhance Spt16 binding, followed by subsequent disassembly on deubiquitylation, allowing elongation to proceed (see Figure 3A) (6). Nucleosomes may then be reassembled in the wake of Pol II progression through the actions of Spt16, and then stabilized on re-ubiquitylation of H2B (6). It was speculated further that the stabilized nucleosome may provide a platform for the recruitment of regulators in addition to Spt16, including the methyltransferases Set1 and Dot1, thereby enabling methylation of H3 and its downstream effects on transcriptional initiation and gene silencing (6).

How does ubiquitylation affect the stability of the nucleosome? Three possibilities have emerged; firstly, the surface charged residues of ubiquitin may promote binding with DNA or other histones. Secondly, H2B.Ub1 may play an indirect role in promoting interaction between H2A-H2B and the H3-H4 tetramer, through subtle rearrangements of the H2B protein (6) (in an analogous situation to that of enhancement of H1 binding to the nucleosome by H2A.Ub1 (89, 118)). Thirdly, it may in fact be due to recruitment of ATP-dependent nucleosome remodeling factors, such as SWI/SNF, to the ubiquitylated histone; double mutants of *htb-K123R* and *snf* delta (a deletion of a subunit of Swi/Snf) exhibit a synergistic slow growth phenotype and a reduction in *SUC2* gene transcription, the latter being independent of the effects on growth, suggestive of a functional overlap (14). Interestingly, in fruit flies and mammals, SWI/SNF itself is reported to have E3 ligase activity directed towards H2B, strongly suggestive of an additional link between histone ubiquitylation and nucleosome remodeling (45). However, to date there remains no clear understanding as to how ubiquitylation affects nucleosome structure, or how this is related (if at all) to Spt16 recruitment, pending further biochemical and structural analysis of the ubiquitylated histone within the nucleosome. In an additional level of complexity, increased stability of the nucleosome through histone ubiquitylation is thought to also affect gene silencing; that is, the same process has gene-differential effects on expression, which we will consider, amongst other reported effects on silencing, in the next section.

6. UBIQUITYLATION, HISTONE DYNAMICS, SILENCING AND GENE REPRESSION

Ubiquitylation of H2B has reported roles in the establishment of telomeric silencing, mediated through its effects on methylation of H3 (10, 33, 34, 47, 63, 65, 74, 75,

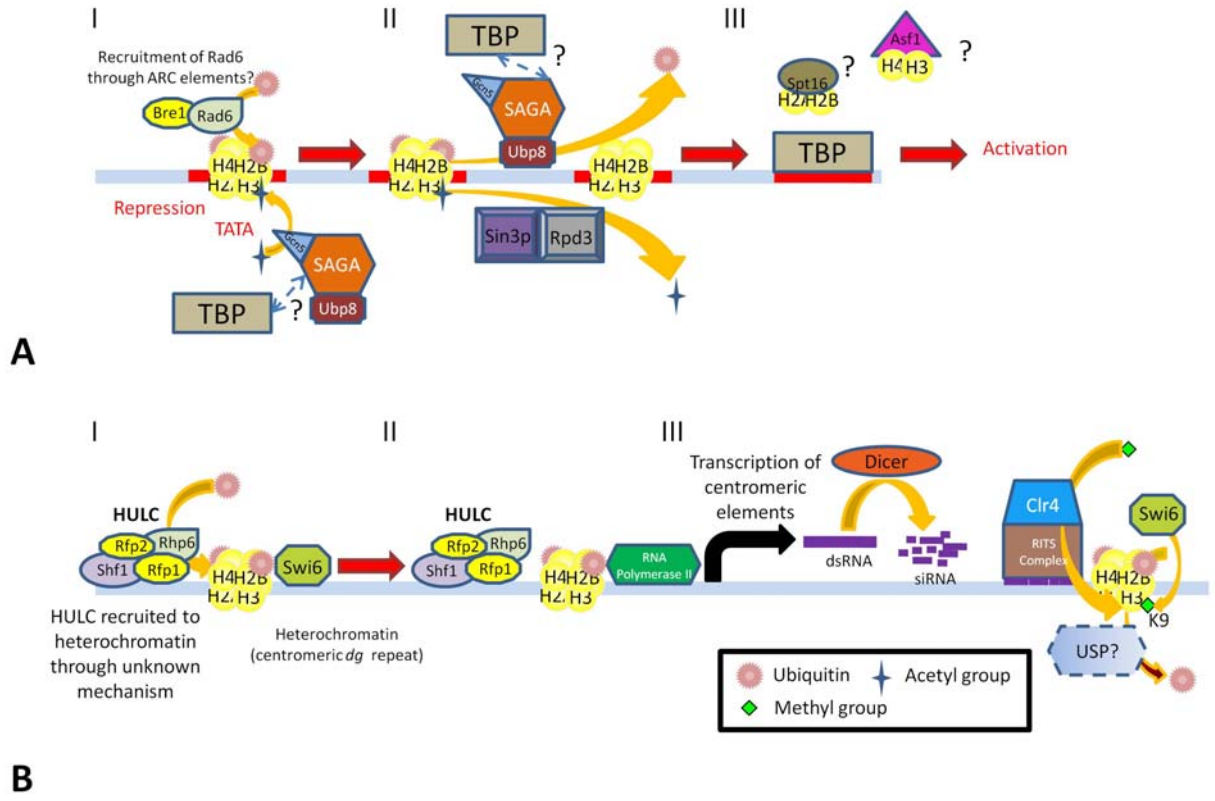

Figure 4. Silencing at repressible genes in *Saccharomyces cerevisiae* and at centromeres in *Schizosaccharomyces pombe* is regulated by distinct mechanisms. (A) The *ARG1* gene in budding yeast is repressed during growth in rich media. This repression is partially mediated by ubiquitylation of H2B by Rad6 at nucleosomes positioned on the TATA box, and also requires acetylation of H3 by the histone methyltransferase Gcn5 (in association with SAGA) (I). Rad6 may be recruited by ARC elements; alternatively, it is possible that H3 acetylation mediates the creation of a suiTable chromatin structure for ubiquitylation. As described for *GAL4*, ubiquitylation is believed to stabilize the nucleosome, preventing binding of TBP. Deacetylation of H3 by Sin3p/Rpd3 may enable deubiquitylation of H2B by SAGA-associated Ubp8 (II) and consequent binding of TBP (which may be recruited through binding to SAGA via the latter's Spt8 subunit), possibly following histone eviction (III). (B) H2B.Ub1 is required for RNA polymerase II (Pol II)-mediated transcription, which is necessary to maintain the structural integrity of centromeric heterochromatin in *Schizosaccharaomyces pombe*. In order for transcription to take place, the heterochromatin needs to be temporarily decondensed to allow passage of Pol II. Rhp6, a homologue of Rad6 in *Saccharomyces cerevisiae*, functions as part of the HULC complex, that also contains two E3 ligases, Rfp1 and Rfp2, and a fourth protein, Shf1. The HULC complex localizes to centromeric *dg*/*dh* repeats, where it mono-ubiquitylates H2B (I). Increased H2B.Ub1 is correlated with decreased binding of Swi6 to heterochromatin, and loss of silencing (II). Transcription of the inverted repeat elements and subsequent processing by the RNA-dependent RNA polymerase complex (RDRC) produces double-stranded RNA. This is cleaved by Dicer, forming small interfering (si)RNAs, which are targeted back to the repetitive elements through interaction with RITS (RNA-induced Initiation of Transcriptional Gene Silencing) complex. This aids recruitment of heterochromatin assembly factors, including the histone methyltransferase Clr4, which methylates H3K9. RITS is anchored by H3K9me, and the methyl tag also recruits Swi6 to the heterochromatin (III). A requirement for deubiquitylation of H2B.Ub1 by a ubiquitin-specific protease may be predicted, although its identity is as yet unidentified.

82, 92, 119-127). Silencing at the telomere is complex and the interplay of histone modifications in its regulation is yet to be fully characterized. In the current understanding, it appears that mono-ubiquitylation of H2B controls di- and tri-methylation of H3K4 and H3K79 through the methyltransferases Set1 and Dot1 (14, 27, 30, 31, 33, 34, 37, 61, 63, 66, 71, 75, 92, 114, 128-130), which act to prevent silencing proteins (Sir2/Sir3/Sir4) from binding to euchromatin (33, 75, 124, 125) (see Figure 3B). In the absence of H2B.Ub1, the resulting decrease in di-and tri-methylated H3K4 and H3K79 is proposed to result in promiscuous binding of the cell's limited supply of

silencing proteins throughout the genome, thereby decreasing those bound to regions of silent chromatin. Thus, it seems that the diffusion of Sir proteins away from heterochromatin weakens silencing (for a review, please refer to Gao and Gross 2006 (131)). The ubiquitin protease Ubp10 (but not Ubp8) is integral to the maintenance of silencing; it is enriched at the telomere and rDNA locus, and physically interacts with Sir proteins at these locations – Ubp10 and the histone deacetylase Sir2 exhibit a mutual requirement for their retention at telomere proximal regions (63, 75, 132, 133). Disruption of Ubp10 levels in yeast reduces silencing at the telomere and causes increased recombination of rDNA, indicative of reduced silencing at this region (74, 75, 132-134). This is correlated with an increase in H3K4 and K79 methylation at telomere-proximal positions (75). However, H3 is also the target of extensive acetylation which is also reported to influence silencing at the telomere (79, 135), and there is contradictory evidence suggesting that the effects of H3 acetylation and methylation on silencing may be independent, interdependent or redundant (79, 135-138). Recent findings from our laboratory have revealed that certain mutations of the H2B C terminus result in increased global H2B.Ub1 and a disruption of telomeric silencing, yet with no change in H3 methylation. This is perhaps indicative of a methylation-independent contribution of H2B.Ub1 to silencing at the telomere (C.-F.Kao *et al.*, unpublished results).

In budding yeast, H2A.Z, a variant of H2A, is also required to prevent the spreading of Sir2-mediated silencing from heterochromatin (139), and furthermore, it is required for the appropriate expression of certain silenced genes under activating conditions (140). Its function in higher eukaryotes is less well-characterized, and it has been found to be associated with both transcriptionally-active and inactive regions, depending on the organism and cells under study (141-144). H2A.Z, like H2A, is modified by mono-ubiquitylation *in vivo*, and this can occur at one of multiple lysine residues as revealed by mutation studies (145). Again, as for H2A.Ub1 (51, 146), H2A.Z.Ub1 is ubiquitylated by RING1b E3 ligase, and antibody-binding studies suggest it is localized to the inactive X chromosome of female mammalian cells (145), in accordance with the reported role of RING1b E3 ligase in regulating X chromosome silencing (147). This implies a role for a mono-ubiquitylated-H2A variant in marking facultative heterochromatin, although its precise role remains unclear. Interestingly, it was also reported that there was often unequal staining for the non-ubiquitylated histone between sister chromatids of the silenced X chromosome (145). This perhaps suggests some requirement for differential regulation of silencing between the two chromatids.

Besides Ubp10, a recent study has reported a putative role for the ubiquitin protease Ubp6 in telomeric silencing (Figure 3B) (79). Although single mutants of *ubp6p* or *sem1p* exhibit only slight reductions in telomeric silencing and H2B.Ub1 levels, a synergistic decrease was reported in the double mutant (79). This would seem to suggest that either the two proteins interact in different deubiquitylation pathways despite their interaction in the proteasome, or that in the absence of Ubp6, a second ubiquitin ligase can interact with Sem1p, and Ubp6 retains some level of deubiquitylation activity *in vivo* even in the absence of Sem1p. This result is unexpected at best. Furthermore, this study reported that whilst the single mutations *ubp6* delta and *ubp10* delta both result in an increase in total H2B.Ub1, in an unanticipated result, wild-type levels are restored in the double mutant (79). The authors speculated that this may be due to activation or up-regulation of a third deubiquitylating pathway in the absence of either ubiquitin ligase (79), but these results remain controversial pending further examination.

In addition to silencing of chromatin regions, a role for H2B ubiquitylation in regulating gene-specific repression has also been reported through a methylation-independent mechanism, thought to involve the stabilization of nucleosomes positioned over promoters (6, 148). The gene *ARG1* (encoding argininosuccinate synthetase) is repressed in the presence of arginine, and this repression is related to both increased acetylation of H3 by the histone acetyltransferase Gcn5p (149), and the ubiquitylation activity of Rad6 (148). The effect of Rad6 on repression is partly mediated through H2B.Ub1, as revealed by mutation studies (70, 148). Repression was found to be correlated with increased levels of H2B on the *ARG1* TATA region, suggesting that ubiquitylation of H2B affects histone deposition in the context of transcriptional repression (6). As increased binding of TBP to the promoter is observed on deletion of *rad6* (148), it has been speculated that the stabilization of histones on the promoter may occlude binding of TBP (TATA-Binding Protein/Spt15), thereby repressing transcription (6). Deletion of Ubp8 or Sgf11 (the latter required for the association of Ubp8 with SAGA and SLIK complexes) partially reduces *ARG1* expression under repressing and activating conditions, indicating that deubiquitylation by Ubp8 is required for both de-repression and activation of the gene (70) (summarized in Figure 4A.). The timing and mechanisms by which SAGA-associated Ubp8 and Gcn5p regulate de-repression remain unclear, further complicated by SAGA's reported ability to target TBP to certain gene promoters (150). A similar situation is also observed at the promoters of other repressed genes, including *PHO5* and *GAL10* (6), although this may be mediated through H2B.Ub1's effect on histone methylation, based on the report that similar phenotypes are observed in *set1* delta mutants (151). Finally, expression microarray analysis of HeLa cells, following knock down of the E3 ubiquitin ligase RNF20/hBRE1 and subsequent near ablation of H2B.Ub1, revealed that transcription of certain genes were up-regulated (152). Further investigation of these genes revealed that whilst H2B.Ub1 is preferentially associated with the coding regions of all genes studied, those up-regulated on knock down of hBRE1/RNF20 showed elevated association levels with the ubiquitylated histone as compared with unaffected genes, which was not a consequence of altered nucleosome occupancy. This was found to be strongly correlated with the presence of H3K4me3 in the 5' region of the open reading frame, but knock down of RNF20/hBRE1 did not affect K4 methylation of all up-regulated genes, suggesting a H3

methylation-independent effect on transcription. Tellingly, despite a reported increase in Pol II occupancy at the transcriptional start sites of these genes, they were also reported to reside within compact chromatin regions, perhaps a consequence of H2B.Ub1-mediated stabilization of nucleosome structure (152).

Studies in *Drosophila* have indicated that deubiquitylation of H2B by the ubiquitin protease USP7 is correlated with homeotic gene cluster silencing and the control of ecdysone targeted genes (82, 83). Deubiquitylation and the observed effects on silencing are dependent on guanosine 5'-monophosphate synthetase (GMPS), which was shown to demonstrate both a genetic and physical interaction with USP7 (82, 83). Mutations of *usp7* or *gmps* were both found to enhance homeotic transformations caused by *Pc* (*Polycomb*) mutations, signifying the involvement of the USP7/GMPS complex in homeotic gene silencing, possibly mediated by the removal of ubiquitin from H2B (although the USP7/GMPS complex also targets p53, which may be involved in this process) (82). A follow-up study by van der Knaap *et al.* (2010) identified that both proteins associate with loci containing early-response genes to the steroid hormone ecdysone, and that mutant alleles of both result in aberrant expression of these genes during development (83). Indirect immunofluorescence revealed that the ecdysone receptor (EcR), GMPS and USP7 (as well as Bre1 and Pol II to a lesser extent) all bind early-response genes prior to ecdysone signaling, but following the ecdysone pulse, USP7 disappears and Bre1 and Pol II levels increase, suggesting that USP7/GMPS functions as a transcriptional co-repressor. In support of this, USP7 was found to interact genetically and physically with EcR, and RNA_i-mediated depletion of USP7 led to de-repression of ecdysone-regulated genes in S2 cells (83). The recruitment of Bre1 supports a role for H2B.Ub1 in transcription initiation, in accord with the data discussed in section 4, and it seems logical that the prevention of ubiquitylation by the constitutive presence of a ubiquitin ligase would be involved in gene repression. However, H2B deubiquitylation is also required for transcriptional elongation (71), emphasizing the intricate relationship of this histone modification in regulating gene expression.

Further evidence for an involvement between H2B.Ub1 and histone eviction in the context of gene silencing comes from a proposed interaction with histone chaperone Chz1p (153, 154). Chz1p is believed to regulate H2A-H2B eviction during the SWR1-catalysed replacement reaction, based on NMR structure analysis (154). Additionally, it was observed that certain genes in *chz1* delta mutant strains were down-regulated, and that many of those were located in subtelomeric regions (153). The mutation was also found to decrease levels of chromatin-associated H2B.Ub1, due to increased association of the ubiquitin ligase Ubp10 with subtelomeric regions (153). Surprisingly, global H2B.Ub1 is increased in *chz1* delta, which the authors suggest may be due to the targeting of Ubp10 away from non-chromatin associated H2B.Ub1 within the cell, although this remains unclear. The effects of decreased ubiquitylation are believed to be mediated through downstream H3K79 dimethylation (also reduced in subtelomeric regions), and the resulting increase in association of silencing proteins Sir3p and Sir4p (153). However, it was not investigated as to whether the effects on silencing were predominantly due to defects in H3 methylation, and it seems plausible that H2B.Ub1 and Chz1p may interact in additional pathways, perhaps relating to nucleosome eviction and reassembly. This remains open to further investigation.

In *S. pombe*, de-repression of heterochromatin is mediated by Rhp6 (an E2 ubiquitin-conjugating enzyme, homologous to Rad6) through its effects on ubiquitylation of H2B, and this was also shown to be independent of H3K4 methylation (as no such effects are observed in *set1* mutants) (155). Rhp6 and its interacting proteins localize to repeat elements within heterochromatin, supporting a direct interaction (155). The de-repression observed appears to be a consequence of the formation of a more accessible chromatin structure, based on the observation that histone density at pericentromeric regions was reduced coincident with an increase in Pol II occupancy on over-expression of *Rhp6* (155). Intriguingly, this is in contrast to the situation in *S. cerevisiae*, in which H2B.Ub1 and Rad6 are absent from the silenced heterochromatic regions (14, 63). This may be explained by the requirement of *S. pombe* Pol II to transcribe heterochromatic repeat sequences, which allows production of small RNAs believed to be essential for RNA_i-mediated modification of heterochromatin and resultant gene silencing (see Figure 4B) (155-159). Paradoxically, it seems that H2B.Ub1 may function by antagonizing the repressive effects of heterochromatin at the repeat elements, which Zofall and Grewal (2007) speculate may be through direct antagonism of the assembly of heterochromatin complexes, or indirectly, through recruitment of Pol II, and the resulting destabilization of the surrounding heterochromatin (155). They also note that the heterochromatin de-repression phenotype observed upon over-expression of *Rhp6* is similar to that observed upon loss of the Clr3 histone deacetyltransferase, with a decrease in tri-methylated H3K9 and defects in localization of the heterochromatin protein Swi6 (155, 160, 161). It was demonstrated that Clr3 restricts Pol II accessibility to heterochromatin repeat elements, with a large increase in Pol II being detected at the silenced *cenH* element in a *Clr3* deletion mutant (161). These similar phenotypes further support the hypothesis that histone modification aids recruitment of the transcriptional machinery, perhaps through altering chromatin structure (155). The reversible nature of histone mono-ubiquitylation in budding yeast, and the requirement for deubiquitylation in transcriptional elongation would seem to suggest that a ubiquitin protease may also be an important factor(s) in centromeric heterochromatin formation in fission yeast. However, thus far the involvement of such a protease has not been reported.

As well as H2B, H2A ubiquitylation is also important for transcriptional silencing in vertebrates. Different mechanisms of gene silencing have been observed depending on the chromatin loci, and are related to the varying E3 ligases that ubiquitylate H2A at K119.

[For an overview, please see reference (16)]; for example, PRC1 (Polycomb repressive complex 1) is targeted to tri-methylated H3K27, where it ubiquitylates H2A, thereby repressing initiation (47), and ubiquitin protease USP21 relieves the H2A.Ub1-mediated repression of H3K4 methylation and transcriptional initiation at silent chromatin (90). Additionally, in a fascinating parallel to the co-operative function observed between H2B and FACT outlined above, ubiquitylation of H2A mediated by the E3 ligase 2A-HUB has been reported to inhibit recruitment of the Spt16 subunit (52). 2A-HUB was shown to mono-ubiquitylate H2A *in vitro* and *in vivo*, and siRNA knockdown of the ligase was shown to affect the expression of certain genes, including a statistically significant overrepresentation of genes involved in the cell cycle, the immune response and pattern specification. In addition, luciferase reporter constructs under the regulation of the promoters of *IP-10* (interferon-inducible protein 10; a chemoattractant for activated T-lymphocytes) and *RANTES* (regulated on activation normal T cell expressed; a chemoattractant for various cell types at inflammatory sites) genes revealed that promoter activity was down-regulated on over-expression of 2A-HUB, suggesting a direct regulation (52). However, over-expression of a mutant 2A-HUB without enzymatic activity resulted in no such effects, suggesting a requirement for H2A ubiquitylation in repression. Knockdown of the N-CoR-HDAC1/3 complex, a transcriptional co-repressor, was observed to decrease 2A-HUB recruitment to the *RANTES* promoter, implicating it in bringing the ligase to a subset of gene promoters. Evidence for the mechanism of repression came from the observed interaction between Spt16 and H2A, but not H2A.Ub1, in HEK cells. Also, induction of immune system genes by treatment with lipopolysaccharides was shown to result in loss of N-CoR, 2A-HUB and H2A.Ub1 from, and FACT recruitment to, the *RANTES* promoter; as expected, FACT recruitment was increased on down-regulation of 2A-HUB. Finally, as predicted from its role in regulating genes encoding proteins of the immune system, 2A-HUB was shown to be important for chemokine-mediated cell migration (52).

7. H2A.Ub1, H1 AND NUCLEOSOME STRUCTURE

H2A.Ub1 also appears to be important for nucleosome assembly through recruitment of linker histones (89, 118). The C terminal tail of H2A is predicted to emerge from the nucleosome at a position near the globular domain of the linker histone H1 (162); as the mono-ubiquitylated K119 of H2A is found in this region, it was supposed that conjugation of the ubiquitin moiety may impact on H1 binding (10). Jason *et al.* (2005) discovered that while H1 could associate with reconstituted chromatin in the presence or absence of H2A.Ub1, ubiquitylation of H2A enhanced binding of H1 to nucleosomes (118). H1 association with nucleosomes is correlated with transcriptional repression (163), and as such, H2A may exert some of its repressive effects through linker histones (118). Indeed, Zhu *et al.* (2007) subsequently identified that mammalian histone 2A deubiquitinase (2A-DUB) destabilizes the interaction between H1 and nucleosomes (89). H2A K119R mutant-containing mononucleosomes possessed greatly decreased levels of histones H1, suggesting that deubiquitylation may be critical for H1 dissociation from the nucleosome. Knockdown of 2A-DUB resulted in a decrease in phosphorylated H1 in HEK293T cells, raising the possibility that H1 eviction is mediated by an increase in phosphorylation, triggered by a decrease in H2A.Ub1 (89). H1 at the *PSA* promoter was reduced on gene induction, and, as expected, this effect was diminished on knock-down of 2A-DUB. The authors propose that the induced rearrangement of chromatin structure may be important for recruiting Pol II, enabling transcriptional initiation (as 2A-DUB augments reporter gene activation) (89). In addition, 2A-DUB was found to interact with the histone acetyltransferase p300/CBP-associated factor (p/CAF), and acetylation status of H2A was found to affect their ability to be de-ubiquitylated by 2A-DUB, with hypoacetylated nucleosomes showing minimal deubiquitylation. As noted by Zhu *et al.* (2007) (89), this is in line with findings that acetylation of histones may facilitate dissociation of H1 (reviewed by Raghuram *et al.* 2009) (164). H1 is important in regulating higher order chromatin structure (165), and as such, it seems likely that a complex network of crosstalk between histone modifications leads to regulation of nucleosome assembly.

8. HISTONE UBIQUITYLATION AND DNA REPAIR

In addition to the previously described roles in gene silencing and activation, histone ubiquitylation impacts on other cellular processes, including DNA repair. Human Rad6 has been observed to redistribute to DNA following damage (166), and Yamashita *et al.* (2004) reported that the meiotic prophase arrest observed in *rad6* null mutants of *S. cerevisiae* is correlated with a decrease in the levels of double-stranded breaks (DSB) at meiotic recombination hotspots, believed to be a consequence of failure to recruit the DSB-formation complex (167). Ubiquitylation of H2B is also implicated in DNA repair and in the DNA damage check point response, as it is necessary for activation of the Rad53 kinase (168). In mammalian systems, H2A.Ub1 is also important in DNA repair, as accumulation of H2A.Ub1 (triggered on DNA damage (84, 169)) is required for the temporary disruption of chromatin necessary for repair (reviewed by Zhou *et al.* 2009) (12). Another histone modification, phosphorylation of the histone variant H2AX, is also triggered on DNA damage, and in the absence of USP3, persists for a prolonged period (84) (reviewed by Ayoub *et al.* 2009 (170)). Phosphorylated H2AX (gamma-H2AX) is important for the recruitment of repair proteins to the damage site, including MDC1, BRCA1 and 53BP1 (171). Gamma-H2AX also acts upstream of ubiquitylation of H2A by the E3 ligase RNF168, through binding of an additional E3 ligase, RNF8 (the action of which is necessary for RNF168 recruitment) to phosphorylated MDC1 (see Table 1 and Figure 5A.) (172-174). RNF8 accumulates at sites of DNA damage, and catalyses the oligo- and poly-ubiquitylation of H2A and H2AX (174, 175). RNF168 binds to H2A.Ub1, 2 and 3 through its MIU (*m*otifs *i*nteracting with *u*biquitin) ubiquitin-binding domains, demonstrated *in vivo* by three recent studies (172, 176, 177). Knockdown of RNF8 reduced H2AX.Ub1 and H2AX.Ub2

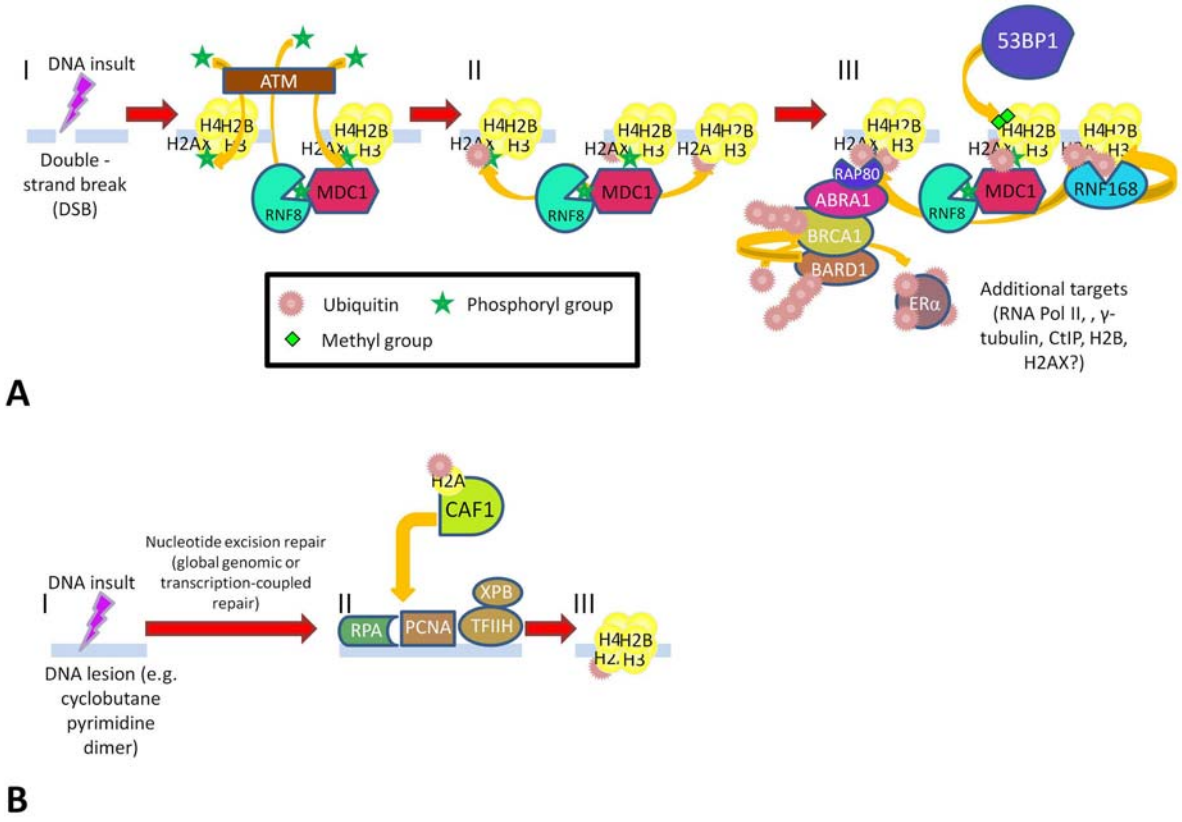

Figure 5. *De novo* ubiquitylation of nucleosome-associated H2A and recruitment of H2A.Ub1 play distinct roles in DNA repair. (A) The ATM (ataxia telangiectasia mutated) kinase is recruited to double-strand breaks in DNA by the MRN sensor complex, enabling the phosphorylation of local H2AX. MDC1 (mediator of DNA damage checkpoint 1) binds to the phosphorylated serine 139 of gamma-H2AX through its BRCT domains, and MDC1 itself is phosphorylated by ATM at an SQ/TQ rich region. This enables the recruitment of the E3 ubiquitin ligase RNF8 to MDC1, via the former's FHA (forkhead associated) domain (I). RNF8 catalyses the initial mono-ubiquitylation of H2A and H2AX (II). A second E3 ligase, RNF168, binds to mono-, di- and poly-ubiquitylated H2A via its MIU (motifs interacting with ubiquitin) domains, and also catalyses ubiquitylation of H2A/H2AX (as well as the generation of lysine 63-linked ubiquitin conjugates). Stabilization and/or amplification of the initial RNF8-mediated histone ubiquitylation signal by RNF168 is required for the sTable recruitment of repair complexes, including 53BP1 (ubiquitylation may relax chromatin structure, exposing H4K20me2 marks required for 53BP1 binding) and the BRCA1/BARD1 E3 ubiquitin ligase (via its interaction with RAP80), the latter subsequently undergoing auto-ubiquitylation, potentiating its ligase activity. BRCA1/BARD catalyses both the mono- and poly-ubiquitylation of several target proteins, including the estrogen receptor ERα as shown (III). (B) In contrast to the *de novo* ubiquitylation of H2A by RNF8/RNF168, H2A.Ub1 is recruited to, and incorporated into nucleosomes following nucleotide excision repair (NER), as part of the process that restores chromatin structure post-repair (I). The histone chaperone, CAF1, is required for recruitment of H2A.Ub1 to sites of DNA damage, and was found to interact with the XPB component of TFIIH (a NER factor), PCNA, a processivity factor for DNA polymerase delta, and RPA p70, the DNA binding domain of the replication protein A complex, as detected by chromatin immunoprecipitation (II). The H2A.Ub1 foci persist for at least 24 hours following DNA damage, suggesting incorporation into chromatin (III).

in HeLa cells, and made the cells more sensitive to ionizing radiation (173). Conversely, knockdown of RNF168 reduced the accumulation of conjugated ubiquitin at sites of DNA damage, preventing the accumulation of repair factors (172). This suggests that whilst RNF8 is required for an initial round of ubiquitylation, subsequent recruitment of RNF168 is required to amplify the amount of H2A.Ub1 on the chromatin to a level required for physiological function (172). The observation that BRCA1/BARD1 is amongst the repair factors that fail to accumulate at DNA double-stranded breaks in RNF168-deficient cells is fascinating, as BRCA1/BARD1 itself has E3 ligase activity (178, 179). As such, Doil *et al.* (2009) propose that the accumulation of ubiquitylated substrates at damaged chromatin may be initiated by RNF8, amplified by RNF168, and subsequently maintained through the action of BRCA1/BARD1 (Table 1) (172). Although BRCA1/BARD1 has been reported to ubiquitylate both H2A and H2B *in vivo* (53), there is no evidence for whether this occurs within the context of DNA damage. Perturbations of this pathway are observed on infection of HeLa cells with herpes simplex virus type I (HSV1); the ICPO E3 ubiquitin ligase of HSV1 was found to ubiquitylate RNF8 *in vitro*, and this is believed to result in

the degradation of RNF8 and RNF168, and resulting loss of H2A.Ub1 and H2AX.Ub1 observed *in vivo* (180). As expected, infection also prevents accumulation of DNA repair proteins at DSBs; the authors suggest that perhaps either RNF8 or ubiquitylated H2A/H2AX are detrimental to viral replication, on the basis that the plaque-forming efficiency of a virus lacking IPCO is partially rescued in RNF8-deficient cells (180). An investigation by Wu *et al.* (2009) identified that the increase in H2A.Ub1 and H2B.Ub1 on DNA damage was correlated with an increase in RAP80 association with these histones. RAP80 binds to H2A.Ub1 and H2B.Ub1 via its UIM (ubiquitin-interacting motif) domains (175), and is required for bringing BRCA1 to DNA damage sites as part of a complex that also includes CCDC98 (181). In order for the cell cycle to resume, the ubiquitylation markers on histones need to be removed, thereby allowing dephosphorylation of H2AX (reviewed by Vissers *et al.*) (85). The human ubiquitin ligase USP3 deubiquitylates both H2A.Ub1 and H2B.Ub1 *in vivo*, and knockdown of this ligase resulted in delays in S phase progression due to the accumulation of DNA breaks and activation of DNA damage repair pathways, underlying the importance of the removal of this marker (84).

Acetylation of H2AX by TIP60 histone acetyltransferase in response to ionizing radiation has also been demonstrated to be necessary for subsequent mono- and poly-ubiquitylation of the same histone by the E2 conjugating enzyme UBC13 (182, 183), suggesting a complex network of histone modifications in response to DNA damage. Interestingly, UBC13 was found to become associated with TIP60 on irradiation, but TIP60 is not necessary for recruitment of UBC13 to H2AX (183). Photo-bleaching of HeLa cells expressing GFP-H2AX revealed that UV-irradiated regions exhibited rapid recovery of fluorescence not seen in un-irradiated regions, that is postulated to be due to release of unbleached GFP-H2AX from chromatin. This release is dependent on both acetylation and poly-ubiquitylation of H2AX, and may be important for enabling repair proteins to access double-stranded breaks (183). Cells expressing a TIP60 mutant without histone acetyltransferase activity exhibited defective formation of repair protein RAD51 foci following UV-irradiation, which may be related to its effects on H2AX release (183), and DT40 cells lacking *UBC13* expression were reported to show greater sensitivity to DNA-damaging agents, through defects in DNA repair (182).

Bergink *et al.* (2006) reported that the UV induced mono-ubiquitylation of H2A is dependent on functional nucleotide excision repair (NER), as ubiquitin accumulation was undetected in cell lines deficient for this pathway (169). It was also observed that the DNA-damage-activated kinase ATR is required for ubiquitylation, but that gamma H2AX was not necessary, suggesting independent activation pathways for the two modifications (169). However, in a recent study, Zhu *et al.* (2009) found that rather than H2A being directly ubiquitylated in response to DNA damage, the histone chaperone CAF-1 is necessary for recruitment of H2A.Ub1 to DNA lesions and that H2A.Ub1 subsequently persists for 24 hours post-irradiation, suggesting an incorporation of the histone into local chromatin (see Figure 5B.) (184). The incongruity between the results was speculated to be due to the different doses of UV irradiation used by the two studies. It is hypothesized that the presence of H2A.Ub1 may act as a marker of prior DNA repair within chromatin (184). As for the earlier study by Bergink *et al.* (2006), the intact NER pathway was shown to be required for recruitment of H2A.Ub1, and the authors note that this may be explained through the finding that NER recruits CAF-1 to the damage site (184, 185).Therefore, it seems that whilst ubiquitylation of H2A and gamma H2AX by RNF8 is important for recruitment of repair proteins at DSBs, targeting of H2A.Ub1 to DNA damage sites is important for subsequent reassembly of nucleosomes and resumption of the cell cycle following UV-induced NER (Figure 5).

Roles for histones H3 and H4 in DNA repair have also been described (59, 60). As mentioned earlier, UV irradiation of cells was found to strongly induce ubiquitylation of H3 and H4 in HeLa cells, an effect abolished on RNA_i-mediated knockdown of the CUL4 component of the CUL4-DDB-ROC1 E3 ligase (see Table 1) (59). Evidence for a direct effect of CUL4 came from assaying ubiquitylation of H3 *in vitro*, using mononucleosomes assembled from either UV damaged or undamaged DNA (59). Furthermore, knockdown of CUL4 was found to reduce the ability of UV-irradiated cells to repair thymine dimers, an effect that is especially marked during the first 1-2 hours. This was correlated with impaired localization of the repair protein XPC to DNA damage sites (59). UV irradiation of HeLa cells was observed to not only increase the amount of H3.Ub1, but to alter the distribution of the histone within the cell; H3.Ub1 in the nuclear pellet fraction decreased, with a corresponding increase in the nuclear extract and cytoplasmic fraction (59). This effect was dependent on CUL4. Based on these observations, the authors proposed a model in which CUL4-DDB-ROC1 is recruited to damaged chromatin, and subsequent ubiquitylation of H3/H4 causes histone eviction. It was hypothesized that this may make the damaged DNA accessible to repair proteins, including XPC (59). Whilst this study found that UV irradiation induced ubiquitylation of H3/H4, but had no effects on H2A.Ub1, a study by Kapetanaki *et al.* (2005) found that UV irradiation of lymphoblastoid cells resulted in a *loss* of H2A.Ub1, and subsequent recovery of normal levels (186). The lack of recovery in cells with mutations in the *DDB2* gene (encoding part of the CUL4-DDB-ROC1 complex) implicated this E3 ligase in ubiquitylation of H2A, supported by the observation that DDB1 (the binding partner of DDB2) can be co-immunoprecipitated with H2A.Ub1 from control, but not *DDB2* mutant lymphoblastoids (186). Wang *et al.* (2006) observe that the discrepancy in the results between the two studies may be a consequence of the different cell lines utilized (HeLa vs lymphoblastoid cell lines); CUL4-DDB-ROC1 has been demonstrated to be capable of ubiquitylating all core histones *in vitro*, and as such, it is perhaps not surprising that ubiquitylation of H2A by this E3 ligase has also been reported *in vivo* (59). The BBAP E3 ligase has also been shown to mono-ubiquitylate H4 at K91 *in vitro* and *in vivo*

(HEK293 cells) (60). The active ligase activity of BBAP is required for its chemoprotective effects on treatment with hydroxyurea or doxorubicin, and there are also fewer 53BP1 repair foci in cells depleted of BBAP. As 53BP1 is recruited to histones through an interaction with H4K20me1 and H4K20me2 (187), the extent of H4 ubiquitylation and methylation in this process were investigated (60). As predicted, doxorubicin-induced DNA damage increased the amount of BBAP in cells, with similar kinetics to an observed increase in H4.Ub1, as well as H4K20me2 (with both histone modifications being lost on siRNA knockdown of BBAP, indicating their dependence on this E3 ligase) (60). Recruitment of the PR-SET7/SET8 methylase (that catalyses mono-methylation of H4K20, a requirement for its di-methylation) to chromatin is also disrupted in BBAP-depleted cells, leading the authors to propose that DNA damage increases BBAP expression, and the resulting increase in H4.Ub1 somehow recruits PR-SET7/SET8 to histones (60). In this way, H4.Ub1 helps mediate DNA repair, through trans-regulation of H4 methylation.

9. ADDITIONAL ROLES FOR HISTONE UBIQUITYLATION

Finally, a requirement for histone ubiquitylation has also been reported in the processing of certain mRNAs, cell cycle progression, gene gating and recombination (73, 152, 188, 189). In addition to the well-documented effects of H2B.Ub1 on histone eviction and deposition described above, it was recently reported that histone ubiquitylation is related to the control of processing of mRNA transcripts encoding histones in human cell lines (152, 190, 191). Initially, it was discovered that RNA_i-mediated knock down of the major mammalian E3 ubiquitin ligase, hBRE1/RNF20, and the resulting loss of H2B.Ub1, was correlated with up- or down-regulation of expression for subsets of genes, in an effect partially independent of H3 methylation (152). The genes found to be down-regulated included those encoding for H2A and H2B, suggesting a role for H2B.Ub1 in *de novo* synthesis of histones (152). Evidence for the mechanism by which H2B.Ub1 may up-regulate a select sub-set of genes despite its ubiquitous presence on active genes, came from the finding that cyclin-dependent kinase 9 (CDK9)-mediated phosphorylation of serine 2 of the C terminal domain of RNA Pol II is essential for maintenance of global H2B.Ub1 levels in human cells (191) (interestingly, not observed in budding yeast). Phosphorylation of serine 2 is important for post-transcriptional events, including elongation and splicing. CDK9 also targets negative elongation factor (NELF) for phosphorylation, and interestingly, NELF, in conjunction with nuclear cap binding complex (CBC), is required for 3' end processing of mRNA transcripts encoding histones (190). Absence of these proteins causes aberrant accumulation of polyadenylated forms of replication-dependent histone mRNAs (that, unlike replication-independent transcripts, end in a conserved stem-loop rather than a polyadenosine tail, which necessitates cleavage by the U7 small nuclear ribonucleoprotein (snRNP)) in HeLa cells) (190). Knock down of CDK9 also increases the polyadenylated form of histone mRNAs, to a similar level observed for NELF, whilst RNF20 or RNF40 knock down causes a smaller increase (indicative of either CDK9-controlled effects on mRNA processing that are independent of H2B.Ub1, or redundant action of multiple ubiquitin ligases). Further evidence for an involvement of H2B.Ub1 in 3' end processing of mRNAs came from the enrichment of RNF20, RNF40 and H2B.Ub1 at the 3' end cleavage site of the *HIST1H2BD* gene (191). A decrease in the processing factors SLBP and LSM11 at this site was observed on knock down of CDK9, indicating the requirement of the latter for recruitment of these factors. As such, it seems that ubiquitylation of H2B.Ub1 is dependent on the action of CDK9, and that this is, in part, required for the recruitment of mRNA processing factors that recognize the 3' cleavage site, possibly through direct binding, or alteration of chromatin structure, thereby facilitating binding of these factors. The observation that histone ubiquitylation is able to control processing of mRNA is interesting, as it would seem to imply a close relationship of the 3' end of mRNA with chromatin, suggesting ties with transcription.

Initial evidence for the involvement of protein ubiquitylation in cell cycle progression came from the finding that murine cells from lines with temperature-sensitive mutations of E1 ubiquitin-activating enzymes underwent cell cycle arrest at restrictive temperatures (192-195). Elevated levels of H2A.Ub1 were detected in transformed human cells as compared to normal cell lines, and a relationship with DNA replication was implied by the findings that levels were further increased on inhibition of replication, and that H2A.Ub1 co-localized with PCNA/cyclin foci (a marker of nuclei involved in DNA replication) (196). A role for H2B.Ub1 in the control of the cell cycle is also implied, through helping to dissociate the phosphatase Cdc14 from chromatin during early anaphase (197). These effects are believed to be mediated through downstream methylation of H3K4, as double mutants of *set1* delta and the gene encoding GTP exchange factor, *lte1* (which regulates the Mitotic Exit Network (MEN)) are synthetic lethal, which is also observed for double mutants of *bre1* delta and *let1* delta (197). This was correlated with defects in Cdc14 release by indirect immunofluorescence (197). The observation that the activator of cell-cycle progression, Myc, recruits the human H2A and H2B ubiquitin protease USP22 to promoters, and that shRNA-mediated knockdown of expression of this enzyme causes G1 arrest of human H1299 cells also implicates histone ubiquitylation in cell cycle control (73). This may be due to its effects on transcription (see sections 3 and 4). Evidence that implicates a requirement for other ubiquitin proteases in mammalian cell cycle control underscores the fact that these enzymes are not redundant in function, although this may reflect the existence of additional non-histone targets (84, 87). For example, knockdown of USP16 (Ubp-M) in HEK-293 cells causes mitotic defects (87), and depletion of USP3 causes delays in S-phase progression, as previously mentioned (84). Transfection of Chinese hamster ovary (CHO) cells with an inactive form of USP16 was also found to cause cells to cease dividing and ultimately undergo apoptosis (88). Furthermore, it was found that USP16 is required for H3S10 phosphorylation, as

H2A.Ub1 prevents the association of the Aurora B kinase with nucleosomes (87). Phosphorylated H3S10 is believed to be required for appropriate condensation of chromatin (198, 199), and as such H2A.Ub1 may maintain an open conformation through its influence on phosphorylation.

There is also evidence to suggest that ubiquitylation of H2B is linked to gene gating (the targeting of genes to the nuclear pore complex (NPC)) (189, 200). Sgf73, the yeast orthologue of human Ataxin 7, is required by the deubiquitylation modules in both SAGA and SLIK to deubiquitylate H2B *in vitro*, and higher global levels of H2B.Ub1 are observed in *sgf73* delta mutant strains (189, 200). Sgf73 is not only associated with the deubiquitylation machinery of SAGA and SLIK, but it is also required for the recruitment of components of the TREX-2 mRNA export complex to SAGA, essential for targeting of TREX-2 to the NPC, and its effects on gene gating (189). *GAL1* gene recruitment to the nuclear periphery was found to be impaired not only in the *sgf73* delta mutant, but also in a catalytically inactive mutant of the ubiquitin protease *ubp8*. A significant reduction in cytoplasmic *GAL1* mRNA is also observed in *sgf73* delta, indicative of a defect in mRNA export, and this is also seen in *ubp8* delta mutants, albeit to a lesser extent. Köhler *et al.* (2008) speculate that the observed defect in gene gating may be due to defective deubiquitylation of H2B.Ub1, due to the strong correlation between the increase in H2B.Ub1 and decrease in cytoplasmic located mRNA, although contributions of other targets cannot be discounted (189).

A recent study has cast further light on the importance of histone ubiquitylation on chromatin structure, by implicating H3.Ub1 in V(D)J recombination in lymphocytes (see Figure 6) (188). V(D)J recombination is critical for immune system function, as it generates a wide repertoire of immunoglobulin and T cell receptors that are necessary for the recognition of challenging antigens (for reviews, please see Jones *et al.* 2009; Swanson *et al.* 2009) (201, 202). This process requires RAG1 and RAG2 (recombination activating genes 1 and 2), that generate double-stranded breaks between recombination signal sequences (RSS) and the flanking gene sequences as the initial step of recombination (202). The N terminal domain of RAG1 contains a conserved RING finger motif, which is common to a large number of E3 ligases, including Bre1 (26, 203, 204). This domain was found to bind to and mono-ubiquitylate H3 *in vitro* and *in vivo* (HEK-293 cells) (188). Point mutations within the RING motif reduce the ability of RAG1 to mono-ubiquitylate H3, without affecting either H3 binding or the endonuclease activity of the core region *in vitro*. However, it was found that abrogation of the ubiquitin ligase activity of RAG1 affected the recombination frequency of an episomal substrate into chromatin, with an increased quantity of RSS DNA break intermediates accumulating in cells transfected with mutated RAG1, compared to those expressing the wild-type protein (188). The requirement for RING motif function was confirmed in a more physiologically relevant context, by expressing wild-type or mutated RAG1 in *RAG1*-/- B cells; fewer cells expressing the mutant protein were observed to undergo D_H-to-J_H rearrangements at the IgH locus (188). Whilst it cannot be concluded that the effects on recombination observed are not in part due to ubiquitylation of other targets by, or auto-ubiquitylation of RAG1, Grazini *et al.* (2010) suggest that H3.Ub1 may mark nucleosomes at broken ends for repair, based on the strong correlation between H3 ubiquitylation activity and recombination frequency observed for RAG1 mutations (188). They consider a number of possible mechanisms through which ubiquitylation may be linked to repair, based on the assumption that H3.Ub1 may act as a 'bridge', to recruit DNA repair proteins directly, or as a 'wedge' (205) to make the area accessible to repair proteins, perhaps through promoting eviction of nucleosomes or the postcleavage complex, or indirectly via recruitment of ATP-dependent chromatin remodeling complexes (188). However, the discovery that H2B.Ub1 increases the stability of the surrounding nucleosome (6) perhaps suggests that ubiquitylation of H3 may have a similar effect; this would support the initial 'bridge' proposal, although any such effects of H3 ubiquitylation on stability and protein binding remain unreported.

10. CONCLUSION

There is accumulating evidence that supports the mono-ubiquitylation of histones as being an integral part of regulating histone dynamics, in terms of eviction and reassembly of nucleosomes, and regulating interaction with linker histones and non-histone proteins. However, despite the leaps made in our understanding of the contribution of histone ubiquitylation to the regulation of gene activity and repair, a number of key issues remain unresolved. Crucially, we do not as yet fully understand the mechanistic basis of how H2B.Ub1 affects transcriptional elongation. Contrary to the previously held belief that ubiquitylation serves to open up chromatin structure, thereby making it more accessible to modifying enzymes, the observed increase in stabilization of nucleosomes on ubiquitylation suggests otherwise (6). As previously discussed, it was hypothesized that the increase in stability may provide a suitable binding site for Spt16 and other factors, before subsequent deubiquitylation of H2B.Ub1 enables Spt16-mediated eviction. However it is not known how H2B.Ub1 increases nucleosomal stability, nor how such changes are mechanistically linked to the recruitment of Spt16 (6). Whilst H2B.Ub1 may be necessary to recruit Spt16 at active genes, the H2B.Ub1-induced stabilization of histones at the *ARG1* gene TATA region is correlated with gene silencing (148), highlighting the requirement for additional gene-specific factors to ubiquitylate histones at promoters or open reading frames. Additionally, it remains unclear as to how histone ubiquitylation affects DNA recombination (188), and the exact nature of the purported interaction between RAP80 and H2A.Ub1/H2B.Ub1 in the DNA damage response (175). Whereas H2A.Ub1 has been reported to exhibit physical interactions with several proteins (118, 172-174), there is currently little evidence for the existence of direct binding partners of the ubiquitylated form of H2B.Ub1, beyond the finding that RAP80 binds both ubiquitylated histones (175). It is interesting that ubiquitylation of H2A and H2B appear to exert their downstream effects through different

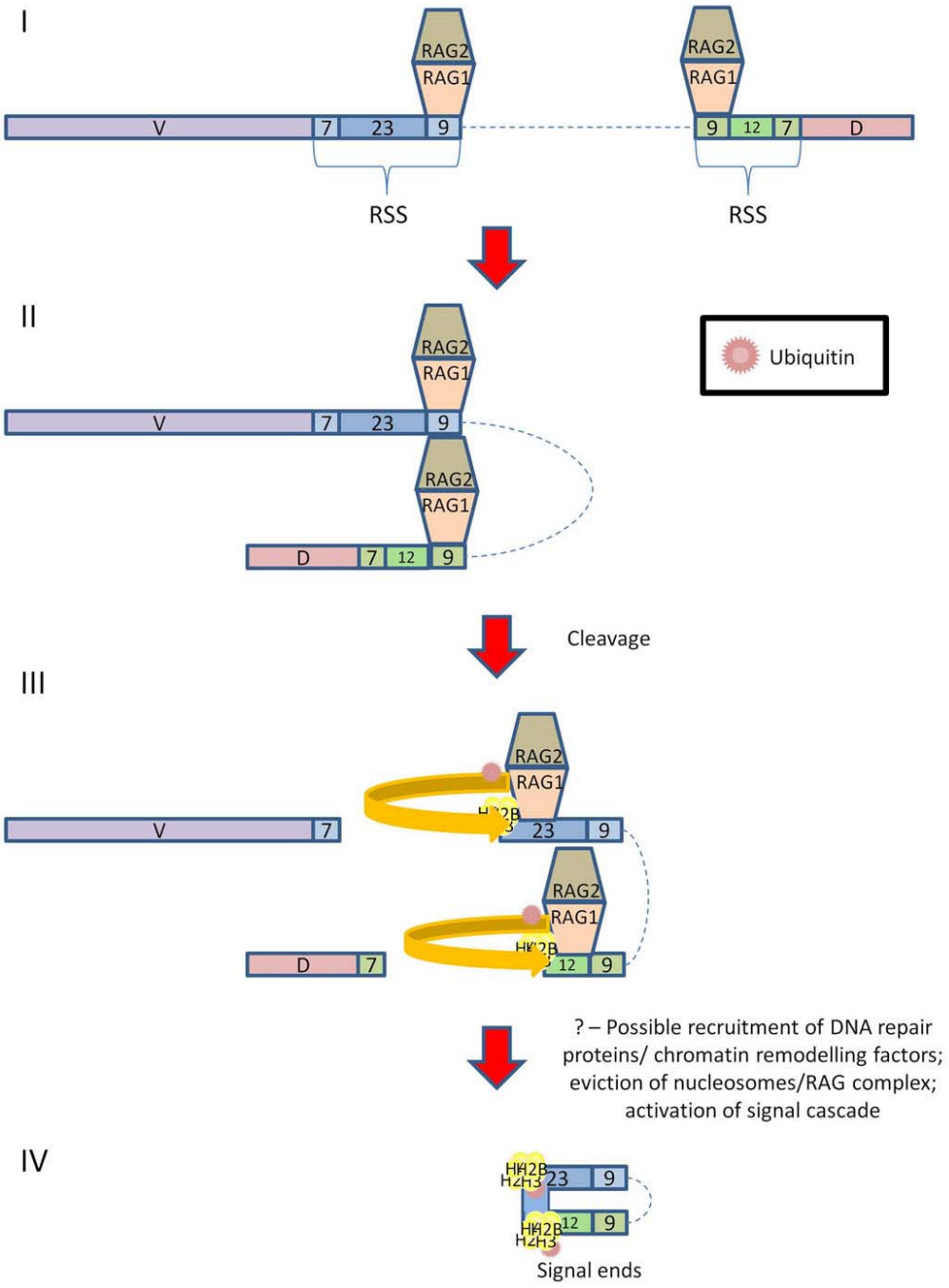

Figure 6. V(D)J recombination (the random recombination of variable (V), diversity (D) and joining(J) exons) is the process whereby diversity of T cell receptor and immunoglobulin genes is generated in the vertebrate nervous system. RAG1/ RAG2 (recombination activating gene 1 and 2) heterodimers recognize and bind to a conserved nonamer sequence within recombination signal sequences (RSS) that flank the V, D and J exons (I). The RSS consist of conserved heptamer and nonamer sequences separated by either a 12 or 23 base pair sequence; only segments flanked by RSS of different lengths can be recombined. The RAG1/RAG2 complexes assemble the 12 and 23 RSS into a synaptic complex, and catalyze double-stranded cleavage at each RSS (II). The N terminus of RAG1 binds H3, and catalyses its mono-ubiquitylation (III). Disruption of the E3 ligase activity of RAG1 disrupts V(D)J recombination and signal end joining *in vivo* (coding end joining is also affected, albeit to a lesser extent), and a strong correlation between levels of H3.Ub1 and recombination efficiency is observed, although this does not exclude the possibility that additional ubiquitylated targets of RAG1 are involved in this process. Substrate ubiquitylation may be important for V(D)J recombination through leading to the eviction of nucleosomes or associated proteins, or providing a landing platform for modifying factors, thereby enabling DNA ligation at the RAG1 bound signal ends (IV).

mechanisms (namely, directly recruiting additional effectors, and somehow altering nucleosome stability), but it seems feasible that additional interacting proteins for H2B.Ub1 may be identified in the future (especially when one considers the number of interacting factors for RAP80 (206)), and this may help in elucidating its mode of function in regulating nucleosome stability and V(D)J recombination.

The extent and biological significance of poly-ubiquitylation of H2B in budding yeast remains uncertain, as does the identity of the ubiquitylation machinery that target sites other than K123 (40). Systematic site-directed mutagenesis of each lysine residue within H2B to arginine, followed by re-introduction of a single lysine and examination of histone ubiquitylation status and gene expression *in vivo*, may serve as a comprehensive, albeit laborious method of identifying the extent and perhaps function of poly-ubiquitylated lysine residues, although there remains the possibility that they have redundant/interacting effects with one another that would complicate any findings. Perhaps more interesting is the possibility that some of the effects previously attributed to H2B.Ub1 may in actuality be mediated in part by poly-ubiquitylated K123, as effector proteins may have greater affinity for a long poly-ubiquitin chain than a single ubiquitin protein, as noted by Geng and Tansey (2008) (40). *In vitro* binding studies using poly-ubiquitylated H2B and proteins such as Spt16 or 19S may begin to answer this question, although this is technically challenging. It would also be interesting to determine the full extent of ubiquitylation-mediated effects on gene transcription, by examining the ubiquitylation status of histones (especially, H2A and H2B) and any associated ubiquitin ligases/proteases at different chromatin regions in yeast and other model eukaryotes, through the use of ChIP (chromatin immunoprecipitation) sequencing and ChIP-on-chip microarrays to characterize selected transcriptionally-active or silenced regions, and ultimately, the entire genomic coding region, as was recently performed for human H2B.ub1 (110). This may enable us to identify regulatory DNA elements marked by histone ubiquitylation, under different physiological conditions. This would possibly spur the identification of additional processes regulated by histone ubiquitylation. Other areas of interest for future investigation include the relationship between gene gating and transcription; a cycle of reiterative ubiquitylation and deubiquitylation of H2B is important for transcriptional elongation, and gene gating has a requirement for the deubiquitylating activity of Ubp8 as well, suggestive of a link between the two processes (30, 189). It seems possible any such interaction may be mediated through RNA Pol II, thereby coupling transcription to targeting of the mRNA. Another emerging area of interest is the relationship between histone modifications and splicing (207-212). Several recent studies have reported increased nucleosome occupancy at exons as compared to flanking introns (207, 210, 212), and certain modifications (methylations of H2B and H3) are enriched on exon-associated nucleosomes (207, 209, 211). In addition, it has emerged that exons with weaker splice sites exhibit greater nucleosome occupancy (211) and alternatively spliced exons have reduced levels of H3K36me3 compared to constitutively spliced neighboring exons (208, 209). Whilst there is currently no published work pertaining to the patterns of histone ubiquitylation on alternatively spliced exons, on the basis that H2B.Ub1 deubiquitylation is required for tri-methylation of H3K36 (30), we speculate that there may be a requirement for histone ubiquitylation in splicing. Additionally, as H2B.Ub1 promotes elongation, and it is proposed that a highly processive Pol II may bypass a weak splice acceptor site (i.e. one that is not well recognized by the splicing factors) in favor of a stronger site downstream (213), H2B.Ub1 may be found to lead to exon skipping. Conversely, as H2A.Ub1 represses elongation (52), it may lead to the inclusion of exons with weak acceptor sites. This area will surely be the subject of intense scrutiny in the future. Finally, there is emerging evidence that H2B.Ub1 also regulates the stability of nucleosomes at DNA replication forks in yeast, in an effect that is independent of downstream methylation of H3 (C.-F. Kao *et al.*, unpublished results), underlining the importance of histone ubiquitylation in regulating processes on the DNA template. Clearly histone ubiquitylation, whether through effects on nucleosome stability and structure, or downstream effects on other histone modifications, plays a fundamental role in regulating cellular processes, and we predict that future studies will continue to elucidate on its importance to chromatin regulation.

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Abbreviations: SUMO: small ubiquitin-like modifier; HRF: HIV-1 resistance factor; Ub1: mono-ubiquitin; Pol II: RNA polymerase II; CTD: C-terminal domain; FACT: facilitates chromatin transcription; SAGA: Spt-Ada-Gcn5-Acetyltransferase; SLIK: SAGA-like; SALSA: SAGA altered, Spt8 absent; PAF: RNA polymerase II-associated factor; DUB: deubiquitylating enzyme; GMPS: guanosine 5'-monophosphate synthetase; ChIP-seq: chromatin immunoprecipitation sequencing; TBP: TATA-binding protein; EcR: ecdysone receptor; PRC1: polycomb repressive complex 1; DSB: double-strand break; UIM: ubiquitin-interacting motif; MIU: motifs interacting with ubiquitin; NELF: negative elongation factor; snRNP: small nuclear ribonucleoprotein particle; MEN: mitotic exit network; RAG1/2: recombination activating genes 1/2; RSS: recombination signal sequences.

Key Words: Histone, Ubiquitylation, Ubiquitination, Deubiquitylation, Deubiquitination, H2A, H2B, H1, Histone modifications, Chromatin, Transcription, Review

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