Histone H2B monoubiquitination: roles to play in human malignancy

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Abstract

Ubiquitination has traditionally been viewed in the context of polyubiquitination that is essential for marking proteins for degradation via the proteasome. Recent discoveries have shed light on key cellular roles for monoubiquitination, including as a post-translational modification (PTM) of histones such as histone H2B. Monoubiquitination plays a significant role as one of the largest histone PTMs, alongside smaller, better-studied modifications such as methylation, acetylation and phosphorylation. Monoubiquitination of histone H2B at lysine 120 (H2Bub1) has been shown to have key roles in transcription, the DNA damage response and stem cell differentiation. The H2Bub1 enzymatic cascade involves E3 RING finger ubiquitin ligases, with the main E3 generally accepted to be the RNF20–RNF40 complex, and deubiquitinases including ubiquitin-specific protease 7 (USP7), USP22 and USP44. H2Bub1 has been shown to physically disrupt chromatin strands, fostering a more open chromatin structure accessible to transcription factors and DNA repair proteins. It also acts as a recruiting signal, actively attracting proteins with roles in transcription and DNA damage. H2Bub1 also appears to play central roles in histone cross-talk, influencing methylation events on histone H3, including H3K4 and H3K79. Most significantly, global levels of H2Bub1 are low to absent in advanced cancers including breast, colorectal, lung and parathyroid, marking H2Bub1 and the enzymes that regulate it as key molecules of interest as possible new therapeutic targets for the treatment of cancer. This review offers an overview of current knowledge regarding H2Bub1 and highlights links between dysregulation of H2Bub1-associated enzymes, stem cells and malignancy.

Key Words
- H2Bub1
- histone H2B
- monoubiquitination
- transcription
- DNA damage
- stem cells
- E3 ubiquitin ligase
- RING finger proteins
- RNF20–RNF40
- deubiquitinases (DUBs)
- USP7
- USP22
- USP44

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Introduction

Reversible post-translational modifications (PTMs) in core histones constituting the nucleosome influence the nature of the chromatin landscape. Histone PTMs occur at specific amino acids on histone tails and include acetylation, ADP ribosylation, deamination, methylation, phosphorylation, proline isomerisation, monoubiquitination and sumoylation (Kouzarides 2007, Dawson *et al.* 2012). Modified histones participate in key cellular processes, dictating the accessibility of chromatin to elements that drive gene transcription and proteins that function in DNA damage signalling. Along with DNA methylation, histone PTMs are major components of the epigenome and may become dysregulated during tumourigenesis.

Although less well studied than many other PTMs, histone monoubiquitination, i.e. addition of a single 8.5 kDa (76 amino acids) ubiquitin molecule to specific lysine residues on histone tails, offers new opportunities

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for targeting of the epigenome for the treatment of malignancy. Histones are the most abundantly mono-ubiquitinated conjugates in the nucleus of mammalian cells, including sites of monoubiquitination at lysines on histones H2A and H2B (Clague *et al*. 2008). In contrast to polyubiquitination that marks a protein for proteasomal degradation, monoubiquitination of histones is associated with the transcriptional control of gene expression and the DNA damage response. Furthermore, cleavage of this single ubiquitin from histones H2A and H2B has been reported to be associated with chromatin condensation prior to packaging of DNA into metaphase chromosomes (Mueller *et al*. 1985).

Lysine 120 (K120) at which monoubiquitination of histone H2B occurs (H2Bub1) is the only site of histone monoubiquitination shown to result in physical disruption of chromatin strands, leading to open and accessible fibre conformations (Minsky *et al*. 2008, Fierz *et al*. 2011). K120 of histone H2B is physically placed at the interface of adjacent nucleosomes and, when monoubiquitinated, probably interferes with nucleosome stacking and thus chromatin structure (Fierz *et al*. 2011). Notably, substitution of ubiquitin at K120 of histone H2B with the bulkier modification of small ubiquitin-like modifier (SUMO) does not functionally replace ubiquitin; therefore, epigenetic reprogramming via PTMs at this site cannot be solely explained by steric effects (Chandrasekharan *et al*. 2009, Fierz *et al*. 2011). Consistent with this, H2Bub1 has been shown to act as a recruiting signal for proteins functioning in histone cross-talk, transcription and DNA damage (Shema-Yaacoby *et al*. 2013). Although lysine 125 on histone H2B can be monoubiquitinated *in vitro* when K120 is artificially mutated, it has no known functional role *in vivo* (Minsky & Oren 2004). Lysine 34 on histone H2B can also be monoubiquitinated and is involved in histone cross-talk; however, it appears to utilise different ubiquitination machinery compared with H2Bub1 and its role in the regulation of chromatin structure, if any, remains to be elucidated (Wu *et al*. 2011). Monoubiquitination of histone H2A at lysine 119 does not influence fibre compaction, probably due to its location in the nucleosome (Jason *et al*. 2001). It is interesting then that monoubiquitination of histone H2A is generally associated with silencing gene expression (Osley *et al*. 2006, Weake & Workman 2008), whereas, for the most part, H2Bub1 is associated with increased levels of gene transcription (Minsky *et al*. 2008).

H2Bub1, therefore, appears to play a unique role, fittingly described as a 'master switch' for gene regulation (Kim *et al*. 2005), with important roles in transcriptional elongation (Pavri *et al*. 2006, Minsky *et al*. 2008, Johnsen 2012), maintenance of replication-dependent histone mRNA 3′-end processing (Pirngruber *et al*. 2009) and the DNA damage response (Minsky *et al*. 2008, Kari *et al*. 2011, Moyal *et al*. 2011). H2Bub1 also has roles in the maintenance of stem cell multipotency (Karpiuk *et al*. 2012), regulation of DNA replication (Trujillo & Osley 2012) and maintenance of centromeric chromatin, revealing significant roles in genome stability (Sadeghi *et al*. 2014). Recently, H2Bub1 has been shown to be lost in advanced cancers (Prenzel *et al*. 2011, Hahn *et al*. 2012, Urasaki *et al*. 2012). This review draws together discoveries related to H2Bub1, including those in the area of H2Bub1-associated ubiquitin ligases and deubiquitinases (DUBs) that may offer new opportunities for the design of epigenomic-based cancer therapeutics.

H2Bub1 and associated machinery in primary tumours

Cancer is frequently described as a disease of aberrant gene expression. Identification of H2Bub1 as a key transcriptional regulator raised the likelihood that it may be altered in cancer development and the possibility that H2Bub1 itself may have tumour-suppressive roles (Espinosa 2008). Loss of global H2Bub1 detected by immunohistochemical staining has been reported for a number of cancers, including breast (Prenzel *et al*. 2011), colorectal (Urasaki *et al*. 2012), lung (Urasaki *et al*. 2012) and parathyroid (Hahn *et al*. 2012). In the case of parathyroid cancer, a mechanistic explanation for the loss of H2Bub1 is provided by the frequent occurrence of mutations in the tumour suppressor *CDC73*, leading to the disruption of the RNA polymerase II-associated factor 1 (PAF1) transcriptional complex that is important for the regulation of H2Bub1 (Hahn *et al*. 2012). CDC73 (also known as parafibromin) is a member of the human PAF1 transcriptional complex that forms a larger complex with the RING finger E3 ubiquitin ligases RNF20–RNF40 that are responsible for the mono-ubiquitination of histone H2B (Rozenblatt-Rosen *et al*. 2005, Yart *et al*. 2005, Hahn *et al*. 2012). Notably, *CDC73* was shown to be WT in benign parathyroid tumours where H2Bub1 levels were maintained (Hahn *et al*. 2012). Furthermore, in breast cancer, basal levels of H2Bub1 were found to be unchanged when comparing normal mammary epithelium with benign breast tumours; however, H2Bub1 levels in malignant and metastatic breast cancer cells were found to be significantly reduced (Prenzel *et al*. 2011). The mechanism of H2Bub1 loss in malignant and metastatic breast cancer, lung cancer and colorectal cancer cells remains to be elucidated.

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Emerging evidence also indicates that RNF20 itself functions as a tumour suppressor. Hypermethylation of the *RNF20* promoter in primary breast cancer cells has been reported (Shema *et al*. 2008, 2011), and *RNF20* transcript levels are lower in metastatic prostate cancer cells relative to benign disease cells (Varambally *et al*. 2005). RNF20 levels are also lower in the testicular germ cell cancer seminoma relative to normal testis (Chernikova *et al*. 2012). A single mutation has been reported in *RNF20* in colorectal cancer cells (Barber *et al*. 2008). A recent study of CpG island methylator phenotype 1 (CIMP1)-associated colorectal tumours has shown an enrichment of mutations in genes encoding proteins associated with chromatin remodelling, including a low frequency of mutations in *RNF20* and *RNF40* (Tahara *et al*. 2014). The effect of hypermethylation, or mutations, on RNF20 protein levels was not investigated in these studies; however, the implication is that these events probably lead to less active RNF20 capable of functioning to monoubiquitinate histone H2B.

Differential levels of expression and/or mutations have been reported in other H2Bub1-associated E3 ubiquitin ligases and DUBs in primary tumours, including *BRCA1* mutation in breast and ovarian cancers (Network 2011, 2012, Alsop *et al*. 2012), ubiquitin-specific protease 36 (*USP36*) overexpression in ovarian tumours (Li *et al*. 2008), *USP22* overexpression in multiple aggressive cancer types (Glinsky *et al*. 2005), and specifically, elevated USP22 protein levels in breast cancer progression and patient prognosis (Zhang *et al*. 2011*a*). A key mechanism of action of at least some of these changes is probably through dysregulation of H2Bub1.

H2Bub1-associated transcription

H2Bub1 preferentially associates with the transcribed regions of highly expressed genes. In a model of p53 overexpression, H2Bub1 was found throughout most of the transcribed region of the p53 target gene *CDKN1A* (encoding p21), but not in the upstream or downstream regions (Minsky *et al*. 2008). This region-specific increase in H2Bub1 was shown to correlate with the recruitment of RNA polymerase II (Pol II) before the increase in *CDKN1A* mRNA levels and to decrease again upon dissociation of RNA Pol II (Minsky *et al*. 2008). The human PAF1 transcriptional complex associates with RNA Pol II on the chromatin of actively transcribed genes, where it participates in transcriptional elongation, recruitment and activation of histone modification enzymes, and recruitment of 3′-end processing factors required to terminate transcription (Jaehning 2010; Fig. 1).

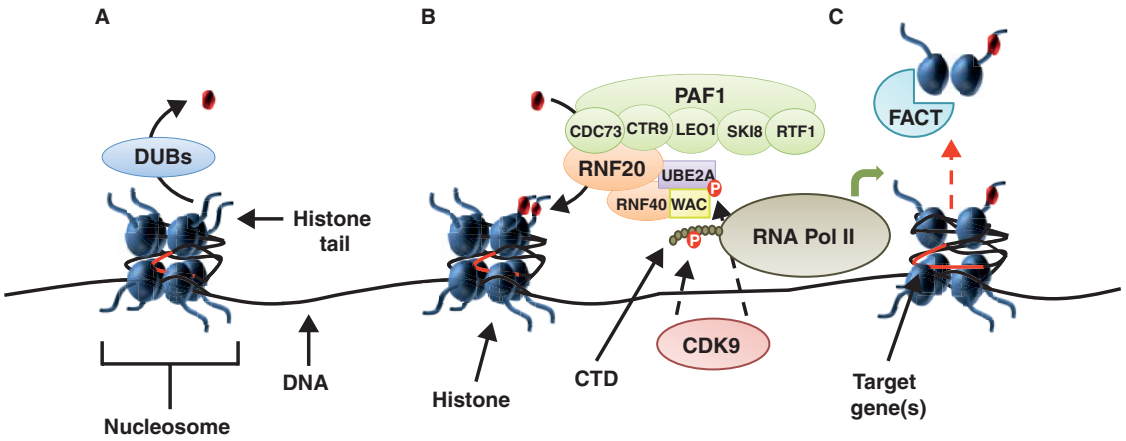

Figure 1

H2Bub1 and associated factors in transcriptional elongation. (A) Nucleosomes function in condensed chromatin to maintain the repression of transcription of target genes (red). The presence of H2Bub1-associated deubiquitinases (DUBs) and consequential absence of H2Bub1 contribute to a closed chromatin configuration. (B) In response to stimuli, pathways that require gene expression are activated. Cyclin-dependent kinase 9 (CDK9) phosphorylates the E2 UBE2A and Ser2 in the carboxy-terminal domain (CTD) of RNA polymerase II (Pol II), creating a binding domain for WAC and the associated RNF20–RNF40 heterodimeric complex that functions as the E3 to monoubiquitinate histone H2B at lysine 120 (ubiquitin represented by a red circle on H2B histone tails). The PAF1 complex (comprising PAF1, CDC73, CTR9, LEO1, SKI8 and RTF1) associates with RNA Pol II at the level of the chromatin and interacts with the RNF20–RNF40 complex to promote transcriptional elongation. Ubiquitin is cleaved by H2Bub1-associated DUBs to return to a closed chromatin structure. (C) H2Bub1 results in the recruitment of the chromatin remodelling factor FACT to facilitate the removal of the H2B–H2A dimer from the nucleosome resulting in the opening up of chromatin and removal of the physical block to RNA Pol II allowing transcriptional elongation to proceed (green arrow) resulting in gene expression.

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The PAF1 complex is composed of LEO1, CTR9, SKI8, RTF1, CDC73 and PAF1 (Jaehning 2010). It is the interaction between this complex and the main H2Bub1 E3 ubiquitin ligase RING finger complex RNF20–RNF40 that is thought to be responsible for the increase in H2Bub1 in actively transcribed genes (Kim *et al*. 2009). Whether it is individual PAF1 complex members or the intact complex itself that is important for H2Bub1 is not entirely clear; however, the PAF1 subunit itself, CTR9 and CDC73 have all been shown to be important for H2Bub1 (Zhu *et al*. 2005, Kim *et al*. 2009, Pirngruber *et al*. 2009, Hahn *et al*. 2012). Furthermore, RNF20, presumably through H2Bub1, interferes with binding of the transcription elongation factor TFIIS to the PAF1 complex, thus affecting the ability to relieve the stalled RNA Pol II and hindering transcriptional elongation (Shema *et al*. 2011). This may be a mechanism whereby RNF20 functions in a tumour-suppressive capacity to selectively repress pro-oncogenic genes that are preferentially residing in compacted chromatin and therefore minimally transcribed under normal conditions.

It has also been reported that the RNF20–RNF40 complex associates with WW domain-containing adaptor with coiled-coil (WAC) and so perhaps indirectly with the PAF1 complex, although the PAF1 complex is required for recruiting the WAC-associated RNF20–RNF40 complex to actively transcribed chromatin (Zhang & Yu 2011). Cyclin-dependent kinase 9 (CDK9) is also involved in this process, required to phosphorylate serine 2 (Ser2) in the carboxy-terminal domain of RNA Pol II, creating a binding domain for WAC at the chromatin level (Pirngruber *et al*. 2009; Fig. 1B).

A number of mechanisms have been proposed to explain the role of H2Bub1 in transcriptional elongation. One compelling model proposes that the histone chaperone FACT is recruited to chromatin at sites containing H2Bub1, resulting in the removal of a histone H2A–H2B dimer from the core nucleosome (Fig. 1C). Disruption of this nucleosomal barrier would then facilitate RNA Pol II to traverse through, enabling transcription (Pavri *et al*. 2006). Furthermore, H2Bub1 and FACT co-operate to influence chromatin dynamics required for DNA repair following the induction of double-strand breaks (DSBs) by affecting the recruitment of DNA repair proteins such as RAD51 (Kari *et al*. 2011).

In contrast, it has been reported that H2Bub1 enhances nucleosome stability (Chandrasekharan *et al*. 2009); however, a more recent study has suggested that the effect of H2Bub1 on nucleosome stability is modest (Fierz *et al*. 2012). Furthermore, in co-operation with the histone chaperone Spt16, H2Bub1 has been indicated to be important for nucleosome reassembly during RNA Pol II-mediated transcriptional elongation in yeast (Fleming *et al*. 2008). To add to the complexity, H2Bub1 has been reported to inhibit the assembly of transcriptional complexes at normally silent promoters, therefore associating H2Bub1 with transcriptional suppression, while also appearing integral for transcriptional elongation of transcribed regions (Batta *et al*. 2011). Perhaps keys to a deeper understanding of the roles of H2Bub1 in transcription, recently described as 'enigmatic' (Johnsen 2012), will be in appreciating the dynamic and reversible nature of this modification, as well as its genomic positioning, cellular type, level of cellular differentiation and function in disease-related or healthy cells.

H2Bub1 functions in DNA damage

Independently from its role in transcriptional elongation, H2Bub1 increases in cells after DNA damage at sites of DSBs. Following such damage, the protein kinase ataxia telangiectasia mutated (ATM) phosphorylates serine sites on both RNF20 and RNF40. This phosphorylated RNF20–RNF40 E3 ubiquitin ligase complex is then recruited to the sites of DSBs where it participates in the monoubiquitination of DNA damage-associated H2Bub1 (Moyal *et al*. 2011). RNF20 functions with NBS1 (a member of the mammalian MRE11–RAD50 DSB repair complex) at sites of DSBs to facilitate repair through SNF2H-mediated chromatin reorganisation (Nakamura *et al*. 2011). This process recruits factors required for both non-homologous end joining (XRCC4 and Ku80) and homologous recombination repair (HRR; RAD51, BRCA1 and BRCA2) (Moyal *et al*. 2011, Nakamura *et al*. 2011, Shiloh *et al*. 2011).

Although treatment of cells with DNA-damaging agents such as doxorubicin has been linked to a global loss of H2Bub1, specific areas of the genome, perhaps those encoding proteins that are key to mounting the DNA damage response, retain or increase H2Bub1 (Minsky *et al*. 2008). As has been mentioned previously, using a model of p53 overexpression, H2Bub1 was identified at the transcribed region of the p53 target *CDKN1A*, which correlated with the recruitment of RNA Pol II and increased levels of *CDKN1A* transcripts (Minsky *et al*. 2008).

Roles for H2Bub1 in histone cross-talk

Evidence indicates that H2Bub1 may be central to processes dependent upon chromatin dynamics such as transcription and DNA damage that are reliant on

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histone cross-talk. H2Bub1 can recruit methyltransferase complexes important for the methylation of lysines on histone H3, specifically H3K4 and H3K79 (Briggs *et al.* 2002, Ng *et al.* 2002, Sun & Allis 2002, Barski *et al.* 2007, Kim *et al.* 2009, Izzo & Schneider 2010, Wang *et al.* 2013*a*). A number of studies have suggested that H2Bub1 is a prerequisite for H3K4 di- and trimethylation (H3K4me2 and H3K4me3), as H2Bub1 recruits the histone methyltransferase complex COMPASS (complex of proteins associated with SET1) involved in the methylation of histone H3 (Wood *et al.* 2005, Kim *et al.* 2009, Smith & Shilatifard 2010). Methylated H3K4 then recruits the chromatin remodelling factor SNF2H (Kouskouti & Talianidis 2005), allowing recruitment of the DNA repair proteins RAD51 and BRCA1 (Nakamura *et al.* 2011). Still, other studies have shown that H2Bub1 may not be a prerequisite for H3K4 methylation (Shema *et al.* 2008, Moyal *et al.* 2011, Vethantham *et al.* 2012), including in primary tumours where H2Bub1 is lost, but H3K4me3 is retained (Hahn *et al.* 2012).

Similar to H3K4me2 and H3K4me3, H3K79me3 has also been identified in transcriptionally active genes. H2Bub1 has been shown to directly stimulate disrupter of telomere silencing 1-like (DOT1L) methyltransferase activity and consequently to facilitate H3K79 methylation in both yeast and human models (Ng *et al.* 2002, McGinty *et al.* 2008). DOT1L is the sole methyltransferase responsible for the methylation of H3K79 (Min *et al.* 2003). H3K79 methylation marks localise preferentially to the proximal regions of actively transcribed genes (Steger *et al.* 2008) and have in fact been found co-localised with H2Bub1 in actively transcribed regions (Jung *et al.* 2012). Although somewhat controversial, the recruitment of the DNA repair protein 53BP1 (TP53BP1) to sites of DSBs has been reported to be dependent upon H3K79 methylation (Huyen *et al.* 2004, Wakeman *et al.* 2012, Kim *et al.* 2014). Increased levels of methylation of both H3K4 and H3K79 have been shown to increase the expression of *HOX* genes in a manner dependent upon H2Bub1, indicating additional and important roles for H2Bub1 in the regulation of development (Zhu *et al.* 2005).

Less well studied than histone H3 methylation complexes, acetylation of K120 on histone H2B (H2BK120ac) is reported to precede H2Bub1 in a temporal fashion (Gatta *et al.* 2011). Earlier research has shown H2BK120ac to be present on the promoters of active genes (Wang *et al.* 2008). These studies suggest that H2BK120ac is an early mark of poised or active chromatin and, further, that this dual histone switch may keep nucleosomes 'hot' for rounds of induction and transcriptional elongation. Additional research is required to elucidate the physiological role(s) of H2BK120ac. K120 on histone H2B has also recently been shown to be methylated by the methyltransferase enhancer of zeste homolog 2, which is the catalytic unit of polycomb repressive complex 2 (Kogure *et al.* 2013). This modification would appear to act as a competitive inhibitor for H2Bub1 and may, at least in part, explain the loss of H2Bub1 observed in cancer cells. The control and role of H2Bub1 in histone cross-talk are clearly both complex and influential. It remains to be determined whether epigenomic-based therapeutics developed to treat malignancy, such as histone deacetylase inhibitors, functionally modify levels of H2Bub1 and consequently the expression of genes and other interactions in which H2Bub1 plays a role.

H2Bub1-associated ubiquitin ligation machinery

Ligation of ubiquitin to a protein requires an activating ATP-dependent ubiquitin enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) that works in conjunction with E2 to covalently attach ubiquitin to a lysine residue of the target protein. Humans have just a few E1 enzymes, around 40 E2 enzymes and over 500 E3 ligases (Lipkowitz & Weissman 2011, Budhidarmo *et al.* 2012). It is perhaps not surprising then that E3s determine substrate recognition and are often members of multicomponent complexes (Braun & Madhani 2012). The most common type of E3 ligases are RING (really interesting new gene) domain proteins, HECT (homologous to E6-AP C terminus) domain E3s being less frequent (Jackson *et al.* 2000, Budhidarmo *et al.* 2012). Histone ubiquitination is a reversible modification, negatively regulated by DUBs (Fig. 2). There are ~100 DUBs classified into six subfamilies, with the majority being cysteine proteases (Clague *et al.* 2013, Jacq *et al.* 2013). DUBs reported to deubiquitinate H2Bub1 belong to the USP subfamily. Recent studies have identified DUBs as having major roles in normal physiological processes and, when aberrantly active, roles in disease including cancer (Nijman *et al.* 2005, Singhal *et al.* 2008, Sowa *et al.* 2009, Clague *et al.* 2013, Jacq *et al.* 2013). Furthermore, as proteases, DUBs are realistic therapeutic targets for the treatment of malignancy (Colland 2010).

Terminology coined recently in the field of epigenomics describes histone modifications that are laid down by chromatin 'writers', removed by chromatin 'erasers', and used as signals to be interpreted and then acted upon by chromatin 'readers' such as molecules described above

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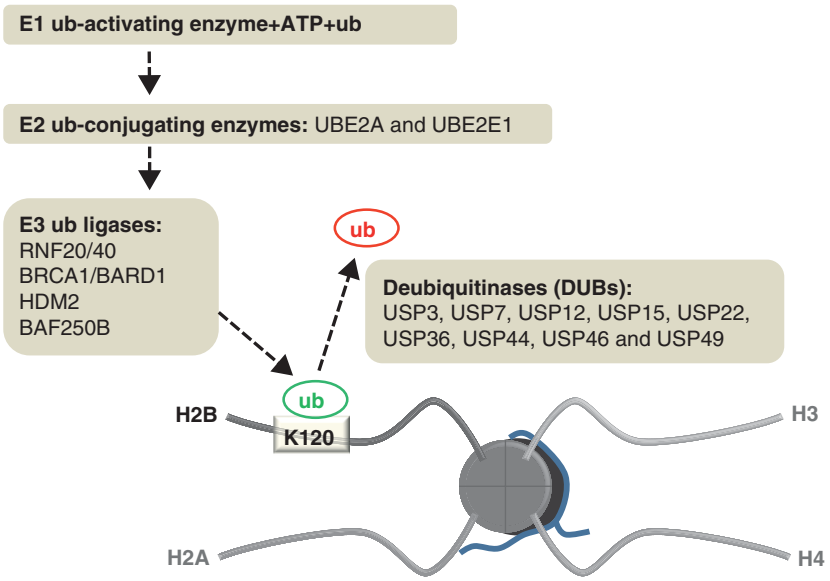

Figure 2

Ubiquitin enzyme cascade associated with the monoubiquitination of histone H2B at lysine K120 (H2Bub1). An E1 ubiquitin (ub)-activating enzyme engages ATP to activate free ubiquitin, which is transferred to the active site of an E2 ubiquitin-conjugating enzyme, two of which have been reported to be associated with H2Bub1. E3 ubiquitin ligases work with E2 to transfer ubiquitin to the target protein. Four E3s, most of them appearing in RING finger complexes, have been identified in the H2Bub1 cascade; however, it is unclear how, or if, they function together for the monoubiquitination of histone H2B. Deubiquitinating (DUB) enzymes, of which nine have been reported to be associated with H2Bub1, cleave ubiquitin from its substrate.

important for histone cross-talk (Dawson *et al.* 2012). Key writers and erasers of H2Bub1-associated ubiquitin machinery are discussed below.

'Writing' H2Bub1: as easy as E1, E2 and E3

E1 is the first enzyme in the ubiquitin pathway, activating free ubiquitin in an ATP-dependent fashion by formation of a thiol–ester bond between its active cysteine site and the carboxy-terminal glycine of ubiquitin. Activated ubiquitin is then transferred to the active site of an E2 ubiquitin-conjugating enzyme (Fang & Weissman 2004). The ubiquitin-conjugating enzyme 2A (UBE2A; yeast homolog RAD 6) is generally believed to be the chief E2 involved in H2Bub1 (Kim *et al.* 2009); however, another E2, UBE2E1, has also been reported to function in H2Bub1 (Zhu *et al.* 2005, Pavri *et al.* 2006). CDK9, in addition to phosphorylating Ser2 in the carboxy-terminal domain of RNA Pol II as mentioned previously, also phosphorylates UBE2A, increasing its activity (Shchebet *et al.* 2012, Zhang & Yu 2012). Interestingly, UBE2A also functions as the E2 for the monoubiquitination of proliferating cell nuclear antigen, a key protein activated in DNA repair (Zhang & Yu 2012). Multiple E3s are involved in H2Bub1, including MDM2 (Minsky & Oren 2004), BRCA1–BARD1 (Mallery *et al.* 2002, Thakar *et al.* 2010), BAF250B (Li *et al.* 2010) and the RING finger protein complex RNF20–RNF40, which is generally accepted as the main E3 for H2Bub1 (Zhu *et al.* 2005, Kim *et al.* 2009, Moyal *et al.* 2011, Fuchs & Oren 2014; Fig. 2).

The RING finger complex RNF20–RNF40 The RNF20 homolog BRE1 (also known as BRE1A) was discovered in the yeast *Saccharomyces cerevisiae*, where it was found to be the major E3 ligase involved in the monoubiquitination of yeast histone H2B at lysine 123 (Hwang *et al.* 2003). In humans, RNF20 forms a hetero-tetrameric complex with RNF40 (also known as BRE1B (Kim *et al.* 2009)), containing two copies of each polypeptide (Zhu *et al.* 2005). The RNF20–RNF40 complex is generally accepted to interact with an E2 conjugating enzyme as the main E3 enzyme to catalyse H2Bub1; however, whether this complex functions as a whole in humans to mediate H2Bub1 is not entirely clear (Moyal *et al.* 2011), as some studies have reported that RNF20 alone is the key functional unit (Kim *et al.* 2005). Down-regulation of either polypeptide leads to depletion of the other, as well as reduction of H2Bub1 (Moyal *et al.* 2011, Hahn *et al.* 2012). Conversely, overexpression of RNF20 results in elevated H2Bub1 levels (Kim *et al.* 2005).

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RNF20 has been described as a putative tumour suppressor (Shema *et al.* 2008), possibly functioning through interaction with p53 and its presence at the promoter of p53 target genes such as *MDM2* (Kim *et al.* 2005). H2Bub1 also increases at the coding regions of p53 target genes upon their activation (Minsky *et al.* 2008). Depletion of RNF20 has been reported to result in a decrease in p53 expression and an increase in cell migration and tumourigenesis (Shema *et al.* 2008). Down-regulation of RNF20 has also been shown to increase various growth promoting pro-oncogenic genes such as *c-Myc* and *c-Fos* (Shema *et al.* 2008). The selective suppression that RNF20 demonstrates against these oncogenes probably occurs by the prevention of recruitment of the elongation factor TFIIS (which interacts with the PAF1 complex to relieve the stalled RNA pol II) to chromatin so that the transcriptional block frequently seen in closed chromatin cannot be relieved, resulting in transcriptional repression (Shema *et al.* 2011). A tumour-suppressive role for RNF40 mediated through H2Bub1 has also been demonstrated in breast cancer cells (Prenzel *et al.* 2011).

A potential oncogenic role for RNF20 has also been reported in a number of studies; however, it is unclear whether this may be cancer type- or stage-specific (Liu *et al.* 2009, Wright *et al.* 2011, Blank *et al.* 2012, Wang *et al.* 2013*a*). Functionally, down-regulation of RNF20 was found to lead to the migration of MCF10A breast epithelial cells, as well as anchorage-independent growth of NIH-3T3 cells and increased tumourigenicity in nude mice (Shema *et al.* 2008). The role of RNF20 in transcriptional elongation and the DNA damage response has been discussed earlier in this review.

The BRCA1–BARD1 complex Similar to RNF20 and RNF40, the tumour suppressor BRCA1 can function as a RING finger E3 ubiquitin ligase, forming a complex with BARD1 that stabilises the BRCA1 protein, facilitating the ubiquitination of histone H2B (Mallery *et al.* 2002, Thakar *et al.* 2010). Breast and/or ovarian cancer-associated mutations in the RING finger domain of BRCA1 abolish ubiquitin ligase activity *in vitro*, although it is unclear whether *BRCA1* mutation influences the loss of H2Bub1 observed in primary breast tumours (Hashizume *et al.* 2001, Thakar *et al.* 2010, Prenzel *et al.* 2011). Loss of BRCA1 has been reported to correlate with the loss of ubiquitinated histone H2A (known to dimerise with H2B) at satellite DNA regions (Zhu *et al.* 2011).

The BRCA1–BARD1 complex has been shown to monoubiquitinate all nucleosome core histones – H2A/H2AX, H2B, H3 and H4, with the exception of the linker histone H1, including the H2AX variant that co-localises with BRCA1 following DNA damage (Mallery *et al.* 2002, Thakar *et al.* 2010). A recent study has demonstrated that the ubiquitination of histones H2B and H2A by the BRCA1–BARD1 complex has differing efficacies, whereby histone H2B is only modestly ubiquitinated in comparison with histone H2A, which may indicate a histone preference for monoubiquitination (Thakar *et al.* 2010). Links between BRCA1 and RNF20 at sites of DSBs following DNA damage have been discussed; however, it is not entirely clear how these E3 ligases may function together *in vivo* to enable H2Bub1.

Human double minute 2 Human double minute 2 (HDM2, human homolog of MDM2) is an E3 ubiquitin ligase that functions to maintain low levels of p53 by polyubiquitination, targeting p53 for degradation via the proteasome. In addition to regulating p53 levels, MDM2 is able to monoubiquitinate histones H2A and H2B (Minsky & Oren 2004). The ability of MDM2 to monoubiquitinate histones may not be in a global sense, but rather in a predominantly p53 target gene manner, given that overexpression of MDM2 increases histone monoubiquitination in the vicinity of a p53 consensus binding sequence within the *p21* promoter (Minsky & Oren 2004). This discovery begins to provide some insight into why multiple E3s seem to have the apparently identical function of monoubiquitinating histone H2B, as we appreciate roles for gene-specific vs global H2Bub1 levels as a means of regulating the transcription of genes in response to cellular stress. It has also been proposed that the H2Bub1-associated role of MDM2 is limited to the monoubiquitination of free histone H2B rather than in the context of the nucleosome (Johnsen 2012).

The BAF250b-elongin C-cullin 2-Roc1 complex
The mammalian chromatin remodelling complex SWI/SNF-A subunit BAF250/ARID1 has also been reported to function as an E3 ubiquitin ligase in a complex with cullin 2, Roc1 and elongin C to target the monoubiquitination of H2Bub1 (Li *et al.* 2010). In this study, down-regulation of BAF250 was found to lead to a decrease in global H2Bub1. Interestingly, BAF250b has been implicated as a requirement for the maintenance of undifferentiated mouse embryonic stem cells (ESCs; Yan *et al.* 2008). The identification of a role for BAF250b in the maintenance of stem cell pluripotency indicates that H2Bub1 may also have a role in this system.

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'Erasing' H2Bub1: DUBs

Empirical evidence indicates that the loss of H2Bub1 is associated with tumourigenesis; therefore, it is important to examine mechanisms by which ubiquitin is cleaved from histone H2B. Six DUBs from the USP subfamily have been reported to deubiquitinate mammalian H2Bub1 – USP3 (Nicassio *et al*. 2007), USP7 (van der Knaap *et al*. 2005), USP15 (Long *et al*. 2014), USP22 (Zhang *et al*. 2008), USP44 (Fuchs *et al*. 2012) and USP49 (Zhang *et al*. 2013) (Fig. 2). USP12 and USP46 regulate H2Bub1 in *Xenopus* development (Joo *et al*. 2011). The *Drosophila* homolog scrawny of a ninth human DUB, USP36, deubiquitinates H2Bub1 in *Drosophila* stem cells (Buszczak *et al*. 2009). It is likely that not all E3s or DUBs are functional in all tissue types and/or during tumour progression, with some requiring catalytic co-factors and/or assembly into large multicomponent complexes, e.g. USP7 and USP22 (Samara *et al*. 2010, Nicholson & Suresh Kumar 2011), to enable full catalytic activity. It is interesting to speculate, however, that the apparent redundancy among DUBs, with multiple DUBs appearing to serve the same purpose, speaks to the fundamental importance of these enzymes in maintaining cellular function. Conversely, many DUBs interact with multiple proteins, such as USP7 that displays both p53-dependent and -independent activity, including deubiquitination of histone H2B (Nicholson & Suresh Kumar 2011).

There is growing interest in therapeutic targeting of DUBs as these key regulators of the ubiquitin–proteasome system occur upstream of the proteasome itself and may offer greater specificity and less toxicity as cancer therapeutics compared with FDA-approved proteasome inhibitors such as Velcade (bortezomib) and Kyprolis (carfilzomib). PR-619 functions as a non-selective broad-range reversible DUB inhibitor (Altun *et al*. 2011), and numerous efforts are underway to identify specific DUB inhibitors, some of which are discussed below.

Ubiquitin-specific protease 22 USP22 is arguably one of the most interesting and significant of all H2Bub1-associated DUBs described thus far. A large body of research has linked this enzyme to tumour progression and oncogenic activity, and USP22 is currently under investigation as a therapeutic target for cancer. Elevated expression has been shown to act as a key indicator of poor prognosis in a variety of different cancers including invasive breast cancer (Zhang *et al*. 2011*a*), colorectal carcinoma (Liu *et al*. 2011), oesophageal cancer (Li *et al*. 2012), papillary thyroid carcinoma (Wang *et al*. 2013*b*), gastric cancer (Yang *et al*. 2011), oral squamous cell carcinoma (Piao *et al*. 2012), salivary duct carcinoma (Piao *et al*. 2013), non-small cell lung carcinoma (Ning *et al*. 2012) and cervical cancer (Yang *et al*. 2014). In addition, inhibition of USP22 has been demonstrated to induce cell cycle arrest and inhibit proliferation in hepatocellular carcinoma (HCC; Ling *et al*. 2012) and bladder cancer (Lv *et al*. 2011). In a seminal study carried out by Glinsky *et al*. (2005), USP22 was identified as part of an 11-gene signature termed the 'death-from-cancer' signature that predicts rapid disease recurrence, distant metastatic sites and poor response to therapy across multiple solid tumours. This 11-gene signature appears to consist of genes with causal roles in human cancer as opposed to just markers of disease. It correlates with a stem cell-like expression profile and appears to be driven by the *BMI1* oncogene, a member of the polycomb group of proteins that function in chromatin remodelling to lead to events such as *HOX* gene silencing. Polycomb/BMI1-driven pathways are active in both normal stem cells and some highly malignant cancers. Recent expansion of this study has confirmed that USP22 is a critical enzyme associated with end-stage disease and that its levels are especially elevated in drug-resistant tumours (Schrecengost *et al*. 2014).

USP22 is a subunit of the human SAGA transcriptional co-factor acetylation complex and, as a member of this complex, functions to deubiquitinate histone H2B (Zhang *et al*. 2008). Ubp8, the yeast homolog of USP22, is also necessary for SAGA-mediated deubiquitination of histone H2B (Henry *et al*. 2003). Given this and the association of expression of USP22 with stem cell-like features in many cancers, it is possible that the deubiquitination of histone H2B would result in reversion to a non-differentiated-like phenotype. Whether the key role of USP22 in late-stage cancers is H2Bub1-related or -independent remains to be elucidated.

USP22 has also been demonstrated to stabilise the histone deacetylase Sirt1, removing polyubiquitin chains that would otherwise lead to this protein's degradation via the proteasome (Lin *et al*. 2012). Sirt1 antagonises the transcriptional activity of p53 by decreasing p53 acetylation, and down-regulation of USP22 leads to the degradation of Sirt1 and increases p53-dependent apoptosis, again flagging strategies to inhibit USP22 as of possible value as a cancer therapeutic.

Ubiquitin-specific protease 7 The ubiquitin protease USP7/HAUSP (Herpes virus-associated USP) was originally isolated as an interactor of the herpes simplex

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virus type 1 immediate-early protein Vmw110 (ICP0) (Everett *et al*. 1998). USP7 has both p53-dependent and -independent activity, with additional substrates, as well as H2Bub1, including PTEN, FOXO4 and PRC1/INK4a (Nicholson & Suresh Kumar 2011). USP7 deubiquitinates the p53 E3 ligase MDM2, inhibiting MDM2 degradation resulting in the polyubiquitination of p53 that leads to its degradation via the proteasome (Li *et al*. 2002, 2004, Cummins *et al*. 2004). In this regard, USP7 acts as a cell cycle regulator through the degradation of p53, which promotes cell cycle progression and consequently cellular proliferation.

In vitro research demonstrated the reliance of USP7 on guanosine-5′-monophosphate synthetase (GMPS) for the deubiquitination of histone H2B (van der Knaap *et al*. 2005). GMPS is a metabolic enzyme required for the synthesis of guanine nucleotides, the levels of which are correlated with proliferating cells (Boritzki *et al*. 1981). Loss of either GMPS or USP7 results in increased levels of H2Bub1 (van der Knaap *et al*. 2010). USP7 also appears to have a role as an enhancer of polycomb-related silencing of homeotic genes *in vivo*, correlating with other observations of a role for H2Bub1 in the expression of *HOX* genes as discussed earlier (van der Knaap *et al*. 2005, Zhu *et al*. 2005). USP7 is further implicated to be part of the H2Bub1 ligation machinery as it forms a complex with the H2Bub1 E2 conjugating enzyme UBE2E1, attenuating its role (Sarkari *et al*. 2013).

Given the eminent druggability of DUBs, and the particular interest in USP7 given its influence on p53 levels, interest in developing small-molecule USP7 inhibitors as therapeutics for some cancers is emerging (Colland *et al*. 2009, Colland 2010, Chauhan *et al*. 2012, Reverdy *et al*. 2012). HBX 41 108, a cyano-indenopyrazine derivative that reversibly inhibits USP7 in a non-competitive fashion, has been shown to stabilise p53, with concomitant increase in the levels of the cell cycle inhibitor p21 and inhibition of cancer cell growth (Colland *et al*. 2009). Other USP7 inhibitors, HBX 19 818 and HBX 28 258, have been identified as possible irreversible inhibitors of USP7 (Reverdy *et al*. 2012). Furthermore, the USP7 inhibitor P5091 has been shown to be well tolerated by animals in human tumour xenograft models of multiple myeloma and to have a synergistic effect with drugs such as HDAC inhibitors that are used in combination with the proteasome inhibitor bortezomib (Chauhan *et al*. 2012). Whether these USP7 inhibition strategies based on p53 stabilisation will be efficacious in *TP53*-mutant tumours where mutations themselves function to stabilise the mutant protein (Muller & Vousden 2014) remains to be determined, and this may depend on the relative importance of other USP7 substrates such as H2Bub1 compared with p53 as drivers of tumourigenesis.

Ubiquitin-specific protease 44 USP44 has been identified as a novel H2Bub1 DUB playing an important role in stem cell differentiation. In a study examining increasing H2Bub1 levels identified during ESC differentiation, a concomitant down-regulation of USP44 was observed (Fuchs *et al*. 2012). USP44 has a number of roles similar to those of other proteins found in the H2Bub1 pathway. An *Usp44*-null mouse model was shown to have defects in mitotic checkpoint regulation and in chromosome lagging, leading to missegregation of chromosomes and aneuploidy and highlighting roles for USP44 in centrosome functioning and mitotic spindle formation (Zhang *et al*. 2012). In the same study, USP44 was also shown to be down-regulated in lung cancer cells and associated with a poor prognosis. The levels of USP44 have also been shown to be elevated in T-cell acute lymphoblastic leukaemia cells (Zhang *et al*. 2011*b*).

Additional H2Bub1-associated DUBs In addition to the three DUBs described, USP3, USP15, USP46, USP12, USP49 and USP36 have all been reported to be involved in the deubiquitination of H2Bub1. Currently, limited research exists on these DUBs; however, based on the diverse roles of DUBs, it is important to understand all the DUBs involved in the moderation of H2Bub1. USP3 has been shown to associate with chromatin and to function as a DUB for both histones H2A and H2B (Nicassio *et al*. 2007, Sharma *et al*. 2014). USP3 was demonstrated to be required for timely progression through S phase and subsequent mitotic entry, and furthermore, its inhibition was found to lead to the accumulation of DNA breaks and activation of the ATM-regulated DNA damage response pathway (Nicassio *et al*. 2007). Nuclear USP15 associates with the RNF20–RNF40 complex, as well as directly with SART3, a component of splicing machinery also known as TIP110 or p110 (Long *et al*. 2014). Interestingly, the loss of USP15 has been reported to be associated with resistance to paclitaxel in ovarian cancer (Xu *et al*. 2009). The *Drosophila* homolog of USP36 (*scrawny, scny*) deubiquitinates H2Bub1 in *Drosophila* stem cells and functions in gene silencing, indicating that these cells use the loss of H2Bub1 to repress the expression of genes involved in differentiation such as Notch target genes (Buszczak *et al*. 2009). These findings are supported by an earlier paper that identified the importance of the *Drosophila* RNF20 homolog for the regulation of H3K4 methylation and

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transcription of Notch target genes (Bray *et al.* 2005). Furthermore, the levels of USP36 are elevated in ovarian cancer cells (Li *et al.* 2008).

USP12 and USP46 have been shown to have roles in *Xenopus* development as DUBs that can deubiquitinate both histones H2A and H2B (Joo *et al.* 2011). Both USP12 and USP46 interact with other proteins for their activation, including WD40 repeat-containing proteins and Usp1-associated factor 1 (UAF1; Kee *et al.* 2010). USP12 has also been implicated as a negative regulator of Notch signalling, an evolutionarily conserved pathway that has key roles in the determination of cell fate (Moretti *et al.* 2012).

Lastly, USP49 has recently been identified as a novel H2Bub1 DUB that functions to regulate exonic splicing. In order to behave as a H2Bub1 DUB, USP49 forms a complex with RuvB-like1 (RVB1), an ATPase with diverse cellular roles, including transcription and chromatin remodelling, and a suppressor for Gal1 (SUG1), a subunit of the 26S proteasome (Zhang *et al.* 2013).

In summary, there exist a large range of H2Bub1-related DUBs that predominately function to regulate development, DNA damage repair, cell cycle progression and stem cell differentiation, all of which are important pathways that affect tumourigenesis. The importance of H2Bub1 vs that of other DUB substrates in tumourigenesis remains to be determined. The discovery of DUB inhibitors is a relatively new field but rapidly gaining momentum, with the potential to identify new therapeutic strategies for the treatment of aggressive tumours.

Roles for H2Bub1 in controlling 'stemness'?

As has been already described in this review, H2Bub1 plays important roles in a variety of cellular processes, including the regulation of histone cross-talk and interactions with various DUBs. Both histone cross-talk and DUBs have been demonstrated to have significant roles in stem cells where they can act to maintain an undifferentiated stem cell state through gene silencing (Buckley *et al.* 2012).

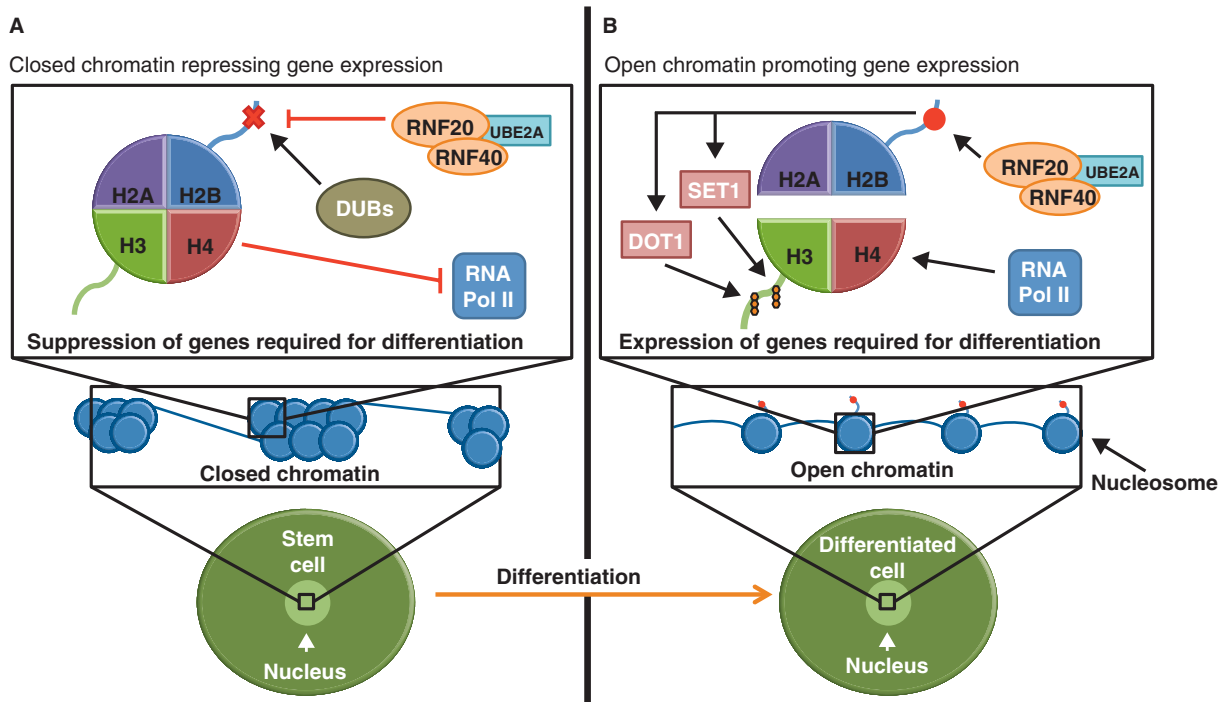

Figure 3

The role of H2Bub1 in a stem cell vs a normal differentiated cell. (A) H2Bub1 is actively repressed in stem cells through the up-regulation of H2Bub1-associated DUBs (indicated by an upward pointing red arrow) resulting in closed chromatin and suppression of genes involved in differentiation. Loss of H2Bub1 (indicated by a red cross) inhibits histone cross-talk, preventing the recruitment of histone methyltransferase complexes containing SET1 or DOT1 (indicated by red crosses) required for the active chromatin marks of H3K4me and H3K79me. (B) H2Bub1 is expressed in the differentiated cell (depicted by a red circle), where there is lower expression of DUBs (indicated by a downward pointing red arrow). Histone cross-talk is facilitated by H2Bub1-related recruitment of methyltransferase complexes containing SET1 or DOT1, promoting H3K4me and H3K79me respectively (indicated by triplicate circles) at lineage-specific genes, resulting in open chromatin and active transcription of genes required for differentiation. The likelihood that H2Bub1 disrupts the nucleosomal unit is depicted.

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Therefore, it is not surprising that a substantial body of evidence that links H2Bub1 to the regulation of stem cells exists. A significant number of studies have reported the role of histone PTMs in ESCs, including H3K4, H3K27 and H3K79, H3K4me and H3K79me known to cross-talk with H2Bub1 (Bibikova *et al*. 2008, Orkin & Hochedlinger 2011). Many enzymes associated with H2Bub1 ubiquitination or deubiquitination have been linked to stem cell differentiation as already mentioned, including BAF250b (Yan *et al*. 2008), USP36 (Buszczak *et al*. 2009), USP22 (Glinsky *et al*. 2005), RNF20 and USP44 (Fuchs *et al*. 2012).

Fuchs *et al*. (2012) demonstrated that H2Bub1 increased during induced differentiation of both human and mouse ESCs. This study was supported by Chen *et al*. (2012), who showed that H2Bub1 levels were very low in mouse stem cells, but increased dramatically after embryoid body and ESC differentiation. H3K4me3 levels were also found to be increased after embryoid body differentiation.

In addition, it was shown that USP44 was present in ESCs where it acted to suppress H2Bub1 levels and thereby to maintain the stem cell phenotype, with concomitant decrease upon differentiation, contributing to the increase observed in H2Bub1. H2Bub1, but not monoubiquitinated histone H2A, levels were also shown to increase significantly during the differentiation of human mesenchymal stem cells into osteoblast or adipocyte lineages, with reliance on CDK9, WAC and RNF40 levels (Karpiuk *et al*. 2012). This study concluded that H2Bub1 is a requirement for the differentiation of multipotent stem cells, flagging key roles for H2Bub1 in the control of stem cell differentiation (Fig. 3). These discoveries, firmly demonstrating the importance of H2Bub1 in stem cell differentiation, have the potential to inform studies of cancer stem cells.

Conclusions

Study of H2Bub1 and its associated enzymatic machinery is rapidly gaining momentum, with specific relevance to our understanding of cancer and the regulation of stem cell differentiation. Control of H2Bub1 is undoubtedly complex, with numerous E3s and DUBs appearing to play similar roles in H2Bub1 enzymatic machinery and many having significant roles external to the mediation of H2Bub1. The rapidly advancing field of DUB inhibitors is developing new ways to manipulate H2Bub1 levels *in vivo*, offering potential opportunities for the future treatment of malignancy. Future studies will undoubtedly need to address H2Bub1 at both the global and gene-specific levels, both *in vitro* and *in vivo*, to continue to unravel the complex manner in which H2Bub1 influences gene expression, plays a role in the DNA damage response and influences the multipotent nature of stem cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

A J Cole and D J Marsh conceived and wrote this review with input and discussion from R Clifton-Bligh. All authors critically reviewed the manuscript.

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