DISSERTATION

GENERATION OF SITE-SPECIFIC UBIQUITINATED HISTONES THROUGH CHEMICAL LIGATION AND CHARACTERIZATION OF HISTONE DEUBIQUITINASES

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ABSTRACT

GENERATION OF SITE-SPECIFIC UBIQUITINATED HISTONES THROUGH CHEMICAL LIGATION AND CHARACTERIZATION OF HISTONE DEUBIQUITINASES

Nucleosome is the basic unit of chromatin and is composed of 147 base pairs of DNA wrapped 1.65 turns around a histone octamer of the four core histones (H2A, H2B, H3 and H4)(Luger et al., 1997). Histones are subject to numerous post-translational modifications. One such modification is the addition of a single ubiquitin (Ub) moiety to a specific lysine residue in the histones, such as H2AK119 or H2BK120 in humans. Depending on the site of Ub attachment, these modifications have distinct functional consequences. Whereas H2A ubiquitination is associated with transcriptional repression and silencing, H2B ubiquitination is associated with actively transcribed regions and has roles in initiation, elongation and mRNA processing. A more recently discovered ubiquitination site in H2A, H2AK13/15, is associated with DNA damage repair. In addition, a number of other ubiquitination sites on all types of histones have been discovered by high throughput mass spectrometry. The functions and regulations of those novel ubiquitinations are not known.

Deubiquitinating enzymes (DUBs) reverse these ubiquitinations and therefore, are involved in a variety of regulatory processes. Mutations in several histone DUBs have been implicated in various diseases, thus they represent potential therapeutic targets. The specificity and regulation of histone DUBs are poorly understood in part because it has been difficult to obtain homogenous ubiquitinated histones and nucleosomes to use as

substrates *in vitro*. Previously, several strategies have been developed to produce chemically defined ubiquitinated histones that use a combination of expressed protein ligation (EPL) and solid phase peptide synthesis (SPPS) techniques. These protocols are technically challenging for a biochemical lab. This dissertation describes our successful approach in obtaining homogenous site-specific ubiquitinated H2A and H2B that were then reconstituted into nucleosomes and used to qualitatively and quantitatively characterize a panel of known histone DUBs *in vitro*. We anticipate that our approach can be applied to generate all types of Ub-histone conjugates regardless of the particular ubiquitination site or histone types. They will significantly facilitate the study of all types of histone ubiquitination.

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CHAPTER 1: INTRODUCTION

1.1 Histone post-translational modifications

Histones undergo diverse and numerous post-translational modifications (PTMs) that mainly target the tails of the core histones: H2A, H2B, H3, and H4, as well as the linker histone H1. Examples of the major histone PTMs include: methylation, phosphorylation, acetylation, ubiquitination, and SUMOylation. These PTMs are important in the regulation of many DNA related processes. In some cases, PTMs function by directly altering nucleosome stability and structure (Bannister and Kouzarides, 2011). For example, acetylation of lysine residues on various histones can neutralize their positive charge and weaken their association with DNA (Hong et al., 1993). In another example, ubiquitination of H2B interferes with chromatin compaction and leads to an open and biochemically accessible fiber conformation (Fierz et al., 2011). The main mechanism by which PTMs exert their function is by recruitment of numerous chromatin associated factors that have been shown to specifically interact with modified histones via distinct domains (Bannister and Kouzarides, 2011). For example, trimethylated H3K4 is bound by the tandem chromodomains within chromodomain helicase DNA binding protein 1 (CHD1), an ATP-dependent remodeling enzyme capable of repositioning nucleosomes (Sims et al., 2005). Other examples of proteins recruited to specific PTMs are those containing bromodomains. These domains recognize acetylated lysines and are often found in histone acetyl transferases (HATs) and chromatin remodeling complexes. For example, Swi2/Snf2 contains a bromodomain that targets it to acetylated histones. In turn, this recruits the

SWI/SNF remodeling complex, which functions to change chromatin to the decondensed form (Hassan et al., 2002).

Interactions between different histone PTMs are known as the "histone crosstalk." For example, H3K27 trimethylation is a prerequisite for monoubiquitination of H2A by RING1B, both marks are associated with gene repression. Another example is that monoubiquitination of H2B is a prerequisite for H3K4 and H3K79 di- and trimethylation, which are known to associate with active transcription (Osley, 2006). In addition, *in vitro* studies indicate that H2AK119Ub represses the di and trimethylation of H3K4, resulting in inhibition of transcriptionl initiation (Nakagawa et al., 2008).

1.2 Non-proteolytic functions of ubiquitin in the histone context

Ubiquitin (Ub) is a 76 amino acid protein of ~ 8.5 kDa. As a post-translational modification, the C-terminal carboxylate of Ub is ligated to the ε-amino group of lysine residues of proteins via an isopeptide linkage through the action of three enzymes E1, E2, and E3 (Fig. 1.1). First, Ub is activated by E1 (Ub activating enzyme) in an ATP dependent manner. Next, the activated Ub is attached through a thioester bond to E2 (Ub conjugating enzyme). Then, Ub is transferred to a lysine residue in the target substrate with the help of E3 (Ub ligase). Proteins can remain monoubiquitinated, or the monoubiquitin can act as a 'seed' for the subsequent conjugation of additional Ub moieties, resulting in a polyubiquitin chain (Fig. 1.1). E2 and E3 enzymatic activities comprise large families, and their associations with each other, cellular localization, and substrate interactions all contribute to targeting a particular protein for either monoubiquitination or polyubiquitination (Pickart and Eddins, 2004).

The ability of Ub to form polymers enables polyubiquitination of proteins, which is typically associated with protein degradation via the 26S proteasome. However, depending on the linkage type, polyubiquitintion marks that don't lead to proteasomal degradation are biologically relevant and important. Mono and polyubiquitination of the core histones, linker histone H1, and histone variants have been reported. For example, in the events of DNA damage response, the E3 ligase RNF168 monoubiquitinates H2A at K13 or K15. Then, a K63-linked polyUb chain is added to the monoubiquitinated H2A. The above ubiquitination events have been shown to be involved in the DNA damage repair pathway (Mattiroli et al., 2012).

Ubiquitinated histones are the most abundant ubiquitinated proteins in the cell. Ubiquitination, when compared to other PTMS, adds a large protein to a histone and this could have substantial effects on chromatin structure and function. The function(s) of the ubiquitinated histone varies depending on the histone and the site of ubiquitination. For example, monoubiquitination of lysine 119 on H2A (H2AK119Ub) is associated with gene repression, while H2BK120Ub is associated with transcription activation (Osley, 2006). H2AK13/15Ub has been shown to be involved in the DNA damage response (Fig. 1.2) (Gatti et al., 2012; Mattiroli et al., 2012).

1.3 H2A ubiquitination and its function in transcription and DNA damage repair

The most abundant histone H2A ubiquitination site has been mapped to the highly conserved residue lysine 119, which occupies 10-15% of cellular H2A and is known to be associated with transcription repression (Fig. 1.2). H2A ubiquitination is mediated by at least two different E3 Ub ligases, RING1B and 2A-HUB, both of which are associated with

transcriptional silencing (Cao et al., 2005; Wang et al., 2004; Zhou et al., 2008). RING1B is a subunit of the polycomb repressive complex 1 (PRC1) (Cao and Yan, 2012). In humans, ubquitinated H2A localizes to large repressed regions within the genome as well as to the promoters of polycomb target genes, such as the HOX genes, in a RING1B dependent manner (Cao et al., 2005; Wang et al., 2004). PRC1 and H2AK119Ub have been shown to localize across the repressed HOX loci of mouse embryonic stem cells. This repression is primarily mediated through the inhibition of RNA polymerase II elongation activity (Stock et al., 2007). Genome wide analyses of PRC1 and H2AK119Ub showed their co-localization at the promoters of transcriptionally repressed genes in mouse fibroblasts (Kallin et al., 2009).

2A-HUB, a component of the N-COR/HDAC repressive complex, is another H2AK119Ub E3 ligase. 2A-HUB was shown to repress a set of chemokine genes in macrophages by inhibiting Facilitates Chromatin Transcription (FACT) recruitment, and elongation by RNA polymerase II (Zhou et al., 2008).

Bergink et al. showed a significant increase in monoubiquitination of H2A upon UV induced DNA damage in nucleotide excision repair (NER). They mapped this monoubiquitination site to lysine 119. In addition, they showed that the E3 ligase RING1B was responsible for ubiquitination of H2A in NER. In addition, deubiquitination of H2A is required for proper repair of DNA double strand breaks (Bergink et al., 2006).

In the early steps of the DNA damage response, the E3 ligase RNF168 monoubiquitinates H2AK13/15. Then a K63 polyUb chain is added on the H2AK13/15Ub. This serves as a signal to recruit BRCA1 and 53BP1(Fig. 1.2) (Mattiroli et al., 2012).

1.4 H2B ubiquitination and its function in transcription and DNA damage repair

1-5% of H2B is ubiquitinated, which makes this modification significantly less abundant than H2A ubiquitination (Fig. 1.2). However, unlike H2AK119Ub, this mark is conserved in yeast as well. The main E3 ligase in humans is the RNF20/RNF40 complex, which is responsible for ubiquitination of H2B at K120 (Zhu et al., 2005). The p53 binding protein Mdm2 is also reported to function as an E3 ligase for histone H2B monoubiquitination in mammals (Minsky and Oren, 2004).

H2BK120Ub is associated with transcription activation (Weake and Workman, 2008). H2BK120Ub and RNF20/RNF40 are localized to promoters of actively transcribed genes (Henry et al., 2003). In addition, H2BK120Ub is required for di and trimethylation of H3K4 and H3K79, marks that are also associated with active transcription (Lee et al., 2007). The RNF20/RNF40 complex travels alongside with the RNA polymerase II transcription machinery (Xiao et al., 2005) and H2B ubiquitination may be important for recruitment of the FACT complex, which plays a role in transcription elongation by displacing nucleosomes in front of the elongating RNA polymerase II (Pavri et al., 2006).

Ubiquitinated H2B has also been implicated in the DNA damage response. The RNF20/RNF40 complex is recruited to DNA double strand break sites where it catalyzes H2B monoubiquitination. Furthermore, depletion of RNF20 disrupts the recruitment of DNA repair proteins in both non-homologous end joining and homologous recombination repair pathways (Moyal et al., 2011).

1.5 H3 and H4 ubiquitination and their functions in chromatin regulation

Histones H3 and H4 have been shown to be monoubiquitinated, although they are less abundant than ubiquitinated H2A or H2B. Wang et al. first reported ubiquitination of H3 and H4 by the CUL4-DDB-ROC1 E3 ligase upon UV-induced DNA damage. This ubiquitination alters the nucleosome stability and causes the histones to be evicted (Wang et al., 2006).

The E3 ligase BBAP, overexpressed in chemotherapy resistant lymphomas, has been shown to ubiquitinate H4K91 upon DNA damage. Disruption of H4K91 ubiquitination affects subsequent H4K20 di and trimethylation and delays the formation of 53BP1 foci at sites of DNA damage (Yan et al., 2009).

Nishiyama et al. showed a role of H3K23 ubiquitination in maintaining DNA methylation during replication. This ubiquitination was carried by UHRF-1, which then recruits the methyltransferase DNMT1. A RING domain mutant of UHRF-1 failed to recruit DNMT1 to replication sites and maintain DNA methylation (Nishiyama et al., 2013)..Recently, H3K18 was also shown to undergo monoubiquitination by UHRF-1 as a prerequisite for DNA methylation (Qin et al., 2015). Although the levels of ubiquitinated H3 and H4 are relatively low, they are important components in maintaining DNA methylation and in DNA repair.

1.6 Histone deubiquitinating enzymes

Deubiquitination is mediated by a group of enzymes that belong to the superfamily of proteases known as deubiquitinating enzymes (DUBs). The human genome encodes

~100 DUBs, which fall into five sub-families based on the type of catalytic domain: ubiquitin-specific protease (USP), ubiquitin carboxy-terminal hydrolase (UCH), ovarian tumor protease (OTU), Josephin (MJD) and JAMM/MPN+ metalloenzyme (JAMM) (Fig. 1.3). The USP comprises the largest family of DUBs and they are cysteine proteases along with UCH, OTU, and Josephin families, whereas the JAMM/MPN+ family members are zinc metalloproteases (Nijman et al., 2005)(Fig. 1.3).

Several DUBs have been identified to target histones and this interaction has great impact on chromatin structure and downstream DNA-based processes (Table 1.1). Most of the identified histone DUBs are members of the USP family, such as USP3, USP16, and USP22. BAP1 belongs to the UCH family, while MYSM1 is a metalloenzyme. Histone DUBs have been mostly identified through knockdown or overexpression of the DUB and monitoring its subsequent effect on the global levels of ubiquitinated histones. On the other hand, *in vitro* studies are usually carried out using histone substrates purified from mammalian cells. These ubiquitinated histones are typically H2AK119Ub or H2BK120Ub, because these are most abundant in mammalian cells. They are also heterogeneous due to numerous other PTMs that may or may not affect DUB specificities (Table 1.1). Due to the difficulty in obtaining chemically-defined ubiquitinated histone substrates, the field is lacking in the studies regarding histone DUB specificities. A more detailed description of previous studies on histone DUBs including *in vivo* and *in vitro* findings is presented below and summarized in Table 1.1.

1.6.1 BAP1

BRCA1 associated protein 1 (BAP1) is a known tumor suppressor dysregulated in multiple forms of cancer. Scheuermann et al. showed that BAP1, as well as its Drosophila homologue Calypso, is specific for H2AK119Ub and not H2BK120Ub nucleosomes *in vitro.* The H2AK119Ub substrate was obtained by ubiquitinating mononucleosomes with E1, E2 (UbcH5c), and E3 (RING1B/BMI1) and the H2BK120Ub substrate was chemically generated (McGinty et al., 2008). The authors showed that BAP1 requires ASXL1 for its deubiquitination activity *in vivo* and *in vitro*. Both BAP1 and ASXL1 comprise a complex named PR-DUB which localizes to HOX genes. Mutations that disrupted BAP1's activity specifically elevated H2AK119Ub levels and impaired HOX gene silencing. Surprisingly, although H2AK119Ub is required for HOX gene silencing, deubiquitination of H2AK119Ub by BAP1 is important for proper HOX gene silencing as well (Scheuermann et al., 2010).

BAP1 is also implicated in the DNA damage response. In contrast to USP3, BAP1 promotes formation of BRCA1 and RAD51 foci at DNA double strand breaks. In addition, recruitment of BAP1 to DNA damage foci correlated with a reduction in H2AK119Ub levels at the DNA lesions (Yu et al., 2014).

Sahtoe et al. recently further refined BAP1 specificity through *in vitro* experiments using homogenous H2AK119Ub and H2AK15Ub nucleosomes. BAP1 is specific for H2AK119Ub, but not H2AK15Ub nucleosomes and this specificity is not due to the amino acid sequences surrounding the ubiquitinated lysines in the histones (Sahtoe et al., 2016).

1.6.2 USP3

Nicassio et al. first identified USP3 as a histone DUB involved in the DNA damage response. Upon overexpression of USP3, levels of ubiquitinated H2A and H2B decreased significantly. Consistently, when USP3 was knocked down in cells, levels of ubiquitinated H2A increased significantly while levels of ubiquitinated H2B increased to a lesser extent. Therefore, the authors concluded that USP3 deubiquitinates both H2A and H2B *in vivo*. However, these studies were not complemented with *in vitro* experiments. The authors also found that cells with USP3 knockdown had spontaneous DNA damage and significant delay in S-phase progression. Therefore, they concluded that USP3 is required for normal S-phase progression and subsequent entry into M-phase. In addition, upon IR, DNA damage foci were formed containing ubiquitinated H2A, Ub conjugates, and γH2Ax. These foci persisted in USP3 knockdown cells for 40 hours post IR, while they cleared in 30 minutes in wildtype cells, which lead the authors to conclude that USP3 is important in the DNA damage response possibly in the termination of the damage signals (Nicassio et al., 2007).

In another study, overexpressed USP3 inhibited the retention of 53BP1, RAP80, and RNF168 foci upon DNA damage (Doil et al., 2009). In search for USP3 substrates in the DNA damage response, Sharma et al. showed that overexpression of USP3 resulted in the removal of Ub from K13/15 of H2A and γH2Ax, and from K118/119 of H2Ax in the DNA damage response. In addition, they show that USP3 overexpression abolishes the 53BP1 and BRCA1 foci resulting from UV and γ-irradiation. The authors concluded that USP3 counteracts ubiquitination by RNF168 and RNF8 (Sharma et al., 2014). However, further *in vitro* studies are needed to demonstrate that USP3 directly acts on ubiquitinated H2A.

1.6.3 USP16

USP16 was first identified in the de-repression of HOX gene expression by deubiquitinating H2AK119Ub. Furthermore, it has been shown to be specific for nucleosomes containing H2AK119Ub, but not H2BK120Ub both *in vivo* and *in vitro*. The specificity of USP16 against H2BK120Ub was tested *in vitro* using reconstituted mononucleosomes containing Flag-human H2A and Flag-yeast H2B that contain ~10% of ubiquitinated histone species. The discrimination of USP16 against H2BK120Ub was not due to the origin of ubiquitiniated H2B, since USP16 failed to deubiquitinate ubiquitinated H2B nucleosomes purified from Hela cells overexpressing Flag-H2B and HA-Ub. It's unknown what determines the specificity of USP16 towards nucleosomes containing ubiquitinated H2A or its mechanistic role in the regulation of HOX gene expression. The authors suggest that USP16 functions in the deubiquitination of H2A at the onset of mitosis during cell cycle progression. In control cells, ubiquitinated H2A begins to decrease at the beginning of M phase, reaches the lowest during M phase, then starts to increase as cells exit from M phase, and it is restored to normal levels when cells enter G1/S. In contrast, in USP16-knockdown cells, ubiquitinated H2A decreases only modestly as cells enter M phase, and progression through M phase is delayed. Ubiquitination of H2A during the cell cycle inhibits H3S10 phosphorylation by directly preventing association of the Aurora B kinase with nucleosomes. This was confirmed *in vitro*, the authors discovered that histone ubiquitination reduced H3S10 phosphorylation and H2A deubiquitination by USP16 restores H3S10 phosphorylation. The mechanism of USP16 recruitment is unknown, however, the authors found that USP16 is phosphorylated and that its phosphorylation

status correlates with *in vivo* H2A deubiquitination and cell cycle progression (Joo et al., 2007).

USP16 has also been implicated in the DNA damage response. In response to DNA damage, USP16 levels increase and this increase depends on an E3 ligase, HERC2. This increase in USP16 is negatively correlated with the levels of DNA damage induced Ub foci (Zhang et al., 2014). The authors also showed deubiquitination of both H2AK119Ub and H2AK15Ub by USP16 *in vitro*, suggesting them as possible USP16 targets.

1.6.4 USP22

USP22 is known to be an oncogene overexpressed in 10 different types of aggressive human cancers (Glinsky et al., 2005). Zhang et al. first identified USP22 as the DUB in the SAGA transcriptional coactivator complex. They showed that USP22 deubiquitinates H2A and H2B *in vitro* (Zhang et al., 2008a). Along with ATXN7L3, ENY2, and ATXN7, USP22 reside in a sub-complex known as the DUB module (DUBm). Knockdown of ATXN7L3 results in the inactivation of the SAGA DUBm and a significant increase of global H2B ubiquitination and a moderate increase of H2A ubiquitination, suggesting ubH2B is the primary target *in vivo*.

The deubiquitination activity of USP22 is required for full activation of SAGA-dependent inducible genes, implicating USP22 in transcription regulation (Lang et al., 2011). It stimulates cellular proliferation by activation of Myc-regulated genes (Zhang et al., 2008b). On the other hand, USP22 was also shown to be involved in transcription repression of Sox2, which is an important regulator of embryonic stem cell pluripotency.

The repression was linked to deubiquitination of H2BK120Ub at the Sox2 promoter (Sussman et al., 2013).

In addition, USP22 has been shown to have multiple non-histone substrates. For example, it's reported to regulate telomere maintenance by deubiquitinating and stabilizing telomeric shelterin component TRF1 (Atanassov et al., 2009).

1.6.5 The yeast SAGA DUB module

The SAGA coactivator complex is conserved in all eukaryotes. Yeast SAGA contains yUbp8, which is homologous to human USP22 and was shown to remove Ub from histone H2B in yeast (Daniel et al., 2004; Henry et al., 2003). Similar to USP22, yUbp8 alone is inactive and requires three binding partners for its DUB activity: Sgf73, Sgf11, and Sus1. These four proteins associate together to form the DUB module complex (Kohler et al., 2008; Lee et al., 2005). The crystal structure of yeast DUB module has been solved (Morgan et al., 2016; Samara et al., 2010). It was revealed that Ubp8, Sgf11, Sus1 and Sgf73 are intertwined so that the conformations of the subunits are largely dependent on their interactions with each other. A comparison of the DUBm structures with and without bound Ub-aldehyde suggests that interactions among the DUBm subunits may stabilize a conformation of yUbp8 that is catalytically competent and able to bind Ub.

Deletion of yUbp8 results in local and global increase in ubiquitinated H2B levels and decreased transcription of SAGA regulated genes (Henry et al., 2003). yUbp8 activity on H2B was mostly observed at the transcriptional start sites. H2B deubiquitination was shown to facilitate recruitment of C-terminal kinase 1 (CtK1), which phosphorylates the

CTD of RNA-polymerase II and promotes transcription elongation (Wyce et al., 2007). Deubiquitination of H2B by yUbp8 has also been associated with DNA damage repair after UV irradiation (Mao et al., 2014).

1.6.6 Other histone DUBs

Other DUBs that have been shown to deubiquitinate histones include: USP27X, USP51, USP44, and MYSM1 USP7? USP49? (Table 1.1). Both USP27X and USP51 have been shown to deubiquitinate H2BK120Ub and their deubiquitination activity is dependent on their association with DUBm subunits ATXN7L3 and ENY2. They compete with USP22 in binding to ATXN7L3 and ENY2 and their depletion results in tumor growth suppression (Atanassov et al., 2016).

USP51 was implicated in the DNA damage response through deubiquitinating H2AK13/15Ub. Overexpression of USP51 inhibits the formation of 53BP1 foci upon ionizing radiation and its depletion results in increased spontaneous DNA damage foci and increased levels of H2AK15Ub (Wang et al., 2016).

USP44 has been shown to be a negative regulator of DNA damage foci assembly, possibly through deubiquitination of histone substrates. USP44 was recruited to RNF168-generated ubiquitination products at DSB sites. Overexpression of USP44 inhibited the 53BP1 foci formation upon ionizing radiation (Mosbech et al., 2013).

MYSM1 is a member of the metalloprotease sub-family of DUBs important in transcription regulation. It was shown to deubiquitinate H2A *in vitro* and *in vivo* and activate expression of androgen receptor target genes through interactions with histone

acetylase pCAF (Zhu et al., 2007). Loss of MYSM1 was associated with increased H2AK119Ub levels (Wang et al., 2013).

Further *in vitro* characterization of the above DUBs with homogenous substrates would shed light on how their specificities are achieved and regulated, which have important implications for their biological substrates and functions.

1.7 Chemical approaches for histone ubiquitination

Chemical synthesis has been used to generate ubiquitinated H2A and H2B relying mainly on: solid phase peptide synthesis (SPPS), native chemical ligation (NCL), or expressed protein ligation (EPL). SPPS is used to synthesize histone fragments of less than 50 amino acids in length. These synthetic peptides subsequently undergo NCL in which a peptide bearing a N-terminal cysteine is joined with the other peptide bearing a C-terminal thioester, to form a native amide bond. EPL is an extension of NCL that overcomes the length limitations in NCL and requires an expressed protein in its C-terminal thioester form, which can be obtained by thiolysis of a C-terminally fused intein. The expressed protein thioester undergoes trans-thioesterification when reacted with a peptide or protein with a N-terminal cysteine. S to N-acyl shift generates a native amide bond between the two proteins (Chatterjee and Muir, 2010).

SPPS relies on selective protection and deprotection of the various functional groups (α-amino, α-carboxy, and side chain functional groups) provided by chemical protecting groups. Protecting group chemistry is also used to block reactive side chain functional groups in full length proteins. Allyloxycarbonyl (Alloc) is one of the most commonly used amino protecting groups. It is specific to lysine side chains and it is used to

block the α-amino group as well. Histidines are un-stably and partially blocked by Alloc. It is orthogonal to other protecting groups such as, Butoxycarbonyl (Boc), i.e. it is stable to the deprotection conditions of Boc. Alloc is easily introduced into the functional group, is generally stable against acids and bases, and can be removed by mild conditions (Isidro-Llobet et al., 2009).

Several groups have devised methods to generate chemically defined Ub-histones, which is summarized in Table 1.2. Muir and coworkers have reported the semi-synthesis and synthesis of monoubiquitinated H2B to study the role of H3K79 methylation by K79 specific methyltransferase, disrupter of telomeric silencing-like 1 (Dot1L) (Chatterjee et al., 2010; McGinty et al., 2008; McGinty et al., 2009) (Fig. 1.4, 1.5 a and b). The first strategy they used to synthesize H2BK120Ub is illustrated in figure 1.4. The authors used SPPS to synthesize amino acids 117-125 in H2B in which the ε-NH_2 in K120 is attached through a glycyl linker to a thiol bearing auxiliary necessary for ligation (green). Amino acid alanine at position 117 is mutated to cysteine and further protected by a photo removable group (red). This synthetic peptide is subsequently attached to Ub through NCL. The photosensitive auxiliary as well as the cysteine protecting group are removed by UV light, resulting in the ubiquitinated synthetic peptide. This peptide is then ligated to recombinant H2B(1-116) with its C-terminal in thioester form. The ubiquitinated H2BA117C undergoes nickel-mediated desulphurization to convert the cysteine back to the original alanine (Fig. 1.4) (McGinty et al., 2008). The end product is identical to native H2BK120Ub. There are several disadvantages of this method. First, the relatively low yield is due to the use of a photolytically removable ligation auxiliary. The preparation of the auxiliary requires a complex nine-step solution phase synthesis that limits the quantity that can be

incorporated into a peptide, which is used in the subsequent ligation reaction. Second, the auxiliary mediated ligation reaction requires a minimum of 5 days to reach 60% completion. Finally, photolytic removal of the ligation auxiliary, and the cysteine protecting group, is poorly scalable and cannot be performed in parallel without multiple irradiation sources. These limitations prompted Muir and coworkers to devise alternative more efficient strategies. Two different strategies (Fig. 1.5 a and b) were adopted that led to efficient syntheses, but compromised the native structure of the ubiquitinated H2B (Fig. 1.5 c). In (a), they substituted the auxiliary mediated ligation (Figure 1.4, green) with a cysteine mediated ligation. They also replaced the photolytically removable cysteine protecting group (Fig. 1.4, red) with a chemically labile group to eliminate the need for irradiation. SPPS was used to synthesize amino acids 117-125 in H2B bearing a A117C mutation. The cysteine at position 117 as well as the ε-NH_2 in K120, were protected with orthogonal protecting groups (shaded red and green in Fig. 1.5 a). A cysteine is selectively linked through an isopeptide bond to the ε-NH_2 in K120 to facilitate ligation to ubiquitin. This cysteine is later converted to alanine by desulfurization resulting in the G76A mutation in Ub (McGinty et al., 2009). The main disadvantage in this scheme is the formation of an isopeptide bond bearing a G76A mutation in Ub. This mutation affects the activities of DUBs. In Figure 1.5 b, another strategy used by Muir and co-workers replaced the native isopeptide bond with a disulfide linkage. Starting with H2BK120C and UbG76C mutants, the thiols in the cysteines generate a disulfide bond between H2B and Ub. This linkage is one bond longer than an isopeptide linkage and is not recognized by DUBs (Chatterjee et al., 2010).

Brik and coworkers generated H2BK34Ub by SPPS and NCL (Fig. 1.6) (Simon et al., 2007). This approach requires the ligation of four H2B peptide fragments to afford the final Ub-histone product at ~15 % yield. The use of four H2B fragments is due to the site of ubiquitination, i.e., in the middle of H2B as opposed to close to the terminals (Siman et al., 2013). Probably the best method to date to generate H2BK120Ub is developed by Brik's group and has 15-20 % yield (Fig. 1.7) (Morgan et al., 2016). This method relies on SPPS to generate a H2B peptide (115-122) bearing a δ-mercaptolysine (mK) residue at position 120 to enable site specific ubiquitination via isopeptide chemical ligation. H2B(2-113) and Ub thioesters were prepared via intein-mediated thiolysis. The two fragments of H2B were ligated using native chemical ligation followed by unmasking the protected thiolysine with methoxylamine, which is then ligated with Ub thioester. The ligation product was subjected to a desulfurization step to yield native H2BK120Ub (Morgan et al., 2016). This specialized chemistry requires knowledge in SPPS which is difficult to achieve in most biochemical labs. In addition, it is more expensive than starting with recombinant proteins. Chemical approaches described in this section as well as others are summarized in Table 1.2.

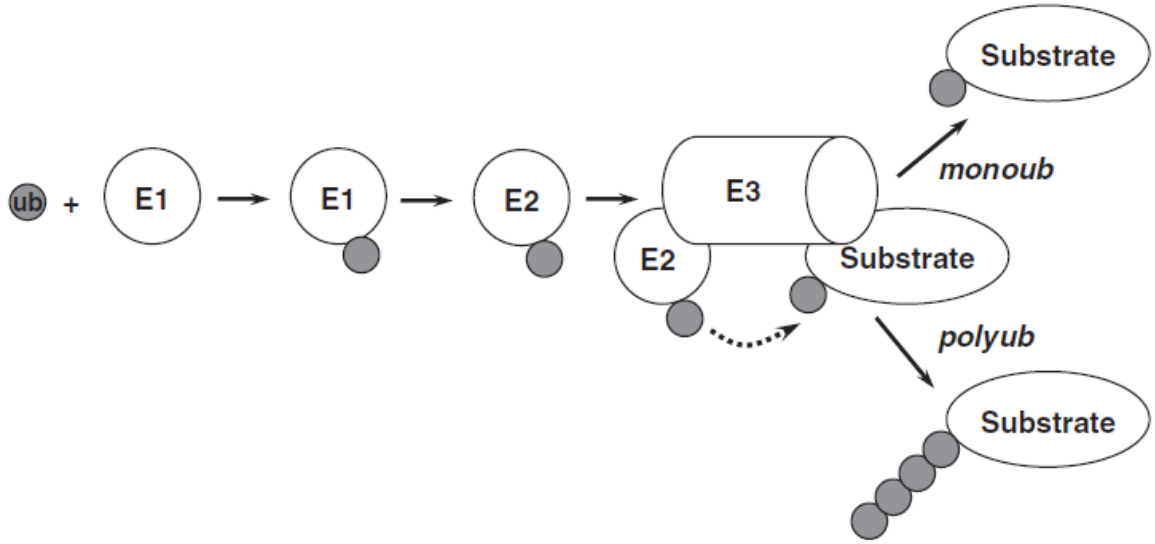


Figure 1.1 Ubiquitination process. Ub (gray) is attached to a lysine in the target substrate through the action of E1, E2, and E3 enzymes. Figure from (Osley, 2006).

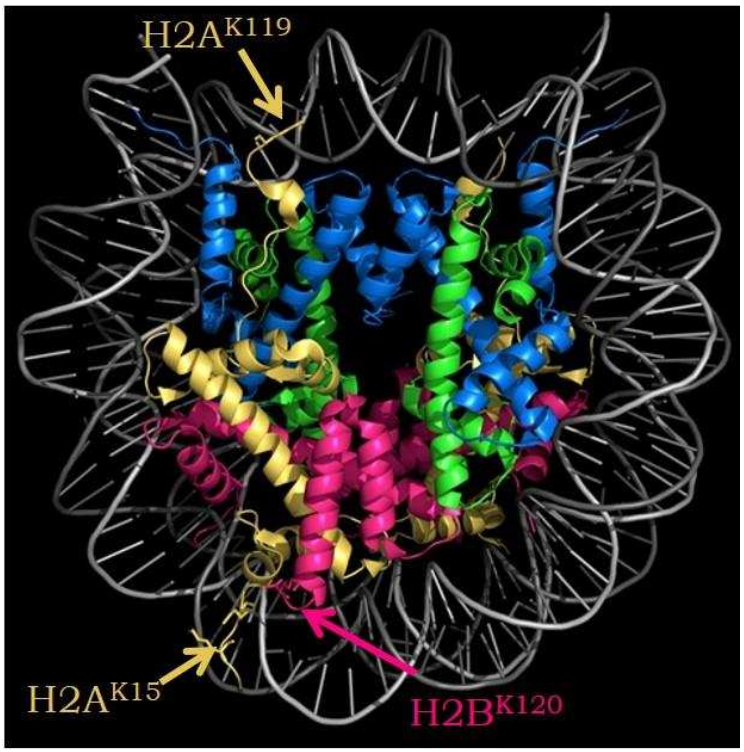


Figure 1.2. . The major sites of Ub attachment in the nucleosome. Arrows indicate the lysine ubiquitinated in: H2B (cyanine), H2A (magenta). H3/H4 tetramer (green) and DNA (gray).

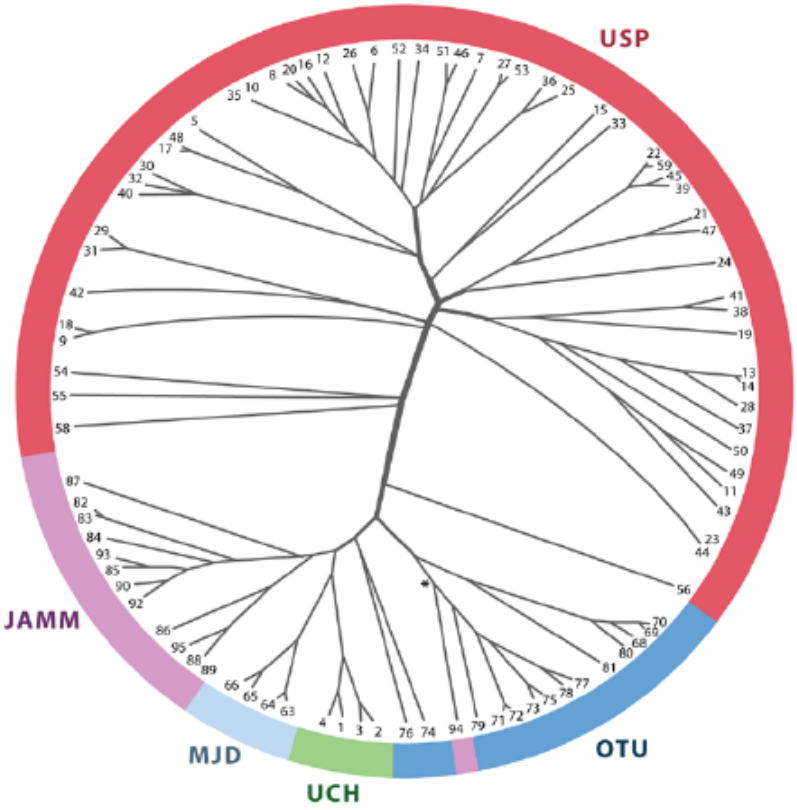


Figure 1.3 Human DUB families. ~100 DUBs are encoded by the human genome and they belong to five subfamilies. Figure from (Nijman et al., 2005).

Table 1.1. Histone DUBs and their biological functions.

DUB	ubH2A or ubH2B as a substrate *in vivo*	Context of ubH2A or ubH2B as a substrate *in vitro*	Biological processes	References
USP3	H2A and H2B; H2AK15Ub and H2AK119Ub increase upon knockdown of USP3.	not determined	DNA damage response, cell cycle progression	(Nicassio et al., 2007; Sharma et al., 2014)
USP15	not determined	H2BK120Ub nucleosome and free histones, with preference for free histones; ubH2A and ubH2B purified from mammalian cells and chemically-defined H2BK120Ub were used as substrates.	pre-mRNA splicing	(Long et al., 2014b)

USP16	H2AK119Ub and H2AK15Ub	H2AK119Ub and H2AK15Ub, but not H2BK120Ub, with preference for nucleosomes; substrates were purified from mammalian and yeast cells.	regulation of HOX gene expression, DNA damage response, cell cycle progression	(Cai et al., 1999; Joo et al., 2007; Shanbhag et al., 2010)
USP22	H2A(K?) and H2B, primarily H2B	nucleosomes and free histones from mammalian cells	transcription activation	(Lang et al., 2011)
USP27	H2A(K?) and H2B	nucleosomes and free histones from mammalian cells	normal cell proliferation	(Atanassov et al., 2016)
USP51	H2AK15Ub and H2B	nucleosomes and free histones from mammalian cells	normal cell proliferation, DNA damage response	(Atanassov et al., 2016; Wang et al., 2016)
USP44	H2A(K?) and H2B	not determined	stem cell differentiation, DNA damage response	(Fuchs et al., 2012; Mosbech et al., 2013)
BAP1	H2AK119Ub	H2AK119Ub, but not H2AK15Ub or H2BK120Ub nucleosomes; ubH2A made by E1, E2, and E3, ubH2B made by chemical semi-synthesis.	HOX gene repression, DNA damage response	(Sahtoe et al., 2016; Scheuermann et al., 2010)
MYSM1	H2AK119Ub	free histones purified from HEK293 cells stably expressing Flag-H2A; ubH2B was not tested.	transcription activation	(Wang et al., 2013; Zhu et al., 2007)
USP7	H2A(K?) and H2B	H2A: nucleosome and free histone. H2B: free histone. Purified from mammalian cells.	regulation of polycomb target genes expression	(Luo et al., 2015; van der Knaap et al., 2005)
USP49	H2B	H2B: nucleosomes purified from a yeast strain expressing Flag-hH2B and free histones and not H2A purified from mammalian cells.	regulation of cotranscriptional pre-mRNA splicing	(Zhang et al., 2013)

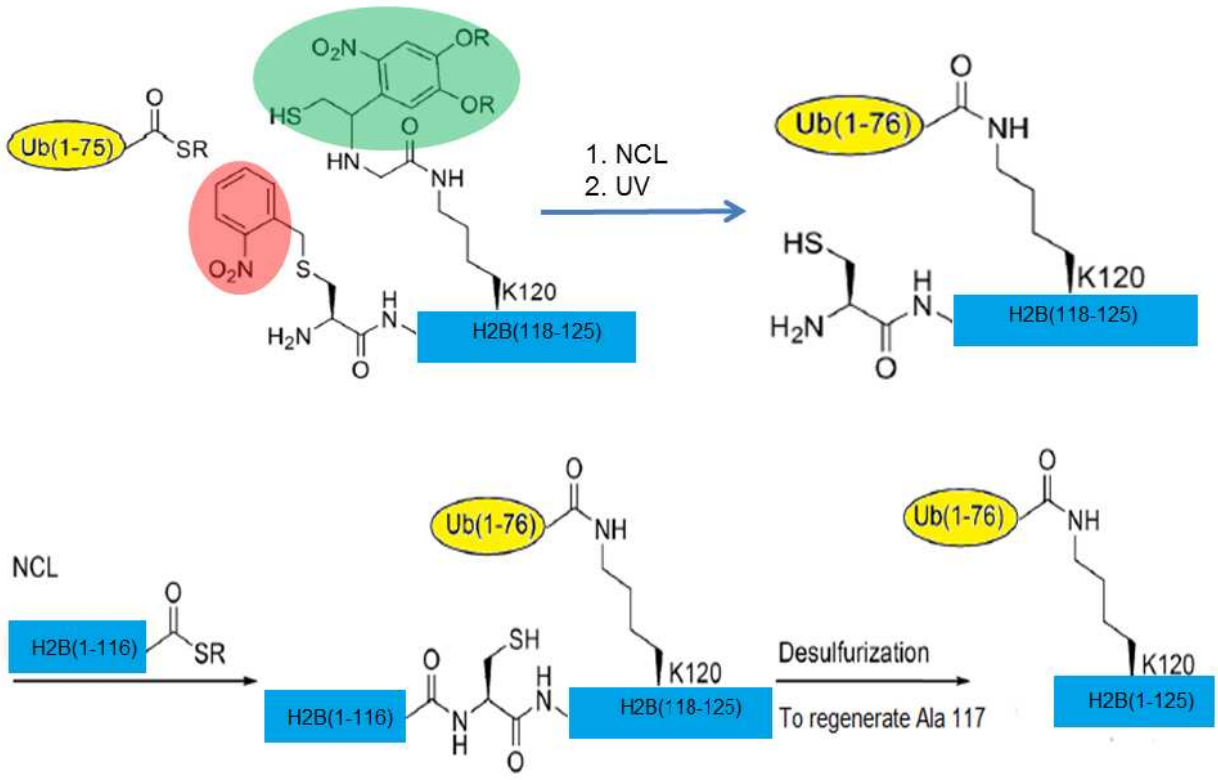


Figure 1.4 Previous method for the semi-synthesis of H2BK120Ub. Scheme used to generate chemically-defined ubiquitinated H2B as described in the text. Figure adapted from (McGinty et al., 2008).

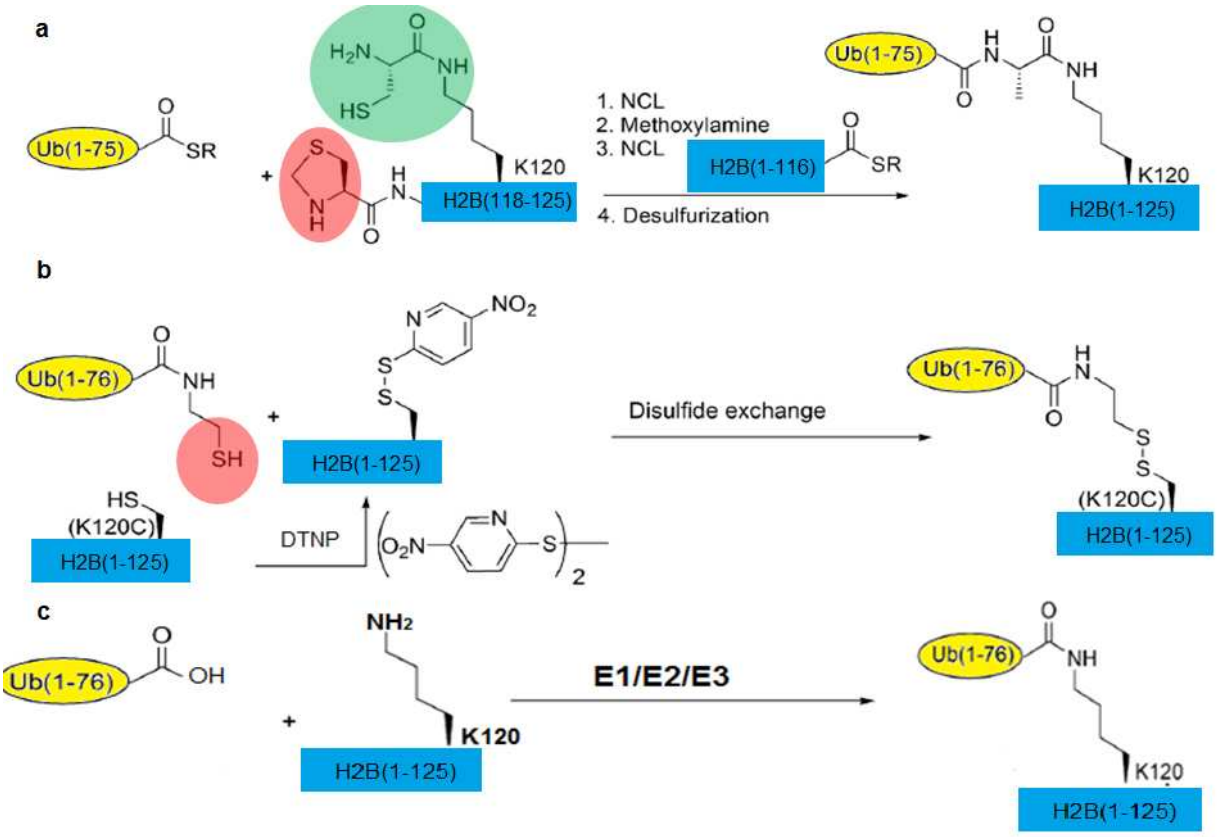


Figure 1.5 Previous methods for the semi-synthesis of H2BK120Ub in comparison with native H2BK120Ub. (a) Semi-synthesis of Ub(G76A)H2B (McGinty et al., 2009). **(b)** Disulfide ubiquitinated H2B (H2BK120ssUb). Figure adapted from (Chatterjee et al., 2010). **(c)** Native H2BK120Ub synthesized by E1, E2, and E3 enzymes.

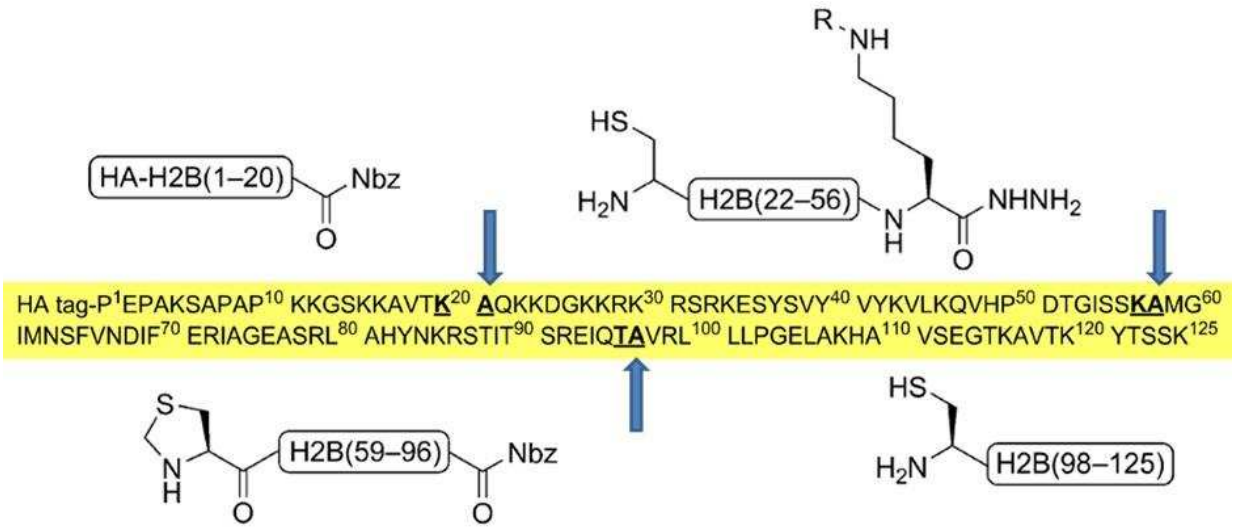


Figure 1.6 Scheme for the semi-synthesis of H2BK34Ub. SPPS was used to generate four H2B fragments since the site of ubiquitination is in the middle of H2B sequence. Figure adapted from (Siman et al., 2013).

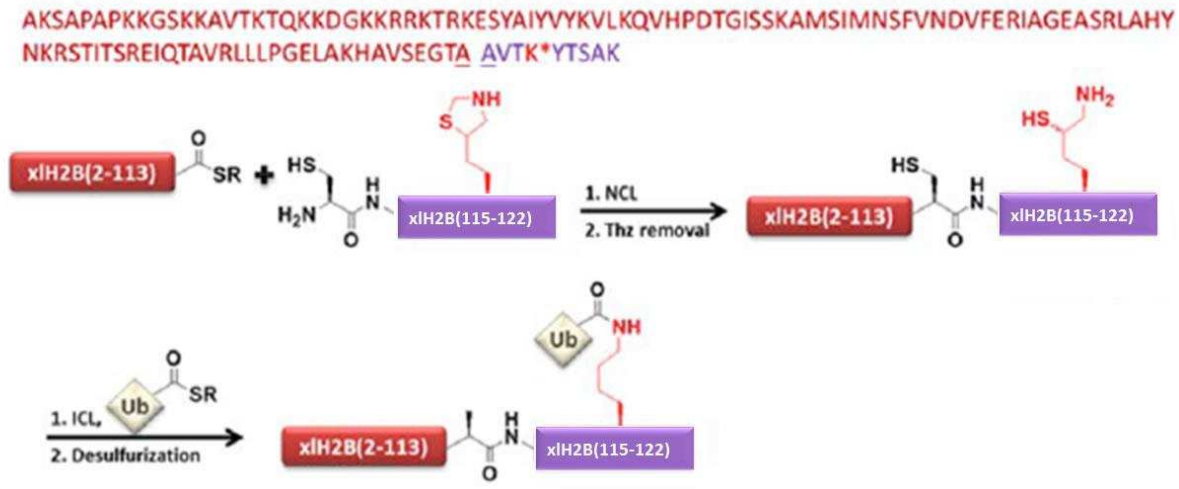


Figure 1.7 Scheme for the semi-synthesis of H2BK120Ub. SPPS and NCL was used to generate H2BK120Ub at 15-20 % yield. Figure adapted from (Morgan et al., 2016).

Table 1.2. Summary of methods used to generate ubiquitinated H2B by chemical synthesis or semi-synthesis.

Substrate	Method used	Nature of the linkage	Limitations	References
H2BK120Ub	SPPS; recombinant protein expression; NCL	Native isopeptide bond	The use of a photolytically removable ligation auxiliary resulted in low yield; the auxiliary mediated ligation reaction requires a minimum of 5 days to reach 60% completion.	(McGinty et al., 2008)
H2BK120Ub	SPPS; recombinant protein expression; NCL	Native isopeptide bond, but contains G76A mutation in Ub	The auxiliary-mediated ligation described above was replaced by cysteine–mediated ligation. The mutation in Ub is known to have inhibitory effects on DUBs.	(McGinty et al., 2009)
H2BK120ssUb	Recombinant protein expression	Disulfide bond, not cleaved by DUBs	The resulting product is highly sensitive to reducing reagents. The linkage is non-native and not cleaved by DUBs.	(Chatterjee et al., 2010)
H2BK34Ub	SPPS; NCL	Native isopeptide bond	Low yield of the final ubH2B product and side product represented in the oxidation of methionine.	(Siman et al., 2013)
H2BK120Ub	SPPS; NCL	Native isopeptide bond	The use of specialized axillary groups makes this method inaccessible to biochemistry labs.	(Morgan et al., 2016)
H2BK120*Ub	Recombinant protein expression	Crosslink, not cleaved by DUBs	The linkage is non-native and not cleaved by DUBs.	(Long et al., 2014a)

CHAPTER 2: SYNTHESIS OF SITE-SPECIFIC UBIQUITINATED HISTONES THROUGH CHEMICAL LIGATION

2.1 Introduction

To understand the mechanistic roles for individual histone modifications as well as their regulation requires the ability to obtain homogeneously modified histones. Despite that histones are very abundant in cells, the highly heterogeneous nature of histone modifications *in vivo* makes them a non-ideal source for biochemical studies. For example, most studies on histone DUBs use ubiquitinated histones purified from mammalian cells as substrates (Belle and Nijnik, 2014). Due to the presence of a variety of naturally occurring PTMs, it remains unclear how these various PTMs could affect the specificity of the tested DUB. This underscores the need to develop strategies to generate homogenous site-specific ubiquitinated histones.

Ubiquitinated histones can in principle be obtained *in vitro* by using recombinant E1, E2, and E3 enzymes. However, this approach has significant limitations. First, the E2 and E3 specific for the histone and linkage type need to be identified, expressed, and purified. Second, *in vitro* conditions have to be optimized to ensure that the enzymatic system reproduces the known *in vivo* specificity. It is not uncommon that *in vitro* the enzymes can attach Ub to non-specific sites and generate polyubiquitinated histones as well. Finally, the yields of these enzymatic reactions vary significantly depending on the specific ubiquitinated histone that one is interested in. For example, the yield reported for the generation of H2BK120Ub is less than 5% (Kim and Roeder, 2011). As a result, several alternative non-enzymatic strategies have been developed to produce chemically-defined

ubiquitinated histones that use a combination of native chemical ligation (NCL) and solid phase peptide synthesis (SPPS). These are summarized in Table 1.2 and described in detail under (1.7). As discussed in chapter one (under heading 1.7), these previous methods have limited yields, and are technically challenging for a biochemical lab. This chapter describes a new approach to produce site-specific ubiquitinated histones starting with full-length recombinant proteins. It eliminates the need for NCL and SPPS. Furthermore, the yield is considerably higher than previous methods and the protocol is well-suited for a biochemistry lab. This approach was applied to generate Ub-histone conjugates where Ub is attached to three major sites of ubiquitination: H2BK120, H2AK119, and H2AK15. Identical procedures can be implemented to attach Ub to any other desired site on any histone type.

2.2 Experimental procedures

To achieve site-specific attachment of Ub on histones, the desired ubiquitination site in the histone was mutated to cysteine by site-directed mutagenesis in order to differentiate it from the other lysines present in the histone. This is possible since histones don't contain cysteines in their amino acid sequences. In addition, a 6xHis-tag was introduced to the N-terminus of Ub to facilitate purification of the Ub-histone conjugate. Recombinant mutant histones and HisUb were expressed in *E. coli* and purified to homogeneity. Using these purified proteins as starting material, seven major steps were performed as illustrated in Scheme 2.1:

(1) The single cysteine in the histone was protected from future alkylation by reacting with methylmethane thiosulfonate (MMTS).

(2) Lysine side chains in the histone were blocked with allyloxycarbonyl (Alloc).

(3) The cysteine was de-protected with tris(2-carboxyethyl)phosphine (TCEP) and reacted with ethyleneimine to give a lysine mimic, S-aminoethylcysteine (Kc).

(4) The C-terminus of HisUb was activated by the E1 enzyme to generate a Ub with a thioester group (HisUbSR). R is an ethanesulfonate group.

(5) Lysine side chains in HisUbSR were blocked with Alloc.

(6) Silver-mediated condensation results in the ligation of Ub and the histone.

(7) All lysine side chains were deblocked by removing Alloc groups.

(8) Purification of the Ub-histone conjugate via the 6xHis-tag, refolding of histone octamers and reconstitution of nucleosomes.

2.2.1 Generation of (Alloc)HisUbSR

HisUb was expressed and purified according to (Long et al., 2014a). Briefly, batch purification of HisUb was performed under native conditions using Ni-NTA agarose according to the manufacturer's protocol. Next, to remove minor impurities, eluates were passed through Q Sepharose Fast Flow resin.

To generate HisUbSR (El Oualid et al., 2010), 1 mM purified HisUb was incubated with 0.1 uM E1 Ub activating enzyme, ATP (10 mM), $MgCl_2$ (10 mM), and sodium 2-mercaptoethane-sulfonate (MESNa) (0.1 M) in NaPi at pH 8 (20 mM). The reaction was incubated at 37 °C for 6 h. The E1 enzyme was precipitated by adding 10 % glacial acetic acid to the solution. Successful generation of HisUbSR was confirmed by ESI-MS. The protein was then dialyzed against 0.4 % TFA/H_2O followed by lyophilization.

The lysines were blocked with Alloc according to (Castaneda et al., 2011). Lyophilized HisUbSR was dissolved in DMSO at 10 mg/ml (1 mM) and incubated with diisopropylethylamine (DIEA, 180 mM), and *N*-(Allyloxycarbonyloxy)succinimide (15 mM). Each HisUb molecule contains 1 N-terminal α-amino group, 7 histidines and 7 lysines, which add up to 15 possible sites to be modified by Alloc. Blocking typically proceeded for 1 h at room temperature and its completion was confirmed by ESI-MS. The protein was then ether-precipitated by mixing with 2x vol of ice-cold ether, vortexing for 15 s, allowing the solutions to settle, and the top organic layer was removed. This was repeated two more times until a white pellet was formed. The final (Alloc)HisUbSR pellet was dissolved in DMSO at 50 mg/ml (5.2 mM) and used in the subsequent chemical ligation with histones.

2.2.2 Generation of (Alloc)histones containing a site-specific lysine mimic

Here I describe the generation of H2B ubiquitinated at position 120 (H2BKc120Ub) as an example (Scheme 2.1). However, this method was applied successfully to ubiquitinate histone H2A at position 119 and position 15 as well. Site-directed mutagenesis was used to introduce the desired K to C mutation in each histone. Histone H2A and H2B lack natural cysteines, therefore, the mutant histones contain a single cysteine that was introduced by mutagenesis. Cysteine side chain is highly nucleophilic and could be readily alkylated by ethyleneimine to generate S-aminoethylcysteine (Raftery and Cole, 1963). S-aminoethylcysteine has a lower pKa value than lysine, however, it has been shown to mimic lysine effectively in Ub conjugation systems (Hofmann and Pickart, 1999; Piotrowski et al., 1997). It is also used as a lysine surrogate in the chemical methylation strategy developed by the Shokat group (Simon et al., 2007). Mutant histones were expressed and

purified according to (Dyer et al., 2004) with the modification that they were dialyzed in 1 mM HOAc before lyophilization.

Cysteine protection in histones by MMTS. MMTS is a sulfhydryl-reactive compound that can reversibly sulfenylate thiol-containing molecules. Reacting with MMTS converts reduced sulfhydryl (-SH) to dithiomethane (-S-S-CH3)(Smith et al., 1975). Treatment with reducing reagents, such as Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), will cleave the disulfide to restore the original sulfhydryl. Lyophilized histones required 0.5% TFA/DMSO to dissolve. MMTS (3.5 mM) was added at 10-fold molar excess over the protein concentration and DIEA (75 mM) was added to adjust the pH to neutral. The solution was incubated at room temperature for 15 min. Protection of the cysteine by MMTS was confirmed by MALDI-TOF MS. The protein was then precipitated by ether as described above. The final pellet was dissolved in DMSO at a concentration of 2-5 mg/ml.

Alloc blocking of lysines in histones. The above histone solution was incubated with 20-fold molar excess of *N*-(Allyloxycarbonyloxy)succinimide over the total amines in the histone. For example, H2BK120C contains 19 lysines, 3 histidines and an α-amino group, which add up to a total of 23 possible reaction groups. At a protein concentration of 0.35 mM, 161 mM *N*-(Allyloxycarbonyloxy)succinimide was added to the protein. DIEA (240 mM) was added to adjust the solution to a basic pH. Blocking took place at room temperate for 2 h. ESI-MS was used to confirm complete blocking. The Alloc-blocked histone was then precipitated by ether. The pellet was dissolved in GdHCl (7 M) and HEPES at pH 8 (100 mM) at a final concentration of 2-5 mg/ml. An aliquot of the Alloc-blocked histone, containing the native cysteine, was saved at this step to serve as a negative control in the subsequent chemical ligation reactions.

Cysteine de-protection and alkylation. TCEP (20 mM) was added to the above histone solution and allowed to incubate at room temperature for 15 min in order to reverse the MMTS protection. The reduced cysteine was then alkylated by ethyleneimine (55 mM) at 37 °C for 1 h (Volk et al., 2005). The de-protection and alkylation were confirmed by ESI-MS. I found that Alloc-blocked histones are insoluble at lower GdHCl concentrations, which allowed its precipitation by simply diluting the protein solution 3-fold with ddH_2O. The protein pellet was collected by centrifugation and then washed with water two times to remove any remaining ethyleneimine. The pellet was then dissolved in 0.5% TFA/DMSO at 20 mg/ml (1.5 mM) for the subsequent chemical ligation with (Alloc)HisUbSR (Scheme 2.1).

2.3 Conjugation of Ub to histones through silver-mediated ligation

Silver-mediated ligation. The ligation procedure was modified from (Castaneda et al., 2011). (Alloc)HisUbSR was incubated with (Alloc)H2BKc120 at a molar ratio of 1:2. Typically, 0.5 mM Ub and 1 mM histone were mixed. DIEA (400 mM), hydroxysuccinimide (H-OSu, 38 mM), and $AgNO_3$ (6 mM) were made fresh in DMSO and added to the Ub-histone mixture. This was incubated at room temperature in the dark overnight. Subsequently the proteins were ether-precipitated and formation of ubiquitinated histones was analyzed by SDS-PAGE after removal of the Alloc groups.

Global Alloc De-blocking. Removal of Alloc groups was based on a modified procedure from (Castaneda et al., 2011), by incubation with chloro-pentamethylcyclopentadienyl-cyclooctadiene-ruthenium(II) ([Cp*Ru(cod)Cl]) and thiophenol at 100x equivalent to the moles of protected amines in the solution, including

all lysine and histidine residues and the N-terminal amine. After the protein pellet was dissolved in 0.5 %TFA/DMSO (v/v) at 5 mg/ml, ddH_2O (10%), [Cp*Ru(cod)Cl] (10 mM), and thiophenol (10%) (v/v), were added. The resulting dark-brown solution was incubated at 50 °C for 4 h. This was followed by at least 5 rounds of ether-precipitation.

Characterization of ubiquitinated histone conjugates. Formation of ubiquitinated histones was confirmed by SDS-PAGE. The same samples were analyzed by immunoblotting with antibodies that specifically recognize the isopeptide linkage in H2AK119Ub and H2BK120Ub (Rabbit monoclonal antibodies, Cell Signaling). The ubiquitinated histone band was cut from an SDS-polyacrylamide gel and sent to the proteomics and metabolomics facility at CSU for trypsin digestion followed by LC-MS/MS analysis. The results confirmed successful generation of S-aminoethylcysteine and correct installation of Ub at the desired position.

2.2.4 Reconstitution of ubiquitinated mononucleosomes

The protein pellet after global Alloc removal was dissolved in GdHCl (7 M), βME (5mM), NaPi at pH8 (0.1M) and imidazole (10mM). The Ub-histone conjugates and unreacted HisUb were purified on a HisTrap column. Elutions from the column were concentrated, mixed with its histone partner(s), and refolded into ubiquitinated H2A/H2B dimer or ubiquitinated octamer (Dyer et al., 2004). For dimer reconstitution, H2BKc120Ub and H2A were mixed at equal molar ratio and were allowed to refold by dialyzing against refolding buffer at 4 ℃: Tris at pH 7.6 (10 mM), NaCl (2 M), EDTA (1 mM), and βME (5 mM). After dialysis, folded histone dimers were purified on Superdex 75 in the refolding buffer. ESI-MS was used to confirm the correct molecular weight of the ubiquitinated

histone species. Similarly, for octamer reconstitution, H2BKc120Ub, H2A, H3 and H4 were mixed at equal molar ratio, allowed to refold by dialysis, and purified on Superdex 200. Histone octamers containing ubiquitinated histones can be readily assembled with 147mer DNA containing the 601 Widom sequence (Lowary and Widom, 1998) to form mononucleosomes by salt dilution (Owen-Hughes et al., 1999). The best DNA to octamer ratio needs to be determined by titration. Typically, DNA and octamer were combined at 1:1.2 molar ratio in 10 µl refolding buffer to reach final concentration of 1.5 µM DNA and 1.8 uM octamer. Dilution buffer (Tris at pH 7.6 (10 mM), EDTA (1 mM), DTT (1 mM), and BSA (0.1 mg/ml)) was added at 30 °C every 15 min with volumes of 3.3, 6.7, 5, 3.6, 4.7, 6.7, 10, 30 and 20 µl. The nucleosomes were then concentrated and stored at 4 °C. The quality of the nucleosomes was evaluated by 6% native PAGE (Long et al., 2014a). Ubiquitinated nucleosomes were incubated with USP2cc to evaluate the quality of the isopeptide bonds. This catalytic core domain of the deubiquitinating enzyme USP2 strips ubiquitin moieties from all ubiquitin-conjugated proteins, including linear or branched chains (Kim et al., 2011).

2.3 Results

2.3.1 Characterization of reaction intermediates and products by mass spectrometry

(Alloc)HisUbSR. HisUb could be produced in large amounts in *E. coli* (~250 mg from 6 L culture). The generation of HisUbSR was confirmed by ESI-MS (Fig. 1a and b) by the addition of 125 Da to the molecular weight of HisUb. The approach developed by (El Oualid et al., 2010) was efficient and complete thioesterification of HisUb was achieved after 6 h incubation at 37 °C. HisUbSR is preserved in 0.4 % TFA/H_2O to stabilize the thiolester.

The efficiency of Alloc blocking was evaluated by ESI-MS. There are 15 potential reacting groups in HisUbSR: 7 lysine side chains, one N-terminus, and 7 histidine side chains. A single Alloc adds 84 Da. ESI-MS results showed that between 10 to 15 Alloc groups were attached to HisUbSR (Fig. 1c and d). This is most likely due to partial protection of the 7 histidines.

(Alloc)H2BKc120. MALDI-TOF MS confirmed the complete protection of the cysteine by MMTS, which adds 46 Da to the molecular weight of H2BK120C (data not shown). This reaction is highly efficient and the protection is maintained during the Alloc blocking step (Fig. 2b). There are 23 potential reacting groups in H2B: 19 lysine side chains, one N-terminus, and 3 histidine side chains. ESI-MS showed between 21 to 23 Alloc groups were added to the protein. This is most likely the result of partial blocking of the three histidines. Next step the cysteine is de-protected by treatment with TCEP, which results in the loss of 46 Da (Fig. 2c). During alkylation of the cysteine by ethyleneimine, it was found that Alloc groups attached to histidine side chains are largely hydrolyzed (Fig. 2d). The conversion of cysteine to S-aminoethylcysteine adds 43 Da and the predominant species observed by ESI-MS corresponds to H2BKc120 with 20 Alloc groups attached (Fig. 2d).

Silver mediated chemical ligation. Formation of ubiquitinated histones (both H2BKc120Ub and H2AKc119Ub) was analyzed by SDS-PAGE (Fig. 3a). Typically, the yield of the ubiquitinated histone is 20-40 % of the input histone. Since all the other steps have nearly 100% yield, the ligation step is most limiting in this approach. To test the specificity of Ub attachment, (Alloc)H2BK120C, whose cysteine was not alkylated, was used in the negative control reaction (Fig. 3 a). I consistently observed a small amount of Ub-histone species generated in the negative control reaction (denoted by #). The chemical nature of

Ub attachment in this species is unclear because this species is refractory to hydrolysis by USP2cc, a DUB that has little specificity and is commonly used to deubiquitinate any Ub conjugates (Kim et al., 2011)(data not shown). Immunoblotting with antibodies that specifically recognize the isopeptide linkage in H2BK120Ub or H2AK119Ub confirmed the specificity of Ub attachment (Fig. 3 b). Notably the Ub-histone species generated in the negative control reactions were not recognized by the linkage-specific antibodies. The bands corresponding to ubiquitinated histones were cut from the gel and sent for trypsin digestion and LC-MS/MS analysis. The obtained spectra showed an addition of 157 Da to the cysteine residue, which confirms alkylation of the cysteine by ethyleneimine (+43 Da) and addition of Gly-Gly from Ub after trypsin digest (+114) (Fig. 4).

2.3.2 Assembly of ubiquitinated mononucleosomes

Unreacted histones were removed by HPLC using a HisTrap column. Although the resulting elutions contained the ubiquitinated histones as well as unreacted HisUb, the latter did not interfere with subsequent refolding of histone dimers or octamers.

Purified histone dimers, H2A/H2BKc120Ub and H2AKc119Ub/H2B, were further analyzed by ESI-MS, which confirmed the expected sizes of the ubiquitinated histones as well as complete removal of Alloc groups (Fig. 5a and b respectively). Purified histone octamers containing ubiquitinated histones were successfully assembled into mononucleosomes by salt dilution. To evaluate the quality of the chemically assembled isopeptide linkages, ubiquitinated nucleosomes were incubated with USP2cc and analyzed by native PAGE. Ubiquitinated nucleosomes migrate slower on a native polyacrylamide gel, giving rise to distinct bands corresponding to the presence of 0, 1, or 2 Ub moieties in the

nucleosome (Fig. 6). I found that a species of the ubiquitinated nucleosomes were refractory to USP2cc, regardless of the site of Ub attachment. This is reminiscent of the species observed in the negative control reactions (Fig. 3a). Given that it is a minor fraction of the final product (Fig. 6 a and b), I expect that this species has little effect on the *in vitro* DUB assays that will be described in Chapter 3.

2.4 Discussion

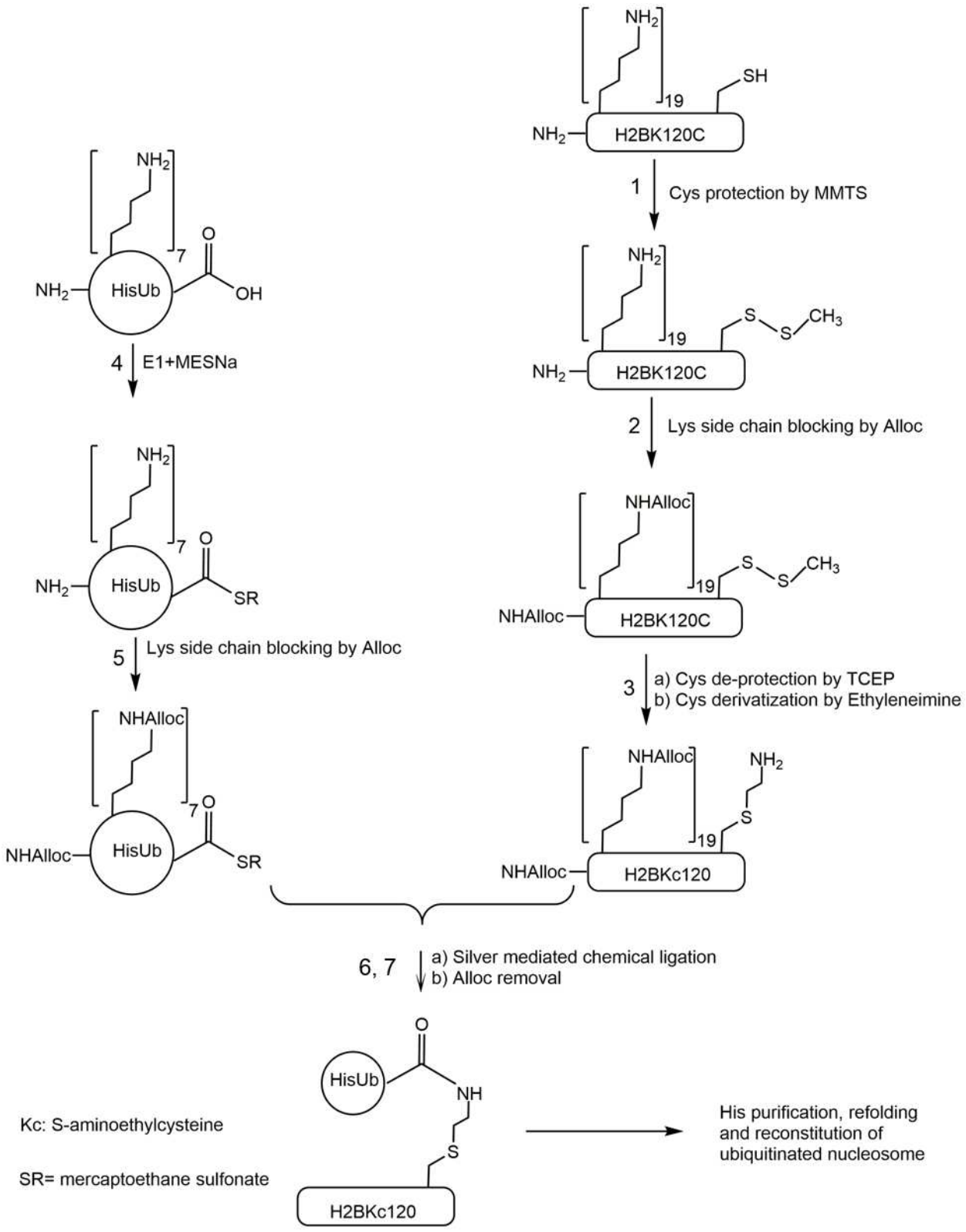
I have successfully developed a non-enzymatic method to generate chemically-defined site-specific ubiquitinated histones with reasonable yields. This approach was adapted from the method developed by Castaneda et al. to assemble Ub chains of controlled length and linkages (Castaneda et al., 2011). Using this method, I have successfully generated H2BKc120Ub, H2AKc119Ub and H2AKc15Ub. I expect that this method could be applied to attach Ub to any desired site in any histone type. Compared to the method developed by Castaneda et al., I optimized the solvent composition as well as the Alloc removal conditions due to differences in solubility of histones and Ub. In addition, I optimized the ratio of histone to Ub in the silver-mediated ligation reaction.

Castaneda et al. used genetically encoded Boc-lysine to specify the site of Ub attachment. Initially I applied this strategy to install Boc-lysine in H2B. However, the low efficiency of Boc-lysine incorporation during recombinant protein expression prevented me from obtaining sufficient amounts of purified histones. Instead the method described here takes advantage of the fact that most histones lack natural cysteines, therefore, a single cysteine mutation can be introduced at the desired site of ubiquitination. Among human core histones, only H3 contains natural cysteines, which can be mutated to alanines

with little effect on nucleosome structure and stability *in vitro*. Cysteines were conveniently protected and de-protected by treatment with MMTS and TCEP. Ethyleneimine was chosen as the alkylating agent because it is highly efficient in converting the cysteine into S-aminoethylcysteine, a well-accepted lysine mimic. Although I could not predict how the substitution of a methylene group with a sulfur would affect DUBs or potential readers of this PTM, the different ubiquitinated substrates I will be comparing all contain this substitution.

Since the un-hydrolysable species is resistant to USP2cc, Ub in that species could be attached to serine, tyrosine, or threonine through a oxy-ester (Wang et al., 2012). Oxy-ester bonds are labile to treatment with hydroxylamine at pH 9-10 (Magee et al., 1984) while amides are resistant to hydroxylamine (Olson et al., 1985). To test this possibility, both the negative control reaction and the reaction containing the linkage-specific Ub-histone conjugate were subject to hydroxylamine treatment. The USP2cc resistant species was resistant to hydroxylamine as well (data not shown). Therefore, the un-hydrolysable species does not contain an isopeptide nor an oxy-ester bond. The un-hydrolysable species might be a side product from the conditions used to remove Alloc i.e. allylation of the free amine upon Alloc removal. Alloc removal by other methods such as, palladium catalyzed reactions, (usually $Pd(PPh_3)_4$) should be tested (Isidro-Llobet et al., 2009). The nature of the un-hydrolysable species is yet to be determined.

In conclusion, this method is more economical and amenable to be performed in biochemistry labs in comparison with previously reported methods that rely on solid phase synthesis. It is easily adaptable to install Ub at any desired site in any histone types. I expect that it will have wide applications in future structural and biochemical studies of histone ubiquitination.



Scheme 2.1. Generation of site-specific ubiquitinated histones by chemical ligation. This scheme uses the production of H2BKc120Ub as an example. See "Experimental procedures" for detailed descriptions of each step.

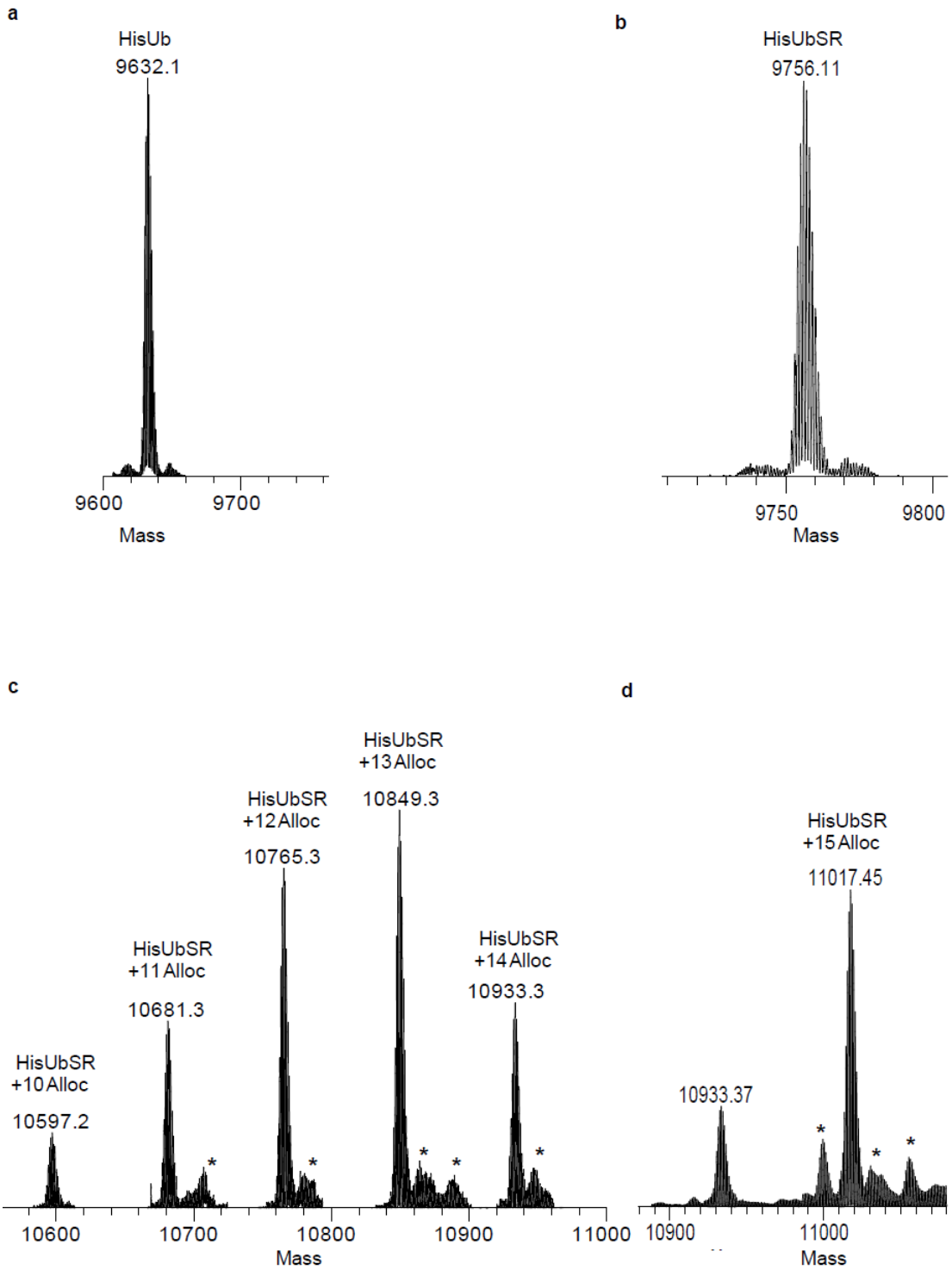


Figure 2.1. ESI spectra of Ub reaction intermediates, Scheme 2.1 (4 and 5). (a) HisUb, calculated mass: 9632.9, observed: 9632.1; **(b)** HisUbSR, calculated mass: 9757.9, observed: 9756.1. **(c and d)** Up to 15 Alloc groups were added to HisUbSR during blocking. Each Alloc group adds 84 Da. The imidazole groups of the seven histidines can react with Alloc, but often not efficiently. Peaks with asterisk are possibly water adducts (+18 Da), which is common in Alloc blocked proteins (Castaneda et al., 2011).

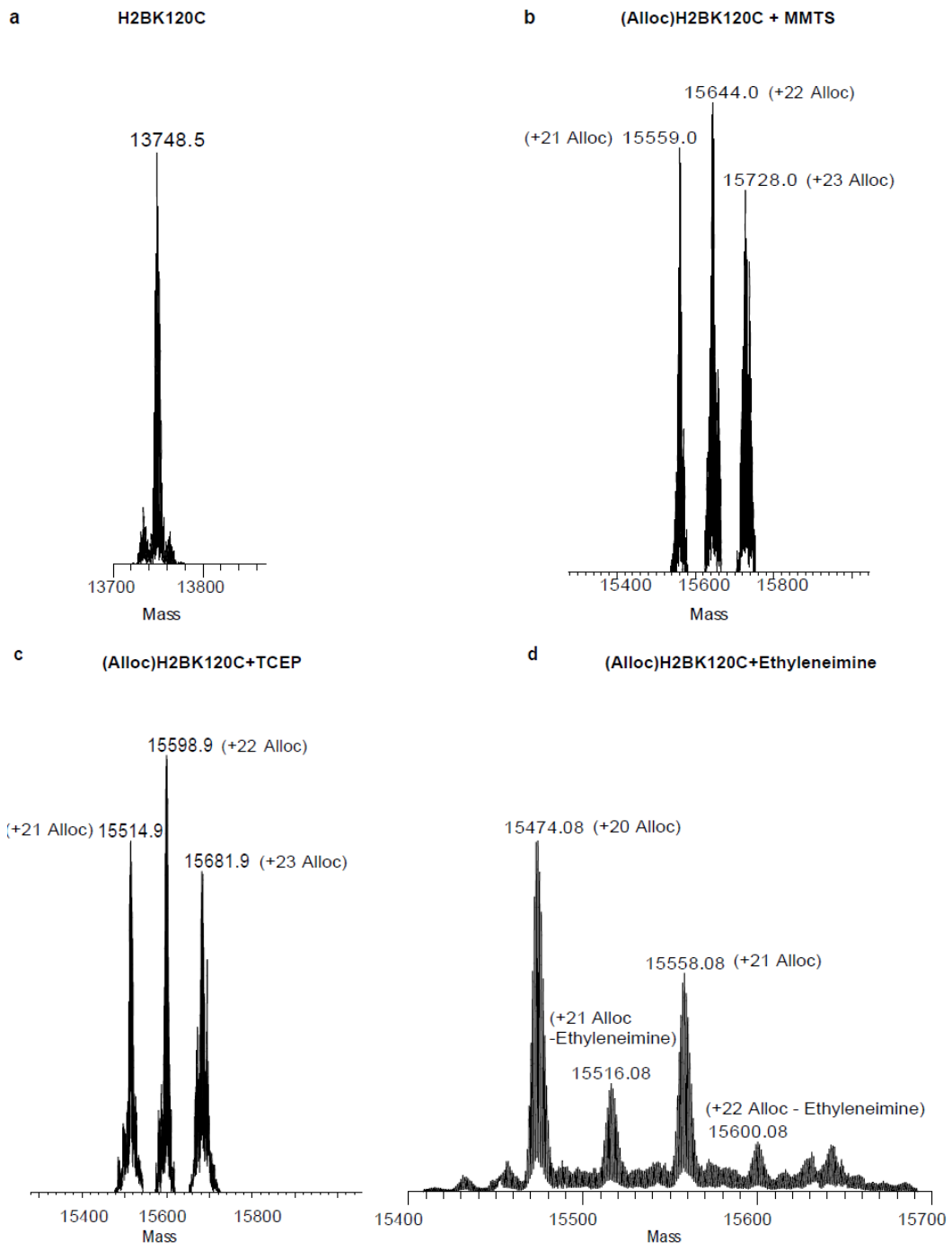


Figure 2.2. ESI spectra of H2B reaction intermediates, Scheme 2.1 (1-3). (a) Purified H2BK120C, calculated mass: 13749.9, observed: 13748.5. **(b)** H2BK120C after treatment with MMTS and *N*-(Allyloxycarbonyloxy)succinimide, calculated mass for addition of 23 Alloc groups: 15727.9, observed: 15728. **(c)** Cysteine de-protection by TCEP results in the loss of 46 Da from all three species. **(d)** Cysteine alkylation by ethyleneimine adds 43 Da. The main species corresponds to the presence of 20 stable Alloc groups. Three Alloc groups, most likely attached to histidines, were partially lost during this reaction. Calculated mass: 15472.9, observed: 15474.08.

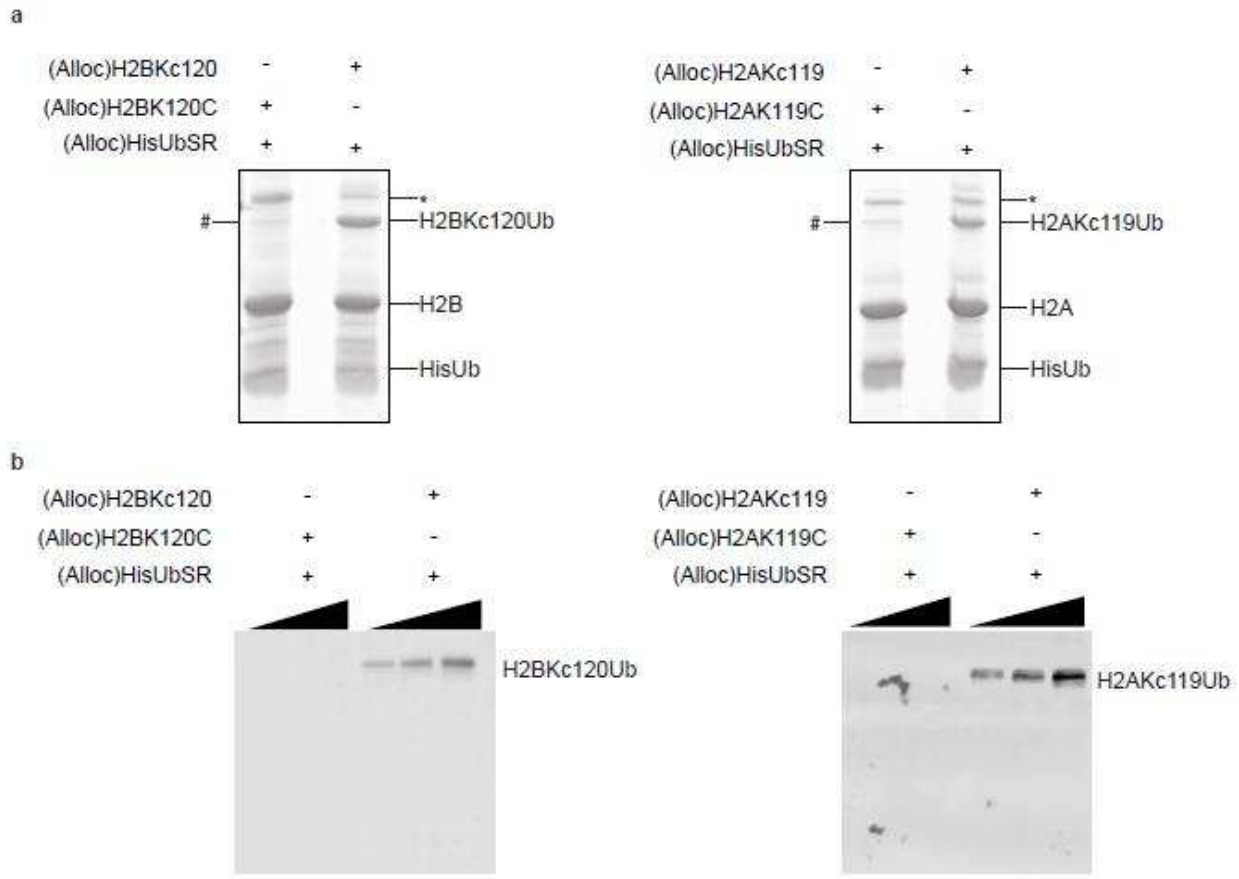


Figure 2.3. Generation of H2BKc120Ub and H2AKc119Ub. (**a**) Coomassie-stained 15% SDS-PAGE gels of the chemical ligation reactions. # denotes an unknown Ub-histone species observed in the negative control reactions with H2BK120C or H2AK119C. (**b**) Western blots of the same samples in (a) with linkage-specific antibodies against H2BK120Ub (left) or H2AK119Ub (right). These results confirm the specificity of Ub attachment.

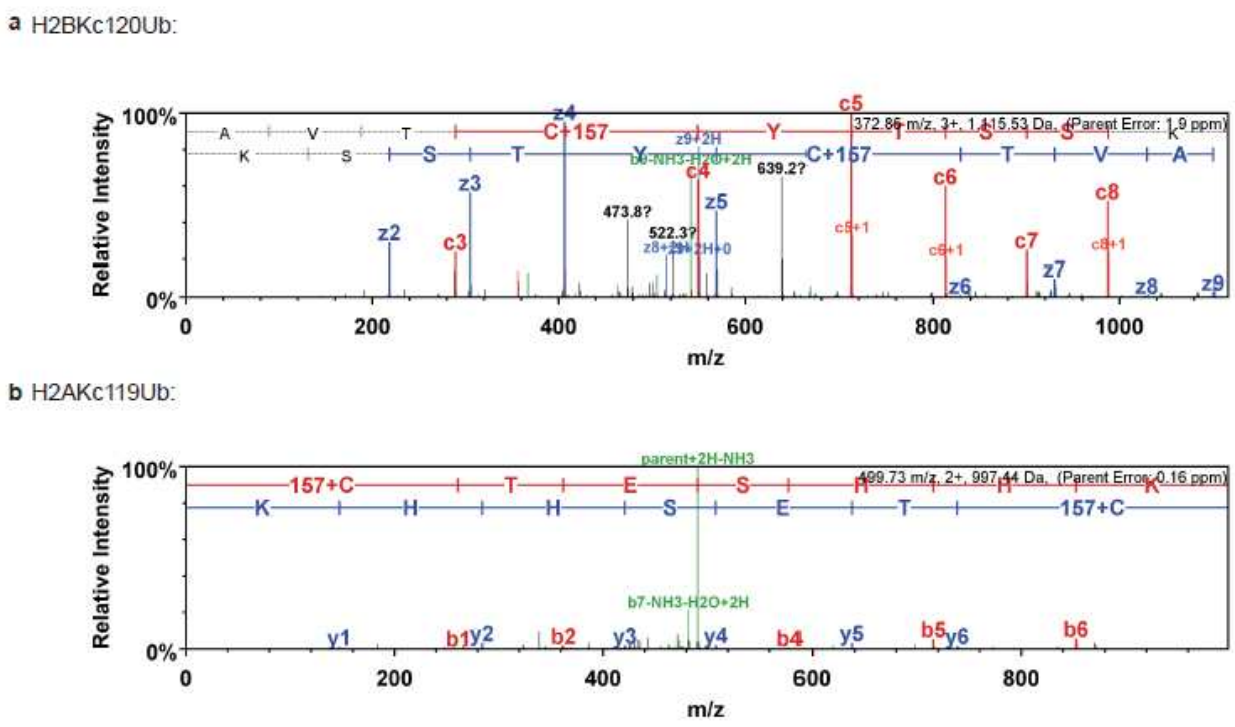


Figure 2.4. LC-MS/MS spectra of trypsin-digested H2BKc120Ub (a) and H2AKc119Ub (b). Shown are the spectra of the tryptic peptides spanning the Ub attachment sites. The +157 Da modification on cysteine represents alkylation by ethyleneimine (+43 Da) and addition of Gly-Gly from Ub (+114 Da).

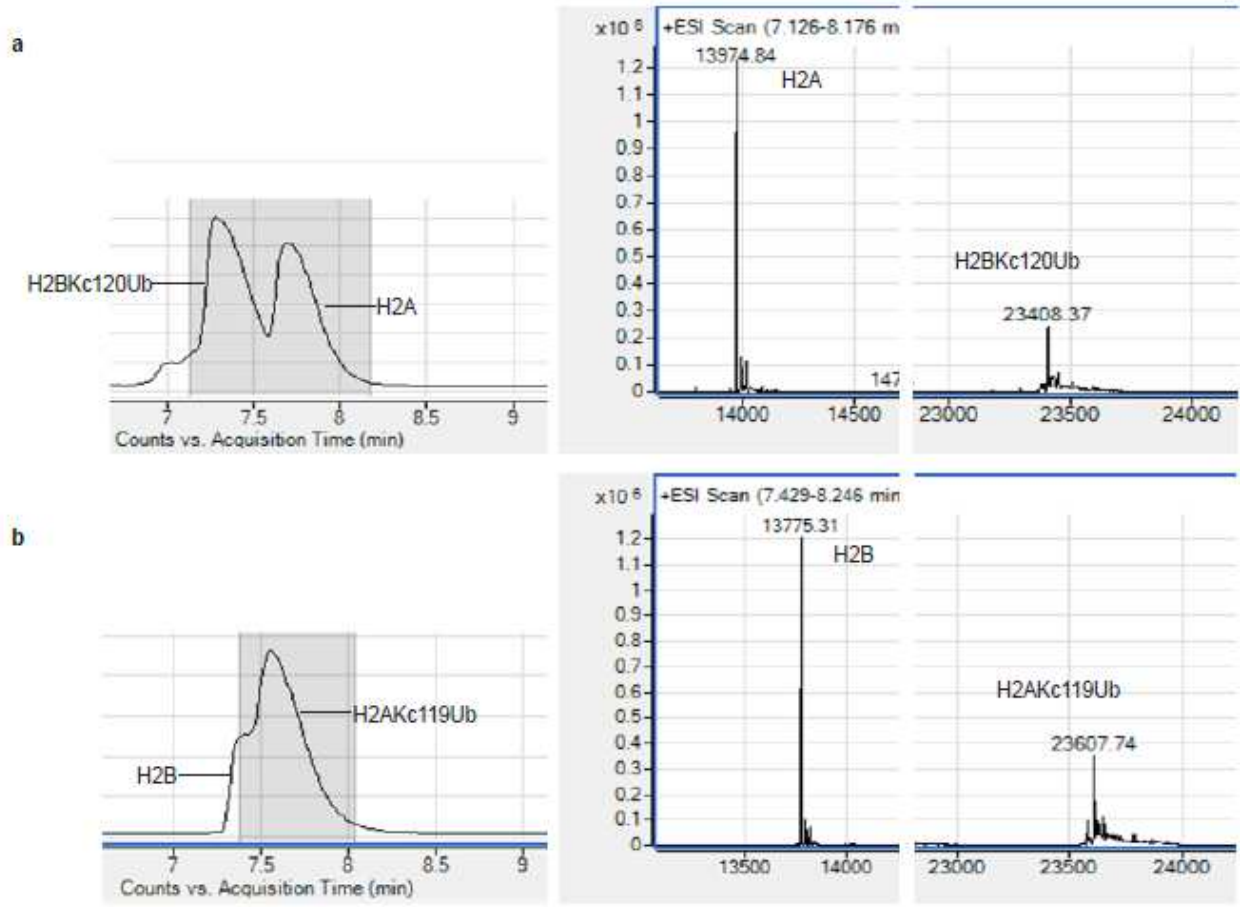


Figure 2.5. ESI-MS analysis of purified histone dimers containing Ub-histone conjugates generated by chemical ligation. Shown are the LC elution profiles and the corresponding MS spectra. (a) H2A/H2BKc120Ub: H2A, calculated mass: 13974.2, observed: 13974.55; H2BKc120Ub, calculated mass: 23407.8, observed: 23408.27. **(b)**H2AKc119Ub/H2B: H2AKc119Ub, calculated mass: 23607.1, observed: 23607.6; H2B, calculated mass: 13774.9, observed: 13775.23.

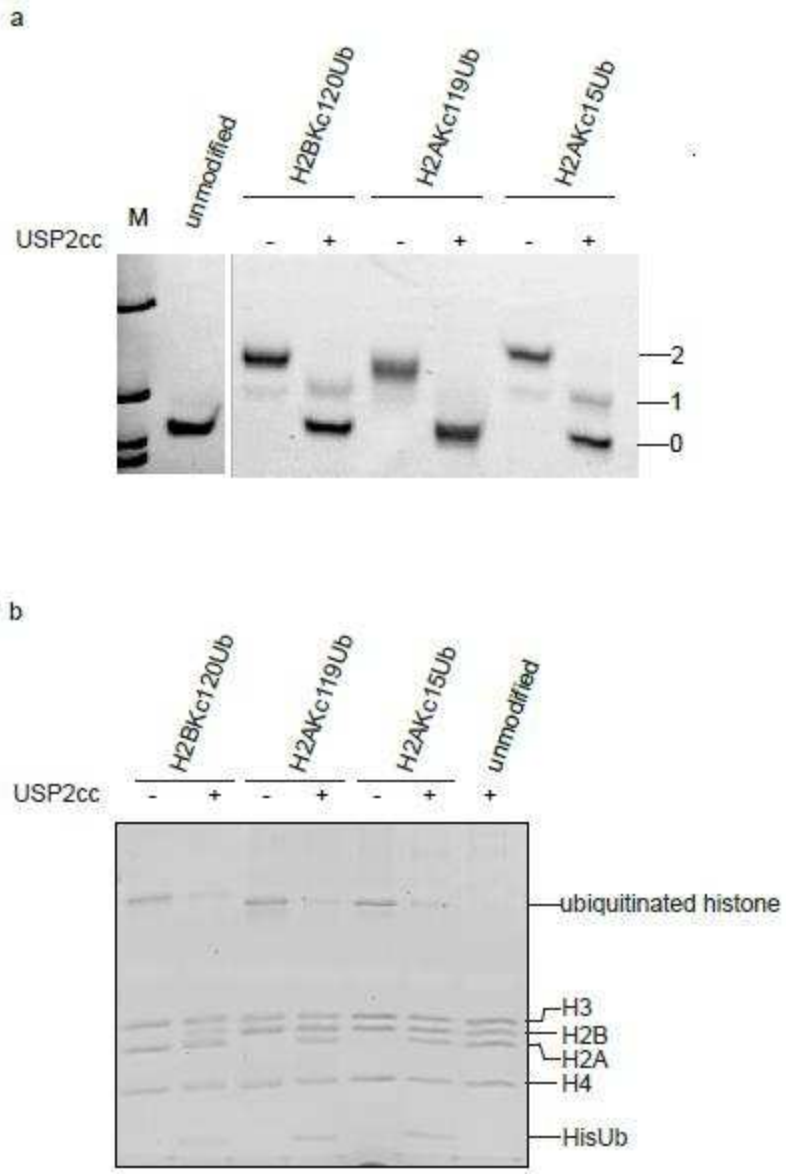


Figure 2.6. Deubiquitination of Ub-histone conjugates generated by chemical ligation. Mononucleosomes containing H2BKc120Ub, H2AKc119Ub or H2AKc15Ub were incubated with USP2cc and reactions were analyzed by native PAGE **(a)** or SDS-PAGE **(b).** Numbers in **(a)** refers to the number of Ubs in the nucleosome.

HisUb

MHHHHHHGGMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQ
QRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG*

MW: 9632.99 g/mol

H2AK15C

SGRGKQGGKARAKACSRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPV
YLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGR
VTIAQGGVLPNIQAVLLPKKTESHHKAKGK*

H2AK119C

SGRGKQGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAP
VYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLG
RVTIAQGGVLPNIQAVLLPKCTESHHKAKGK*

MW: 13949.24 g/mol

H2BK120C

PEPAKSAPAPKKGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQV
HPDTGISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAV
RLLLPGELAKHAVSEGTKAVTCYTSSK*

MW: 13749.91 g/mol

Figure 2.7. Full primary amino acid sequences of HisUb, H2A, and H2B used in this work. The K to C point mutations are underlined.

CHAPTER 3: SPECIFICITY AND KINETICS OF HISTONE DUBS

3.1 Introduction

There has been substantial interests in DUBs responsible for deubiquitinating histone substrates and their roles in the regulation of transcription and DNA damage response (Table 1.1). However, the field is lacking quantitative data to address the specificities of these DUBs, which is fundamental to mechanistic understanding of their biological functions. The challenge of *in vitro* quantitative studies largely lies in the difficulty to obtain homogeneous ubiquitinated substrates. This challenge has been met by the development of a new approach, as described in Chapter 2, which allowed me to obtain homogenous site-specific ubiquitinated histone substrates in milligram quantities. In addition, it is highly desirable to develop a sensitive real-time assay to follow the kinetics of deubiquitination. In collaboration with Dr. Yun-Seok Choi in the Cohen lab, we developed an assay that uses a fluorescently-labeled free Ub sensor to detect Ub released by DUBs. This chapter describes substrate specificities of a set of previously reported histone DUBs: the yeast SAGA DUB module (DUBm), human DUBs including USP3, USP16, BAP1, USP22, USP27X, and USP51. Detailed kinetic characterizations were performed with a subset of those DUBs by Dr. Yun-Seok Choi.

3.2 Experimental Procedures

Human BAP1, USP3, USP16, USP22 and their associated proteins were purified from mammalian cells that stably express 3xFlag-tagged versions of each DUB. The recombinant DUB modules containing USP22, USP27X or USP51 were expressed and purified from

insect cells by our collaborator Dr. Sharon Dent (MD Anderson Cancer Center) as described in (Atanassov et al., 2016). The yeast SAGA DUB module was a gift from Dr. Cynthia Wolberger (Johns Hopkins University) (Samara et al., 2010).

3.2.1 Cloning, transfection, and generation of stable cell lines

The coding sequences of BAP1, USP3, USP16, and USP22 were amplified from human cDNA and confirmed by sequencing (Uniprot accession number Q92560, Q9Y6I4, Q9Y5T5, and Q9UPT9, respectively). They were inserted into the pcDNA5FRT vector downstream of a 3xFlag tag. Site-directed mutagenesis was used to introduce mutations at the catalytic site of each DUB: BAP1(C91S), USP3(C168S), USP16(C205S), and USP22(C185S). Plasmids that encode either wild-type or catalytically-inactive DUBs were transfected by Lipofectamine 2000 into parental Flp−In T-REx 293 cells (Invitrogen). Stable cell lines were established following manufacturers' instructions. Cells stably expressing the DUBs were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% calf serum and 1% penicillin / streptomycin /glutamine. Doxycycline at 1 μg/mL was used to induce DUB expression 48 hours before harvest.

3.2.2 Affinity purification of DUBs from mammalian cell extracts

Cells grown to ~90% confluency were harvested by scraping in PBS. To obtain whole cell extract, cell pellets from 5x 15 cm dishes were resuspended in 1.5 ml cold lysis buffer: Tris at pH 7.5 (20 mM), NaCl (300 mM), 4-benzenesulfonyl fluoride hydrochloride (AEBSF,1 mM), and Triton X-100 (0.5 %), and incubated on ice for 30 minutes. Cell lysates were centrifuged at 20,000 rcf for 20 min and the clear supernatant was incubated with

100 μl equilibrated anti-Flag M2 agarose (Sigma) for 3 hours at 4 °C with rocking. After the unbound fraction was removed, the resin was washed five times with lysis buffer. Bound proteins were eluted with elution buffer: Tris at pH 7.5 (20 mM), NaCl (75 mM), AEBSF (0.25 mM), Triton X-100 (0.125 %), 3xFlag peptide (1 mg/ml). Elution was carried out for 30 min at 4 °C and repeated three times. Subsequently DTT (2 mM) was added to the eluates before they were concentrated (Amicon Ultra-0.5 centrifugal 3K filter device) and stored at -80 °C.

3.2.3 Ub-AMC assay

For Ub-AMC hydrolysis, Ub-AMC (0.5 μM) was incubated with the DUB in assay buffer: Tris at pH 7.6 (100 mM), $MgCl_2$ (2 mM), DTT (2 mM), Brij-35 (0.05 %), BSA (0.1 mg/ml), and NaCl (100 mM). The catalytically-inactive mutant DUBs were also tested to monitor possible contaminating DUBs from affinity purification. Ub-AMC hydrolysis was monitored continuously for 1 h at 30 °C on a Synergy 4 plate reader (Biotek, λ_{ex} = 340 nm and λ_{em} = 440 nm).

3.2.4 Nucleosome deubiquitination assays

Nucleosome concentrations were determined by diluting the nucleosomes in 0.1 M NaOH and its absorbance at 260 nm was measured using the NanoDrop Spectrophotometer and (260 nm absorbance was converted to ng/ul and 100 kDa was used as the molecular weight of Ub-nucleosome).

For qualitative nucleosome deubiquitination assays, nucleosomes (50 nM) were incubated with the DUB in assay buffer: Tris at pH 7.6 (100 mM), $MgCl_2$ (2 mM), DTT (2

mM), Brij35 (0.05 %), BSA (0.1 mg/ml), and NaCl (100 mM). The amounts of DUBs and their respective catalytically-inactive mutants were normalized by immunoblotting for the Flag-tag, but their exact concentrations are not known. The reactions were allowed to proceed for 30 min at 37 °C, then stopped by adding N-ethylmaleimide (NEM, 20 mM). 6 % native polyacrylamide gel (37.5:1 acrylamide:bis-acrylamide) was pre-run at 4 °C for 15 min at 130 V in 0.2xTBE (45 mM Tris-borate, 1 mM EDTA). Then, 5 ul of the reactions were loaded and ran for 2 h at 130 V at 4 °C. The gels were stained with ethidium bromide to visualize the nucleosomes. In the reactions with BAP1, NaCl was added to a final concentration of 0.3 M prior to loading on the gel in order to dissociate the enzyme from DNA.

For quantitative DUB assays, a fixed concentration of the DUB was added to increasing concentrations of nucleosome substrates in the same assay buffer as described above. In addition, a fluorescently-labeled free Ub sensor (tUI) was added to each reaction to allow monitoring of the released Ub in real time. tUI binds free, unconjugated Ub with a k_d of 0.5 nM. Labled with Atto 532, its fluorescence increases upon Ub binding (*manuscript in preparation*). Upon addition of the DUB, fluorescence was monitored continuously on a Jobi Yvon Fluoromax 4 fluorimeter at 30 °C (λ_{ex} = 530 nm and λ_{em} = 550 nm). Arbitrary fluorescence units were converted to HisUb concentration by using a standard curve constructed with HisUb standards. Initial velocity was determined using linear parts of the progress curve at each substrate concentration and K_M and V_{max} values were obtained by fitting the initial rates with the Michaelis-Menten equation using Prism.

3.3 Results and Discussion

3.3.1 Expression and purification of the histone DUBs

Human USP3, USP16, USP22 and BAP1 were successfully overexpressed in 293 cells in a doxycyclin-inducible manner, although their expression levels vary. Affinity purified fractions were analyzed by both anti-Flag immunoblotting and silver staining after SDS-PAGE (Fig. 3.1). Consistent with previous reports, each DUB is associated with other cellular proteins that co-purified (Lang et al., 2011; Scheuermann et al., 2010; Sowa et al., 2009), but currently we do not know the identities of the co-purified proteins and some of them are likely to be contaminants. It is also possible that multiple DUB complexes exist after one-step affinity purification. The conclusions we draw from activity assays rely on the comparison between wild-type and catalytically-inactive mutant DUBs purified in parallel. Silver-staining profiles of the eluates show strong similarities of proteins co-purified with wild-type DUBs and their mutant counterparts (Fig. 3.1).

3.3.2 Activities of purified histone DUBs against Ub-AMC

The human genome encodes ~ 80 DUBs. I used Ub-AMC as a generic substrate to monitor possible contaminating DUB activities in the affinity-purified fractions. Ub-AMC hydrolysis by wild-type (WT) BAP1 or USP16 were significantly higher than their catalytically-inactive counterparts (MUT) (Fig. 3.2 a and b). No appreciable Ub-AMC hydrolysis was detected with USP3 WT or MUT (data not shown). Both WT and MUT USP22 preparations showed comparable Ub-AMC hydrolysis, indicating contamination of other DUBs (Fig. 3.2 c). Importantly, USP22 MUT did not cleave nucleosomes containing

H2BKc120Ub, H2AKc119Ub, or H2AKc15Ub (Fig. 3.3 d). It is likely that many cellular DUBs can cleave a generic substrate such as Ub-AMC, but not ubiquitinated nucleosomes.

3.3.3 Qualitative deubiquitination assays

BAP1. Human BAP1 efficiently deubiquitinated H2AKc119Ub nucleosomes, but not H2BKc120Ub and H2AKc15Ub nucleosomes (Fig. 3.3a). This is consistent with previous reports using recombinant BAP1 in complex with its binding partner, ASXL1 (Sahtoe et al., 2016). It's likely that ASXL1 also co-purified with BAP1 in our preps. Although I did not quantitatively measure the enzyme kinetics with each substrate, I estimated that BAP1 hydrolyzes H2AKc119Ub ~50-fold more efficiently than H2BKc120Ub. Thus, BAP1 shows strong selectivity for its cognate substrate. Whether this is largely a K_M or k_{cat} effect is not known and future studies are needed to determine how this specificity is achieved. Note that there were low levels of contaminating DUB activities from both WT and MUT preps (Fig. 3.3a). The contaminating activity did not differentiate the three nucleosome substrates.

USP16. Human USP16 was highly active with all three nucleosome substrates I tested. This is not due to contaminating DUBs since the catalytically-inactive mutant prep showed no activity (Fig. 3.3b). When a time course was performed, all three substrates were equally deubiquitinated by USP16 (data not shown). Thus, I concluded that affinity-purified UPS16 has no intrinsic selectivity against H2AKc119Ub, H2BKc120Ub, and H2AKc15Ub nucleosomes.

However, USP16 was intially identified as an H2AK119Ub-specific DUB with preferences for nucleosomal, but not free histone substrates (Joo et al., 2007). Although

later reports confirmed that USP16 deubiquitinates both H2AK119Ub and H2AK15Ub *in vivo* (Shanbhag et al., 2010; Zhang et al., 2014) H2BK120Ub had not been investigated in those studies. There are two main differences between my studies and the previous report by Joo et al.: First, Joo et al. performed serial purifications by conventional chromatography to obtain the purified enzyme, whereas I performed one-step affinity purification. Second, Joo et al. reconstituted nucleosomes using ubH2B purified from yeast via Flag-tagged H2B. It's unclear which factor, or both, may account for the differences we observed. Future studies using recombinant USP16 will be needed to clarify the intrinsic specificity of this enzyme. Whether H2BK120Ub is a substrate for USP16 *in vivo* has not been addressed either. Interestingly, knockdown of USP16 leads to cell cycle defects that prevent M-phase progression (Joo et al., 2007). This may account for our observation that catalytically-inactive USP16 was expressed at ~5-fold lower levels than wild-type. Both H2AK119Ub and H2BK120Ub are deubiquitinated during mitosis (Matsui et al., 1979; Mueller et al., 1985). Based on our results, we think that it is likely that USP16 is responsible for deubiquitination of both substrates.

USP3. To our surprise, affinity purified human USP3 has no detectable deubiquitination activity against Ub-AMC, ubiquitinated histone or nucleosome substrates (Fig. 3.3c and data not shown). Previously, there were no reports on USP3 activity *in vitro*. However, overexpression *in vivo* significantly decreases endogenous levels of H2AK119Ub, H2BK120Ub and H2AK15Ub (Nicassio et al., 2007; Sharma et al., 2014). To validate our constructs, we overexpressed WT or MUT USP3 by transient transfection in 293T cells. We observed a decrease of H2AK119Ub in cells overexpressed WT USP3 (Fig. 3.2d). This is consistent with previous reports (Nicassio et al., 2007). Therefore, USP3 purified under the

current conditions possibly lost factor(s) required for its deubiquitination function. It will be interesting to identify those factors in future studies.

USP22. Human USP22 showed comparable deubiquitination activity against both H2BKc120Ub and H2AKc15Ub nucleosomes, yet little activity against H2AKc119Ub nucleosomes (Fig. 3.3d). USP22 had been shown to deubiquitinate both H2BK120Ub and H2AK119Ub *in vivo* with a preference for H2BK120Ub (Lang et al., 2011; Zhang et al., 2008a). Whether H2AK15Ub is a substrate for USP22 has not been investigated. Lang et al., showed that USP22 is part of the DUB module (DUBm) associated with the SAGA histone acetyltransferase complex. The subunits in the DUBm: ATAXN7, ATAXN7L3, and ENY2, are required for the activity of UPS22 (Lang et al., 2011). It's likely that these proteins co-purified with USP22 in our preps.

It is interesting that USP22 is able to deubiquitinate H2AKc15Ub nucleosomes with an efficiency comparable to H2BKc120Ub substrate. In the nucleosome structure, the N-terminus of H2A emanates from the nucleosome in close proximity to the C-terminus of H2B (Fig. 1.2). Therefore, the locations of Ub on H2AKc15Ub and H2BKc120Ub nucleosomes are likely to be very similar. USP22 is associated with transcriptional regulation of SAGA-dependent genes through deubiquitination of H2BK120Ub. In a recent study, USP22 along with ATXN7 and ENY2, were shown to be required for deubiquitination of H2BK120Ub following DNA damage, optimal formation of γH2Ax, and class switch recombination (CSR) (Ramachandran et al., 2016). H2AK15Ub is another mark important in the first steps of DNA damage response. Whether and how DUBs differentiate these two substrates are interesting questions, which are further investigated with quantitative deubiquitination assays described below. However, proteins that target USP22 to different

biological processes *in vivo* may play important roles in determining its specificity; these are factors that we cannot address with *in vitro* assays.

(y)SAGA DUBm. The SAGA DUBm is highly conserved from yeast to man. Yeast SAGA DUBm can be expressed in *E.coli* by co-expressing Ubp8, Sgf11, Sus1, and Sgf73 (1-96) (Samara et al., 2010). Ubp8 is the catalytic subunit that is homologous to human USP22. I found that (y)SAGA DUBm was highly specific to H2BKc120Ub nucleosomes (Fig. 3.3e). Unlike its human homologue, (y)SAGA DUBm was inactive against H2AKc15Ub nucleosomes (Fig. 3.3e). It's unclear what accounts for the differences in their specificities. Future studies with recombinant human SAGA DUBm are needed for direct comparison. It's worth noting that yeast does not have endogenous H2AK119Ub or H2AK15Ub. USP22 may have evolved to handle additional substrates that appear later in evolution.

Preliminary quantitative DUB assays were carried out to determine the K_M and k_{cat} of (y)SAGA DUBm (data not shown). It appears that the recombinant (y)SAGA DUBm is highly unstable under our assay conditions. I obtained rough estimates of its kinetic parameters with the H2BKc120Ub nucleosome substrate: K_M = 800 ± 500 nM; k_{cat} = 80 ± 30 min^{-1}. It has been proposed that targeting of (y)SAGA DUBm to different places on chromatin solely depends on its affinity to H2BK120Ub and it acts on the whole transcribed genome in a very fast manner (Bonnet et al., 2014). The parameters we determined support that (y)SAGA DUBm is an efficient enzyme (Daniel et al., 2004; Henry et al., 2003; Ingvarsdottir et al., 2005).

3.3.4 Kinetic characterization of USP22 family of DUBs

Among ~80 human DUBs, USP22 is closely related to USP27X and USP51. USP22 shares 82% and 70% identity with USP27X and USP51, respectively. Recently, Atanassov et al. discovered that these three DUBs are interchangeable subunits of the DUBm (Atanassov et al., 2016). They compete for binding to ATXN7L3 and ENY2, subunits of the DUBm, and incorporation into the DUBm is required for their catalytic activities. However, USP27X and USP51 do not associate with the SAGA acetyltransferase complex. Depletion of each DUB had subtle effects on global H2BK120Ub levels and affected transcription of overlapping, but non-identical sets of genes. Another recent report demonstrated that USP51 targets H2AK15Ub during DNA damage response (Wang et al., 2016). These recent developments raise the question whether these highly related DUBs have the same substrate specificities and how their specificities may instruct their respective regulatory functions *in vivo*.

To address these questions, we developed a fluorescence-based, real-time DUB assay using a sensor designed to bind free, unconjugated Ub. The design and characterization of this sensor, tUI, will be described elsewhere (Choi et al., manuscript in preparation). Using this assay, we determined the enzyme kinetics of affinity purified USP22, recombinant DUBm containing USP27X or USP51 in complex with ATXN7L3 and ENY2, against all three ubiquitinated nucleosome substrates (Fig 3.4). Because we don't know the concentration of active DUBs in each prep, we could not directly compare the V_{max} values of different DUBs. However, we could compare the K_M values and substrate selectivity for each DUB.

The catalytic efficiency of each DUB was calculated by dividing V_{max} /K_M, which was then normalized to that of H2BKc120Ub. For example, in case of 3XFlag-USP22 IP, the

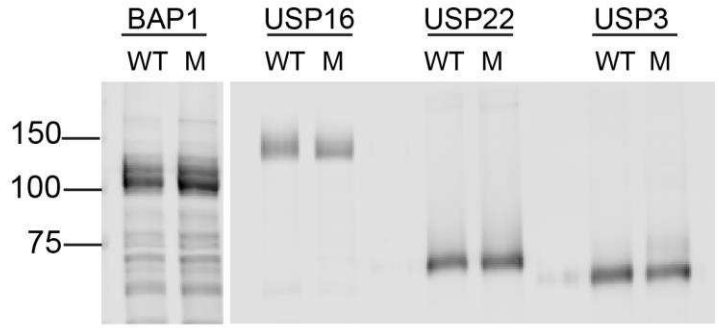
V_{max} /K_M for H2BKc120Ub equals 0.04 min^{-1} ± 0.002, which was considered 100 % (Fig. 3.4). All three DUBs showed comparable normalized catalytic efficiencies against H2BKc120Ub and H2AKc15Ub nucleosomes. In contrast, H2AKc119Ub nucleosomes were poor substrates and affinity purified USP22 showed strongest selectivity among the three DUBs (Fig. 3.4). It's possible that USP22 immunoprecipitated from mammalian cells were co-purified with factor(s) that enhance its selectivity. We are in the process of characterizing recombinant USP22 DUBm that includes ATXN7L3, ENY2 and an additional unique subunit, ATXN7.

For USP22 and USP27X, the selectivity against H2AKc119Ub nucleosomes primarily lies in V_{max}. There are less than 2-fold differences in K_M for H2BKc120Ub and H2AKc119Ub nucleosomes, suggesting both DUBm may bind to nucleosomes independent of Ub. For USP51, it was striking that it has significantly lower K_M and V_{max} for H2AKc15Ub among the three substrates. Since the same batch of substrates was used in all the assays, this observation cannot be attributed to the quality of the substrate. This finding is significant because a recent report showed that H2AK15Ub is a substrate for USP51 in DNA damage response *in vivo* and *in vitro* (Wang et al., 2016). Based on the low K_M, USP51 could be recruited to DNA damage site directly via binding to H2AK15Ub. The low V_{max}, however, suggest that either USP51 is designed to be a slow enzyme so that H2AK15Ub does not prematurely disappear during the repair process, or modifications and/or regulatory factors that are absent in the recombinant DUBm can activate USP51 upon DNA damage. Future studies should: (i) directly determine the binding affinities of USP51 DUBm and the nucleosome substrates; (ii) determine enzyme kinetics of affinity purified USP51 from cells

with and without DNA damage treatment; (iii) investigate whether H2AK15Ub is a substrate of USP22 or USP27X *in vivo*.

In conclusion, our *in vitro* results confirm that H2BK120Ub is the most likely substrate for USP22, USP27X, and USP51, as previously reported. They also show that H2AK15Ub could be an *in vivo* substrate for USP22 and USP27X, whereas H2AK119Ub is not likely a substrate for all three DUBs.

a



b

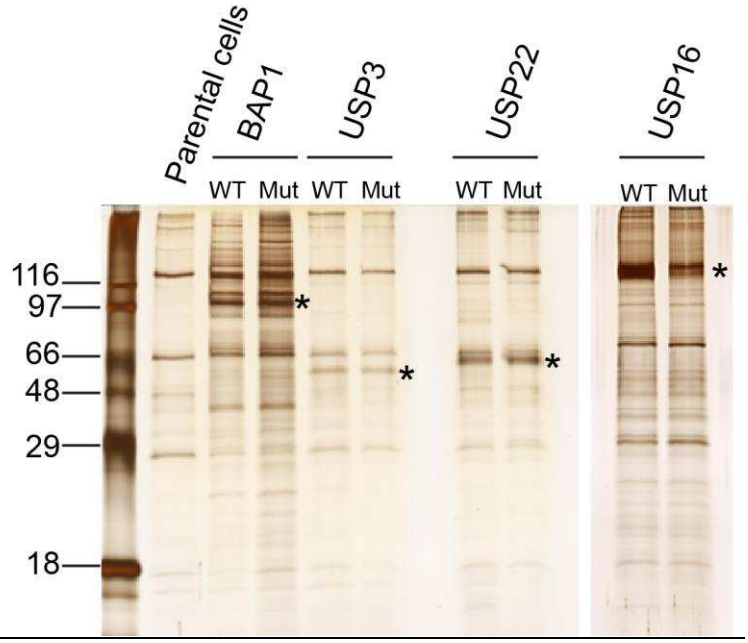


Figure 3.1. Affinity-purified human DUBs. (a) The amounts of affinity-purified DUBs, wild-type (WT) and catalytically-inactive mutants (MUT), were normalized based on immunoblots with an anti-Flag antibody. The predicted molecular weights of the DUBs are: BAP1 (83 kDa), USP16 (100 kDa), USP22 (60 kDa), and USP3 (60 kDa). **(b)** Silver-stained gel of affinity-purified DUBs. "Control" is prepared from parental un-tagged cells. *Asterisks* indicate the bands that correspond to the 3xFlag-tagged DUBs. In the case of USP16, the catalytically-inactive mutant was expressed at ~5-fold lower levels, possibly due to toxicity to the cell.

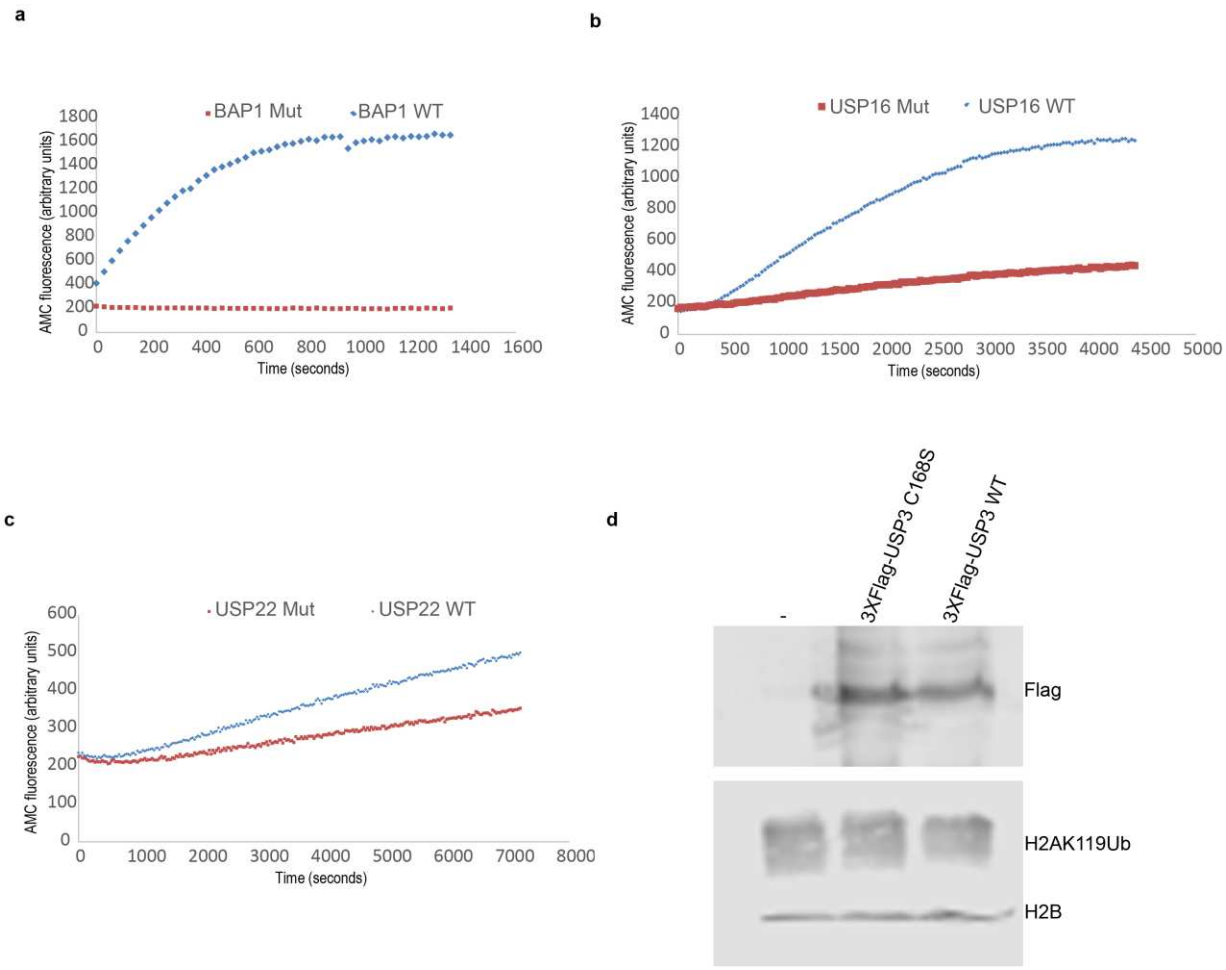


Figure 3.2. Ub-AMC hydrolysis by affinity-purified DUBs. Ub-AMC hydrolysis by affinity-purified wild-type (WT) and catalytically-inactive mutant (Mut) **(a)** BAP1, **(b)** USP16, and **(c)** USP22, were monitored over time to monitor possible contamination of other cellular DUBs. **(d)** 293T cells were transiently transfected to overexpress either WT or MUT USP3. Total cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting with anti-Flag (top), anti-H2AK119Ub and anti-H2B (as loading control).

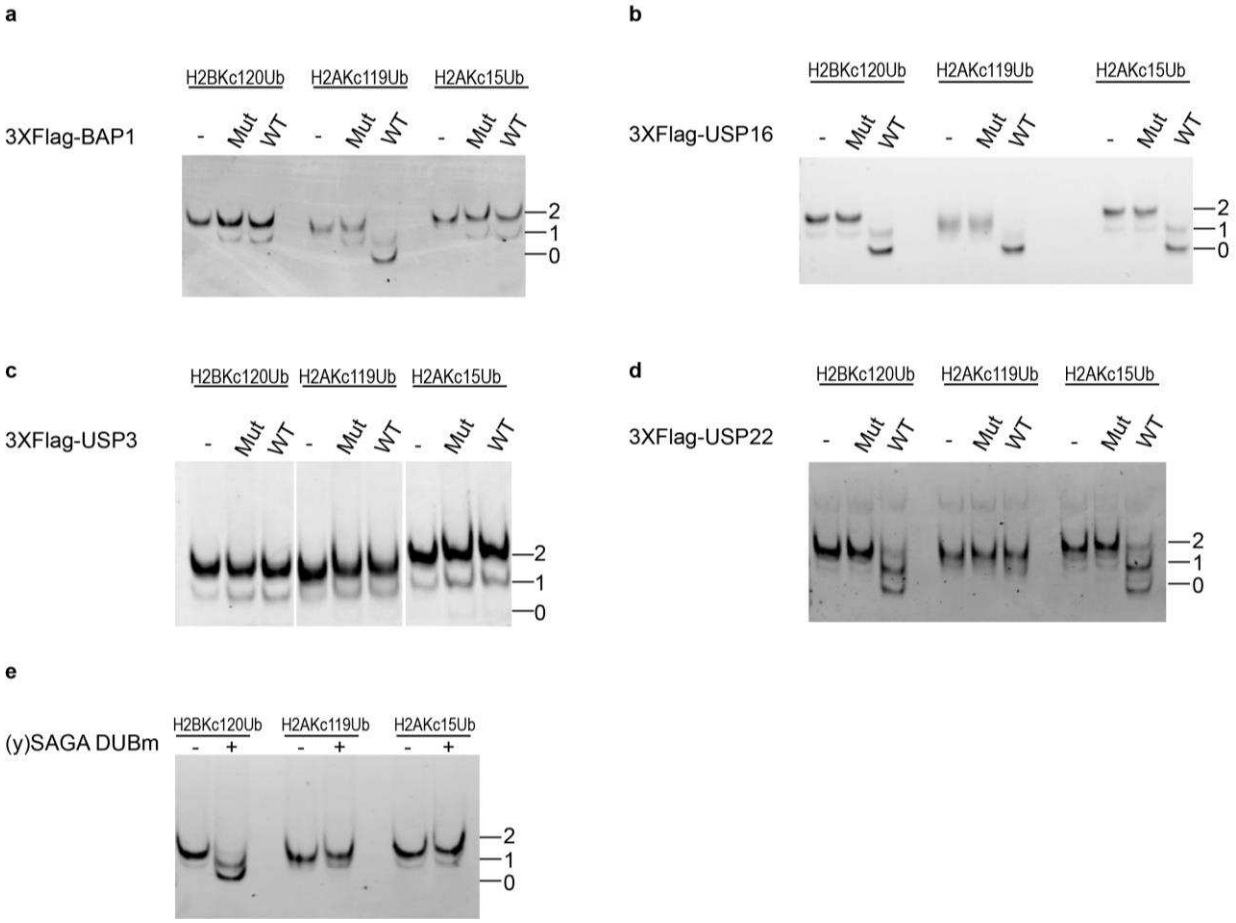
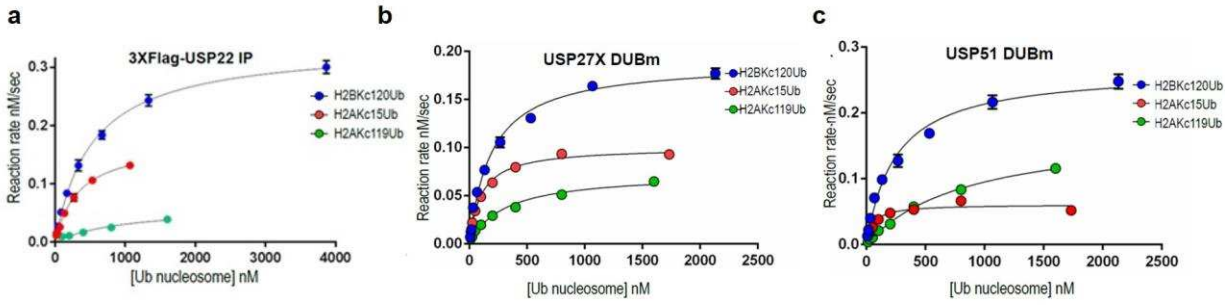


Figure 3.3. Specificities of histone DUBs toward H2BKc120Ub, H2AKc119Ub, and H2AKc15Ub nucleosomes. (a) BAP1, **(b)** USP16, **(c)** USP3, and **(d)** USP22 were affinity-purified from mammalian cells via a N-terminal 3xFlag tag. For each DUB, the concentrations of the wild-type (WT) and catalytically-inactive mutant (MUT) enzymes were normalized by anti-Flag immunoblotting. WT and MUT enzymes were incubated with the indicated nucleosome substrates (50 nM) at 37 °C for 30 min and the reactions were analyzed by native PAGE. Numbers refer to the number of Ubs in the nucleosome. **(e)** Purified Recombinant (y)SAGA DUBm was expressed and purified from E.coli. (Samara et al., 2010). 40 nM enzyme was incubated with 60 nM indicated nucleosome substrates at 30 °C for 5 min and the reactions were analyzed by native PAGE.



d

Complexes	H2BKc120Ub		H2AKc15Ub		H2AKc119Ub	
	V_{max} (nM/min)	K_M (nM)	V_{max} (nM/min)	K_M (nM)	V_{max} (nM/min)	K_M (nM)
USP22 IP	20.3 ± 0.3	500 ± 20	10.4 ± 0.7	300 ± 20	3.7 ± 0.3	1000 ± 400
USP27x DUBm	11.3 ± 0.3	200 ± 20	6 ± 0.1	100 ± 10	4.4 ± 0.3	300 ± 50
USP51 DUBm	15.9 ± 0.7	200 ± 30	3.6 ± 0.2	50 ± 10	10.6 ± 0.5	900 ± 80

Complexes	Normalized V_{max}/K_M		
	H2BKc120Ub	H2AKc15Ub	H2AKc119Ub
USP22 IP	100%	70%	8%
USP27x DUBm	100%	100%	25%
USP51 DUBm	100%	100%	20%

Figure 3.4. Michaelis-Menten enzyme kinetics of 3XFlag-USP22 IP, USP27X DUBm, and USP51 DUBm. The enzyme kinetics of **(a)** 3XFlag-USP22 immunoprecipitated from mammalian cells, **(b)** recombinant USP27X DUBm, and **(c)** recombinant USP51 DUBm, were determined with a fluorescence-based real-time assay using tUI, a free Ub sensor. **(d)** Table showing V_{max} (nM/min), K_M (nM) and V_{max} / K_M normalized to that of H2BKc120Ub. These experiments were performed by Dr. Yun-Seok Choi and each titration was performed in duplicates.

CHAPTER 4: DISCUSSION AND PERSPECTIVES

4.1 The ubiquitinated histones are homogenous and are useful in *in vitro* studies

Several detection and characterization methods were used to confirm the type of the Ub-histone linkage. First, a ligation reaction with a histone containing native cysteine had significantly less ubiquitinated histone product (5-10 fold) when compared to a reaction with a histone containing alkylated cysteine. Thus, the alkylated cysteine was the major target for Ub attachment while lysines were efficiently blocked by Alloc.

Second, both reactions mentioned above were blotted with antibodies specific to H2BK120Ub and H2AK119Ub linkages. The western showed reactivity towards the antibody only in the reaction containing alkylated cysteine, therefore, Ub attached at the alkylated cysteine was recognized as a native Ub-histone linkage. Third, the ubiquitinated histone band was cut and sent for trypsin digestion followed by LC-MS/MS and the linkage was confirmed to be site-specific at the alkylated cysteine.

Although western blot and mass spectrometry confirmed the presence of the site-specific linkage, they do not exclude the presence of other un-specific Ub attachments. Therefore, DUBs known for their *in vitro* specificity towards H2AK119Ub (BAP1) or H2BK120Ub (yeast SAGA DUBm), were used to confirm the nature of the linkage. i.e. BAP1 hydrolyzed H2AKc119Ub and not H2BKc120Ub or H2AKc15Ub nucleosomes. Similarly, yeast SAGA DUBm hydrolyzed H2BKc120Ub and not H2AKc119Ub and H2AKc15Ub nucleosomes. This is consistent with previous *in vitro* characterization studies (Morgan et al., 2016; Sahtoe et al., 2016).

Finally, the chemically generated Ub-histones could be used in *in vitro* studies as ubiquitinated histone monomers, in dimers or octamers, and as nucleosomes as all Ub-histone forms have been shown to be relevant *in vivo*.

4.2 Generation of less abundant Ub-histone conjugates and *in vitro* studies of histone crosstalk

H2AK119, H2BK120, and H2AK15 are the major and most abundant sites of ubiquitination. Ubiquitination at other sites of histones, although less abundant, have been reported (Tweedie-Cullen et al., 2009). In order to investigate the role of ubiquitination at other sites and to study the interplay of multiple modifications, it is necessary to obtain these homogenous site-specific ubiquitinated histones at a scale appropriate for *in vitro* studies.

For example, it has been reported that ubiquitination of H2B at lysine 34 directly regulates H3K4 and K79 methylation through trans-tail crosstalk both *in vitro* and *in vivo* (Sun and Allis, 2002; Wu et al., 2011). Therefore, synthesizing such substrate will help in the understanding of how this modification regulates H3 methylation and potentially affects the structure of chromatin, as well as to help shed light on the mechanisms of K34 ubiquitination and deubiquitination.

Similar to full length histones, histone peptides can be ubiquitinated using this method and can be used in *in vitro* studies to gain a better understanding of the mechanisms involved in the regulation of histone ubiquitination. For example, Sahtoe et al. used a N-terminal H2A peptide obtained by SPPS to study BAP1 deubiquitination. The H2A

peptide was ubiquitinated on K13 by thiolysine-mediated conjugation (El Oualid et al., 2010). They showed that the specificity of the BAP1/ASXL1 complex for H2AK119Ub is not determined by the amino acids surrounding the isopeptide bond, but at regions outside the ubiquitinated tail (Sahtoe et al., 2016).

4.3 USP3 is a H2A DUB *in vivo* which is not reflected in our *in vitro* assays

USP3's role in the DNA damage response is well documented (Belle and Nijnik, 2014). Several groups suggested monoubiquitinated histones as substrates for USP3. However, as discussed in Chapter 3, USP3 IP was inactive towards all the ubiquitinated histones or nucleosomes tested. In addition, it was inactive in Ub-AMC hydrolysis assays. To test the possibility that USP3 might have lost factor(s) required for its activity during the IP, Ub-Vinyl Sulfone (Ub-VS) activity assays were carried out with whole cell lysates extracted at different salt concentrations (Griffin et al., 2015). Initial results of these experiments showed that USP3 shows activity when lysed at low salt concentration. Further investigation and characterization of potential USP3 activators, which might have been lost during the IP, will shed light on its biological function and regulation.

4.4 USP16 as a possible H2A and H2B DUB during mitosis

USP16 is phosphorylated at the onset of mitosis and its activity is required for cell cycle progression into mitosis (Cai et al., 1999; Joo et al., 2007). Both H2A and H2B are deubiquitinated during mitosis, however, DUBs responsible for their deubiquitination are unknown (Matsui et al., 1979; Mueller et al., 1985). In this study, USP16 deubiquitinated H2BKc120Ub, H2AKc119Ub, and H2AKc15Ub nucleosomes similarly *in vitro*. This suggests

that they could all be *in vivo* substrates for USP16, possibly during mitosis. H2BK120Ub has not been tested as a possible substrate *in vivo* (Cai et al., 1999; Joo et al., 2007; Shanbhag et al., 2010). Therefore, it is necessary to investigate whether H2BK120Ub is a substrate for USP16 *in vivo* to complement the *in vitro* results reported here. Finally, since USP16 is important in mitosis, purification of USP16 from synchronized mitotic cells might add additional information to the specificity of USP16 against histone substrates.

4.5 Further studies of USP22, USP27X, and USP51

The *in vitro* DUB assays presented in this study showed that USP51 has ~5 fold higher affinity to H2AK15Ub when compared to H2BK120Ub. This is interesting since USP51 has been shown to deubiquitinate H2AK15Ub during DNA damage (Wang et al., 2016). Since USP51 is implicated in the DNA damage response, it would be appropriate to purify USP51 from cells that have been subject to DNA damage and compare it with USP51 purified from cells without DNA damage in *in vitro* DUB assays. Purification of USP51 from cells subject to DNA damage might preserve possible PTMs and interacting protein required for USP51 activity as a response to DNA damage.

Our *in vitro* DUB assays also showed that USP22, USP27X, and USP51 as H2AK15Ub DUBs. Only USP51 has been shown to deubiquitinate H2AK15Ub *in vivo* and *in vitro* (Wang et al., 2016). Further studies are required to test whether H2AK15Ub is a substrate of USP22 and USP27X *in vivo.* In addition, future experiments should address how these DUBs are regulated and whether or not they compete for H2AK15Ub.

In vitro structural and functional studies are required to shed light on the mechanistic details of DUBs specificity. With the use of chemically generated Ub-histone conjugates, crystallization of the catalytically-inactive mutant DUB along with its Ub-histone substrate can be attempted to understand intrinsic properties of the enzyme that might contribute to the enzyme-substrate specificity.

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