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Enzymatic assays for assessing histone deubiquitylation activity

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Abstract

While the post-translational modification of histones by the addition of ubiquitin was discovered decades ago, it has only recently been appreciated that the dynamic regulation of histone ubiquitylation patterns is an important mechanism for controlling a variety of biological processes. The processes include transcription, the recognition and repair of genomic damage and DNA replication, among others. Enzymes that catalyze the addition of ubiquitin to histones, such as the polycomb family, have been well-studied. In contrast, the enzymes that remove ubiquitin from histones are less well understood. The assay strategies described here provide a platform for the thorough *in vitro* and *in vivo* analysis of histone deubiquitylation. In some cases, these poorly characterized enzymes are likely to provide new opportunities for therapeutic targeting and a detailed understanding of their biochemical and biological activities is a prerequisite to these clinical advances.

Keywords

Histone; Ubiquitin; Deubiquitylation; DUB; Chromatin; USP family

1. Introduction

1.1. Overview of histone ubiquitylation

Over the past fifteen years, it has become increasingly clear that the posttranslational modification of nucleosomal histones plays a major role in transcriptional regulation [1]. These modifications include acetylation, methylation, sumoylation, phosphorylation, ubiquitylation and others. In essentially all cases, enzymes have been identified which either add or remove these modifications. Furthermore, the addition or removal of these modifications can take place in a highly dynamic manner such that rapid changes in transcriptional output can be accomplished. These modifications play roles in the loading of general transcription factors onto promoters, in transcription initiation, transcription elongation, splicing and potentially termination. While many of these modifications are small, (e.g. acetylation, methylation and phosphorylation), the addition of ubiquitin to histones nearly doubles the size of the protein. In mammals, significant fractions of histone H2A and histone H2B are ubiquitylated (5–15%), while very minor fractions of histone H3 and histone H4 are ubiquitylated (<1%) [2,3]. Unlike polyubiquitylation, which targets proteins for proteasome-mediated degradation, histones are primarily monoubiquitylated. Monoubiquitylation appears to serve as a regulatory or signaling mark rather than as a tag for protein destruction.

1.2. H2A ubiquitylation

The addition of ubiquitin to human histone H2A can be accomplished by at least two distinct cellular enzymes. The first is the Polycomb family enzyme RING1b/RNF2. RING1b exists in a complex with RING1a and BMI1, although these latter two proteins do not appear to have direct catalytic activity [4–6]. They do, however, modulate the catalytic activity of RING1b. Together with a protein called HPH2, these three proteins form the Polycomb complex PRC1 [7]. PRC1 is recruited to specific chromosomal regions by recognition of H3K27 trimethylation [8], which is added by EZH2, a component of the other major Polycomb complex PRC2. Ubiquitylated H2A (ubH2A) and PRC1 colocalize to large repressed regions of the genome, including the HOX gene cluster, the inactive X chromosome and areas of facultative heterochromatin [9]. Interestingly, RING1b is also present in a complex referred to as E2F6.com [10]. E2F6.com is important for the repression of genes normally activated by the E2F and MYC family of transcription factors. More recently, a second protein in addition to RING1b was shown to catalyze the ubiquitylation of histone H2A. This protein, termed 2A-HUB, associates with the N-CoR corepressor complex [11].

1.3. H2A deubiquitylation

Like the addition of ubiquitin to histone H2A, the deubiquitylation of H2A can occur dynamically and is catalyzed by a number of different proteins. Enzymes that deubiquitylate H2A in humans include USP3, Ubp-M/USP16, USP21, and USP22 [12–15]. In addition, an unrelated protein termed 2A-DUB also deubiquitylates human H2A [16]. The critical role these enzymes play in regulating chromatin-linked processes is highlighted by their conservation across many disparate species (Table 1). USP3 has been implicated in maintaining genomic integrity and loss of USP3 blocks S phase progression [13]. These effects may be related to a functional interaction between USP3 and the isoform g-H2AX which accumulates at doubled stranded breaks. USP16 is also important in cell cycle regulation because its ability to deubiquitylate human H2A is required for histone H3S10 phosphorylation [12], an event critical for chromosome segregation during mitosis. Consistent with this, USP16 is required for the G2/M transition. USP16 activates transcription of HOX genes in a manner consistent with reversing the repressive activity of the Polycomb complex. USP21 is important for transcriptional initiation based on its ability to deubiquitylate human H2A, and this has been linked to activating histone H3K4 methylation [9]. USP22 deubiquitylates histone H2A *in vitro* (although *in vivo* activity has not been demonstrated) [14,15,17]. It is interesting to note that the *Drosophila* homolog of USP22, termed nonstop, can play a role in position effect variegation [18], a function linked to the Polycomb family and presumably H2A ubiquitylation by PRC1. Human USP22 is also important for the G1/S cell cycle transition [17]. While structurally unrelated to the USP family, 2A-DUB is also capable of deubiquitylating human H2A [11]. Interestingly, 2A-DUB associates with PCAF, a histone acetyltransferase subunit of the hSAGA complex. This is similar to findings with USP22. However, unlike USP22, 2A-DUB appears to associate only with the PCAF subunit and not with any other components of the hSAGA complex. It is also interesting to note that 2A-DUB deubiquitylation of H2A is affected by the level of histone acetylation within the nucleosome, which is presumably regulated by its association with PCAF [16]. In contrast, studies of Ubp-8, the yeast homolog of USP22, found no association or requirement linking acetylation and deubiquitylation of H2B [19]. (Yeast lack detectable H2A ubiquitylation.)

Relevant to human disease, it is noteworthy that levels of ubH2A are significantly lower in prostate cancer cells than in normal prostate [11]. This effect correlates with recruitment of 2A-DUB to genes activated by the androgen receptor. The presence of 2A-DUB at these genes presumably explains the lower levels of ubH2A. In addition to X chromosome

inactivation and transcriptional repression of the HOX gene cluster, the p16^{INK4a} locus is repressed by PRC1, or at least the BMI1 subunit of PRC1 [20–22]. While it has not been formally proven that this repression is accompanied by an increase in ubH2A at the p16^{INK4a} locus, this is a likely mechanism.

The functional specificity of the H2A deubiquitylating enzymes remains unclear. It is possible that one or more of these enzymes are involved in transcriptional activation (or repression), on a global level, while others are involved in transcriptional activation (or repression) at individual loci. In addition, it is possible that one or more of these enzymes play a more important role in DNA damage repair responses. Our lack of a complete understanding of the functional distinctions between this set of enzymes is highlighted by the fact that depletion of either USP16 or 2A-DUB results in a global increase in ubH2A [11].

1.4. Functional consequences of H2A ubiquitylation and deubiquitylation

The FACT complex is important for transcriptional elongation because it functions as a histone chaperone to displace nucleosomes in front of the elongating RNA polymerase_{II} complex [23]. At least one recent study has shown that the ubiquitylation of histone H2A blocks FACT complex recruitment and therefore blocks transcriptional elongation by RNA polymerase_{II} [11]. Second, ubH2A blocks transcription initiation by blocking the di- and trimethylation of histone H3K4 by the MLL/Trithorax complex [9]. A third functional consequence of histone H2A ubiquitylation has been suggested by studies showing that this isoform of H2A is more efficient at binding the linker histone H1 [16,24]. This has led to the model that ubH2A may help facilitate the formation of higher order chromatin structures that are repressive to transcription. Understanding the functional relevance of these distinct biochemical events that represent potential effectors of H2A ubiquitylation is among the central challenges facing the field.

1.5. H2B ubiquitylation

The major E3 ligase responsible for ubiquitylation of histone H2B in yeast is BRE1 [25]. In humans it appears that RNF20/40 is the functional homolog of BRE1, at least in terms of histone H2B ubiquitylation [26]. Multiple E2-conjugating enzymes have been reported to function with RNF20/40 in humans for H2B ubiquitylation, including hRAD6 and hUbcH6 [26,27].

1.6. H2B deubiquitylation

Unlike other histone modifications, it appears that ubiquitylation of histone H2B must both be added and then removed in order for efficient transcriptional initiation and elongation [19]. In addition to USP22 and its yeast homolog, H2B can be deubiquitylated by USP3 and USP7 in humans. As mentioned above, USP3 has been implicated in genomic integrity and DNA damage induced by replication stress [13]. An interesting observation was recently made during studies of USP7 function in *Drosophila* when it was found that USP7 could enhance Polycomb function. While it is possible that this is because USP7 can deubiquitylate H2A, in addition to H2B, it also remains possible that the USP7-mediated deubiquitylation of H2B has some regulatory interaction with Polycomb function. Human USP7 is also known as HAUSP, an enzyme that can also deubiquitylate the tumor suppressor p53 [28].

1.7. Functional consequences of H2B ubiquitylation and deubiquitylation

H2B ubiquitylation, like H2A ubiquitylation, has been implicated in many different mechanistic events related to transcription. For example, subunits of the 19S proteasome are

recruited to promoters in a manner that depends on the ubiquitylation of H2B [29]. It is currently unclear what role these proteasome subunits might play at the promoter. Some models suggest that they might be involved in the degradation of activators or polymerase molecules during the cyclic transcription events [30]. It is also interesting that at least two of the subunits of the 19S proteasome have been shown to interact with the hSAGA complex [31], which we and others have now shown can remove the ubiquitin from H2B and H2A via its USP22 subunit [15,17]. There is also evidence that H2B ubiquitylation may be important for recruitment of the FACT complex to promoters [32]. As mentioned, FACT is important for transcriptional elongation because it functions as a histone chaperone to displace nucleosomes in front of the elongating RNA polymerase_{II} complex. ubH2B can also stabilize the FACT complex at yeast coding regions [33]. It appears logical that the repressive ubH2A mark would repel FACT while the activating ubiquitylated H2B (ubH2B) mark would help recruit FACT.

Somewhat paradoxically the presence of ubiquitin on H2B represses transcriptional elongation at some yeast genes [19]. Here, mechanistic insight was provided by a recent study showing that the presence of ubiquitin on H2B blocked the recruitment of the RNA polymerase_{II} serine 2 kinase Ctk1 [34], the homologue of human CDK9. *In vivo* evidence suggests that the yeast SAGA complex and its USP22 ortholog subunit Ubp8, are required for removing this ubiquitin mark and allowing Ctk1 recruitment, RNA polymerase_{II} phosphorylation at serine 2 and transcriptional elongation. An interesting observation made as part of this study was that the Ubp8 subunit of SAGA traverses into the coding region of the gene while most of the SAGA subunits remain tethered at the promoter with the activator. Translocation into the coding region appears to be mediated by direct binding to the elongating RNA polymerase_{II} complex. A similar phenomenon occurs with the human SAGA subunit USP22 [17].

1.8. Polycomb proteins and histone ubiquitylation in normal and cancer stem cell function

A great deal of evidence has implicated the Polycomb proteins in the function of both normal and cancer stem cells in recent years [35,36]. For example, Polycomb genes are overrepresented in expression profiling signatures from normal and cancer stem cells [37–42]. This has been a consistent finding from many labs. Interestingly, USP22 appears in at least one of these Polycomb cancer stem cell signatures [43]. Polycomb proteins have been most thoroughly characterized in *Drosophila*, where they function as transcriptional repressors to antagonize the activity of the Trithorax group proteins. The mammalian Polycomb protein BMI1 was originally identified as a gene which cooperates with MYC in cellular transformation [44,45]. The specific targets of BMI1 that mediate this cooperation have not been identified although both MYC and BMI1 are able to regulate some common genes important for cell cycle progression and transformation. These include the human telomerase reverse transcriptase (hTERT) gene, and the p16^{INK4a} tumor suppressor [20,46–49]. BMI1 is important for G1/S transition [21]. An additional observation from *Drosophila* connects Polycomb function to MYC. These studies have shown that many loci in *Drosophila* are co-occupied by Polycomb and MYC proteins and that these proteins have a genetic interaction at these loci [50,51].

Recently, the Oren group showed that in human cells, ubH2B generally occurs within the coding region of actively transcribed genes [52]. They were able to conduct these studies after generating an antibody that is highly specific for the ubiquitylated form of histone H2B. Yeast appear to lack histone H2A ubiquitylation and it therefore remains unclear which specific functions of histone H2B ubiquitylation in yeast are evolutionarily linked to ubiquitylation of either H2A or H2B in mammalian cells. In yeast, Ubp8 function is required for the transcription of some SAGA targets, but not for others. Furthermore histone H3K4 methylation is affected by loss of Ubp8 at some genes but not at others [53]. Therefore it

seems that different genes, presumably depending on their chromatin context, have different requirements for H2B ubiquitylation in yeast.

The homeotic HOX genes typically induce differentiation and block cell cycle progression. Therefore repression of the HOX genes by Polycomb proteins can enhance cell cycle progression [54]. BMI1 is a bona fide oncogene, as over expression with MYC is sufficient to cause leukemia in mice [44,45]. Presumably, the common over-expression of BMI1 in human cancer increases the activity of PRC1 for the ubiquitylation of H2A. However since RING1b is the actual ubiquitin ligase for H2A it has still not been formally proven that BMI1 over expression in cancer is important because of an increase in the activity of RING1b.

2. Sources of deubiquitylating enzymes

Recent years have seen the development of methodologies for assaying the activity of histone deubiquitylation enzymes, both *in vivo* and *in vitro*. Clearly, specific issues related to biological process controlled by these enzymes are best examined *in vivo*, while addressing specific biochemical questions about the catalytic activity of these enzymes or their substrates can best be accomplished *in vitro*. Here we describe some of the current methodologies for pursuing these questions and discuss how aspects of the methodologies impact the interpretation of the biological data.

2.1. Enzyme isolation from heterologous expression in bacteria

The genetic makeup of *E. coli* is easy to manipulate, making it a popular host system for heterologous protein expression. *E. coli* can produce large amounts of recombinant protein in a short time period and culturing bacteria is both inexpensive and simple. Some proteins can actually be secreted into the culture medium, which can then be collected for protein isolation [55]. However, there are disadvantages to using *E. coli* as a host system, mainly the presence of the periplasm, which is a reducing environment that does not allow for most mammalian proteins to fold properly or form disulfide bonds. An additional limitation is that *E. coli* cannot produce large heterologous protein complexes or proteins containing unpaired thiols [56]. Furthermore, *E. coli* lack the machinery necessary for performing post-translational modifications and enzymes that target histones are often regulated by such modifications [57]. Facilitating the use of this system, there are many purification kits commercially available for the isolation of recombinant proteins from *E. coli*.

The following is a general protocol for the isolation of recombinant DUBs from *E. coli*. cDNA for the DUB of interest is cloned into a Lac operon containing vector which also contains a gene for antibiotic resistance.

1. Competent *E. coli* are transformed and a single colony is selected for expansion into a large-scale liquid culture. At the end of the culture period, (typically 5–8 h), a small volume (1–3 mL) can be saved as a non-induced control.
2. The production of the recombinant protein is then induced by addition of isopropyl beta-thiogalactoside (IPTG, an inducer of the Lac operon which cannot be metabolized) and incubation at 37 °C on a shaker for an additional 4–5 h.
3. The culture is centrifuged at 5000g for 15 min at 4 °C, and the pellet is resuspended in 10 mM TRIS–HCl [pH 7.5] containing 0.5 mM EDTA and 1 mM PMSF.
4. Cells are lysed either by mechanical disruption or enzymatic digestion.
5. For mechanical disruption:
 - a. Split the solution into two tubes and freeze–thaw in liquid nitrogen.

- b. Sonicate samples (6 rounds in 15 s bursts) on ice.
 - c. Add 1.25 mL of 20% Triton-X-100 (final concentration 0.5–1%) per tube and rock for 30 min at 4°C.
 - d. Centrifuge at 12,000g for 15 min at 4°C.
6. For enzymatic lysis:
 - a. Add 1/10 volume of fresh lysozyme (10 mg/mL) and shake at 37 °C for 30 min.
7. Freeze in liquid nitrogen and then thaw at 37 °C for 15 min. Add 1 mg/mL DNase (1/10 vol) and 1 M MgSO₄ (1/10 vol) and shake for 15–30 min.
8. These cleared lysates are added to a chromatography column for purification. For His-tagged proteins, (like USP21 and USP4 which were purified by Nakagawa et.al. [9]), equilibrate 2 mL of 50% Ni-NTA media slurry by flowing 20 mL of His₆-Wash Buffer through a column (Qiagen).
9. Add beads to the lysate mix and rock for 1 h at 4 °C.
10. Add the lysate/bead mixture to the column followed by two 12 mL washes with His₆-Wash Buffer (50 mM NaH₂PO₄, 200 mM NaCl, 2 mM imidazole, adjust to pH 8 with NaOH).
11. The column is eluted with Elution Buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, at pH 8.0).
12. Concentrated enzyme can then be detected semi-quantitatively by western blot and used in enzymatic assays as described in section 4.

2.2. Enzyme isolation from insect cells

Recombinant proteins are often isolated from insect cells infected with baculovirus expression vectors. As an example, the protocol was adapted from one used by Zhao et al. to isolate the USP22 module of the human TFTC/STAGA/hSAGA complex [15]. A baculovirus expressing FLAG-epitope tagged USP22 was transduced into Sf9 insect cells. Whole cell extracts were generated and the proteins were immunopurified using anti-FLAG antibody. Endogenous proteins can also be isolated using insect cell extracts, as was shown by Van der Knaap et al. to isolate endogenous USP7 from *Drosophila* nuclear extracts [58]. Insect, yeast and mammalian nuclear extracts are isolated using the following protocol from our lab.

1. Harvest cells in PBS and pellet for 5 min at 12,000 rpm.
2. Aspirate supernatant and resuspend pellet in 5x pellet volume Buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl with fresh 1x protease inhibitor and 1 mM PMSF).
3. Incubate on ice for 15 min.
4. Lyse cells by passing through at 26-gauge needle 5 times.
5. Pellet nuclei for 20 s at 2500 rpm (this should be done carefully. If needed, a second spin for 20 s at 3000 rpm can be done).
6. Remove supernatant (cytoplasmic portion).
7. Resuspend nuclear pellet in 2x pellet volume Buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA with fresh 1x protease inhibitor, 1 mM DTT and 1 mM PMSF).

8. Incubate on ice for 30 min, vortexing every 5 min.
9. Pellet debris for 5 min at 14,000 rpm.
10. Collect supernatant (nuclear portion).
11. Dilute in 5x Buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 100 mM KCl, 0.2 mM EDTA with fresh 1x protease inhibitor, 1 mM DTT and 1 mM PMSF) to lower salt concentration.

Typically experimental procedures for isolation of both endogenous and exogenous epitope-tagged enzymes from insect cells are carried out at 4 °C. Using the baculovirus expression system circumvents many of the issues found when expressing high levels of recombinant protein in bacteria. The following protocol was adapted from [59] for recombinant protein purification using baculovirus infected insect cells.

1. Infected cells were harvested by centrifugation for 8 min at 2000 rpm.
2. Supernatant was discarded and the pellet was washed with ice cold PBS.
3. Samples were then spun at 4 °C for an additional 8 min at 2000 rpm.
4. The supernatant was discarded and the pellet was resuspended in 1.5 mL of lysis buffer (25 mM Hepes [pH 7.5], 5 mM KCl, 0.5 mM MgCl₂ with fresh 0.5 mM DTT and 1 mM PMSF), per original T150 flask of cells.
5. Samples were then incubated on ice for 10 min.
6. Samples were mechanically homogenized and NaCl was added to a final concentration between 200 and 300 mM.
7. Samples were then centrifuged at 2000 rpm for 5 min at 4 °C.
8. Samples were centrifuged again at 25,000 rpm for 30 min at 4 °C in low protein binding tubes (e.g. polyallomer).
9. Glycerol was added to a final concentration of 10–20% and samples were loaded onto purification column as described below [58].
10. Nuclear cell extracts were prepared and fractioned on an 18 mL POROS-Heparin column (Applies Biosystems) equilibrated with HEMG buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM MgCl₂, and 10% glycerol supplemented with 0.5 mM MBS, 0.2 mM PMSF, 1 mM DTT, 1 µg/mL leupeptin, and pepstatin A).
11. A 100 mM KCl flowthrough was used to wash off any non-specific proteins.
12. The flowthrough was then applied to a 22 mL Q Sepharose FF column (Amersham) developed with a linear gradient of HEMG/100–600 mM KCl containing PMSF, DTT and Leupeptin as described [58].
13. Endogenous protein was eluted using KCl supplemented with (NH₄)₂SO₄ up to 800 mM.
14. Samples were applied to a Phenyl Superose HR 5/5 column (Amersham) developed with a linear gradient of 800–0 mM (NH₄)₂SO₄ in HEG (25 mM HEPES-KOH, [pH 7.6], 0.1 mM EDTA and 10% glycerol). (The molarity at which the enzyme elutes varies and must be determined empirically by western blotting or other methods.)
15. Samples were then loaded onto a HiPrep 16/60 sephacryl S-300 column (Amersham) developed with buffer T (25 mM TRIS [pH 8.0], 0.1 mM EDTA and

10% glycerol) plus 0.01% NP-40 and 100 mM KCl. (As true for molarity, the volume of buffer T needed to elute the enzyme varies.)

16. Fractions were then supplemented with insulin and applied to a Mono Q HR 5/5 column (Amersham) developed with a linear gradient from T/100–500 mM KCl. (Again, the molarity of KCl needed to elute the protein varies.)
17. The protein could then be immunopurified using protein A Sepharose beads (CL-4B, Amersham) conjugated to an antibody raised against the protein of interest.

Using the baculoviral expression system allows for proper folding, post-translational modification and oligomerization in a manner that is often similar to what occurs in mammalian cells. Unlike bacterial cells, the cytoplasm of insect cells allows for proper disulfide bond formation as well. Many mammalian proteins generated using this system display proper post-translational modification patterns, including N- and O-glycosylation, acylation, amidation, carboxymethylation, phosphorylation, and prenylation. The baculoviral system allows for expression of single polypeptides or of multimeric protein complexes. By using co-infection or multi-cistronic baculovirus vectors, investigators have reconstituted multi-protein complexes with 5 or more subunits [60]. Furthermore, the expression of these heterologous genes is controlled by the polyhedrin promoter which allows the level of target protein expression to be 30–50% of total cell protein content, yielding as much as 1 gram of isolated protein per liter of insect cell culture [61].

There are a number of issues associated with using a baculovirus system that may present technical challenges. However, many of these can be overcome by optimizing specific conditions for the protein of interest and the final analysis to be performed on that protein. For example, insect cells may be inefficient at secretion, and this can sometimes be overcome by the addition of a secretion signal (i.e. honeybee melittin sequence) [62]. Other issues such as improperly folded proteins and proteins that occur as intracellular aggregates may be due to expression late in the infection cycle. If the expression is not sufficiently high, the experiment may need to be optimized by timing harvests to capture maximal expression or by increasing the multiplicity of infection. For example, an m.o.i. of 5 and harvest at 48 h after infection was used by Van Der Knaap et.al., for USP7 immunopurification [58]. Some potential N-linked glycosylation sites may become fully glycosylated or not glycosylated at all, unlike the range of glycoforms found in mammalian cells. Another disadvantage to the baculoviral system is non-specific association of the recombinant protein with insect proteins that are orthologs of its mammalian binding partners. This can result in an improper assay of enzymatic activity of the monomeric DUB of interest, and instead reflect the activity of the DUB as part of a protein complex. This can sometimes be overcome by using a more stringent salt solution or even mild detergent to dissociate insect-cell binding partners.

2.3. Enzyme isolation from yeast

In yeast, histone H2B is monoubiquitylated. As discussed above, Ubp8, the yeast ortholog of the human DUB USP22, is part of the yeast SAGA complex. Daniel et.al., isolated yeast SAGA and GST-tagged Ubp8 from the yeast strains CY396 and EJ758 respectively using the following protocol [63].

1. Protein complexes were purified from whole-cell extracts from yeast which were allowed to grow to mid-log phase in yeast extract-peptone-dextrose (YPD) medium.

2. To isolate monomeric DUBs or DUB complexes from yeast, a series of chromatography columns were used beginning with a Mono Q HR 16/10 column (Amersham).
3. Bound proteins were eluted with a 500 mL 100–500 mM NaCl linear gradient.
4. Fractions were diluted to 100 mM NaCl, and loaded onto a Mono Q HR 5/5 column (Amersham).
5. Bound proteins were eluted with a 100–500 M 25 mL linear gradient of NaCl.
6. Peak fractions were diluted to 100 mM NaCl and loaded onto a Mono S HR 5/5 column (Amersham).
7. Proteins were eluted using a 100–500 M 10 mL linear gradient of NaCl and then fractions were diluted to 100 mM NaCl again.
8. Fractions were then loaded directly onto a 1 mL histone agarose column (Sigma).
9. After washing, proteins were eluted using a 100 mM–1 M 10 mL linear gradient of NaCl and fractions were again diluted to 100 mM NaCl and loaded onto a 1 mL native DNA cellulose column (Amersham).
10. Proteins were again eluted with a 100 mM–1 M 10 mL gradient of NaCl.
11. Elutions were concentrated to 0.7 mL with a Centriprep-30 concentrator (Millipore) and loaded onto a Superose 6 HR 10/30 column (Amersham), equilibrated with 350 mM NaCl.
12. Superose 6 fractions were diluted to 100 mM NaCl and loaded onto a Mini Q PC 3.2/3 column (Amersham).
13. Finally, proteins were eluted using a 4.8 mL gradient from 100 to 500 mM NaCl.
14. Purified DUBs could then be analyzed by western blotting and DUB complexes could be visualized by silver staining. Purified DUBs and DUB complexes could then be used in enzymatic assays as described in section 4.

2.4. Purification of deubiquitylating enzymes from mammalian cells

Another common way to isolate deubiquitylating enzymes is to use epitope-tagged enzymes in mammalian cell culture. Zhang et.al., generated a stable cell line expressing a FLAG-tagged version of USP22 in H1299 cells [17]. FLAG-USP22 was then affinity purified from nuclear extracts and eluted with FLAG peptide. Similarly, Zhu et.al., used HEK293T cells to create a stable cell line expressing FLAG-tagged 2A-DUB [16]. Other epitope tags include HA, MYC, V5, MBP, HIS and GST, or even GFP. Cai et al. used a GST-fused Ubp-M/USP16 to isolate the enzyme from CHO cells [64], and Nicassio et.al. used a MYC-tag for isolation of USP3 from transiently transfected HEK293T cells [13]. The following protocol is used by our group for isolation of epitope tagged proteins from either stably-expressing cell lines or transiently transfected cells, and is carried out at 4 °C as follows:

1. Nuclear extracts are diluted 1:5 in NP-40 style lysis buffer (20 mM NaH₂PO₄, 150 mM NaCl, 0.5% IGEPAL, 5 mM EDTA, 50 mM NaF, 30 mM Sodium Pyrophosphate and 10% Glycerol) with 1:1000 fresh protease inhibitor (PI: 1000x).
2. At this point, 1/10 volume can be removed and stored as input.
3. Prewashed (5x in NP-40 lysis buffer with fresh PI) FLAG M2 beads (Sigma) are added to the diluted nuclear extract and rotated overnight.

4. Beads are added to Bio-Spin chromatography columns (BioRad) and washed twice with 2 mL BC 100 (100 mM KCl, 10% glycerol, 20 mM TRIS [pH 8.0] and 0.2 mM EDTA) supplemented with fresh Protease Inhibitor and DTT
5. Wash once with 2 mL BC-500-NP40 (500 mM KCl, 0.1% NP-40, 10% glycerol, 20 mM TRIS [pH 8.0] and 0.2 mM EDTA) containing fresh PI and DTT.
6. Subsequently, a wash is done with 2 mL BC-500 (without NP-40) with fresh PI and DTT.
7. The column is washed twice more with BC100 supplemented with fresh PI and DTT.
8. Non-specific proteins are eluted off with 2 washes of 100 μ l of myc peptide (Sigma: diluted to 400 μ g/mL in BC-100) followed by three final washes with 2 mL of BC-100 with fresh PI and DTT.
9. FLAG-tagged proteins are competitively eluted three times from the beads using 100 μ l of FLAG peptide (Sigma: diluted to 400 μ g/mL in BC-100).

Using epitope tagged proteins for enzyme isolation from mammalian cells has a number of advantages. The most obvious is that protein expressed in mammalian cells is folded in its native environment and there are no issues with post-translational modifications or cross-interactions with host proteins. Furthermore, the antibody-antigen binding is highly selective due to its high affinity. In many situations, using antibodies against the epitope for western blotting and other experiments after isolation is very useful, especially if there is no commercially available antibody against the DUB of interest. However, although the FLAG epitope is relatively small (8 amino acids long), it is still adding a fused peptide to the DUB of interest. There are ways to remove the tag after isolation using proteases but the efficiency varies. In certain experiments, having the addition of a tag to the protein may serve as a disadvantage. Although many cancer cell lines can rapidly divide and produce protein, creating a stably-expressing cell line can take time and mammalian cells do not yield as much protein as insect cell lines. Finally, generating mammalian cells with higher than physiological levels of a DUB can result in a non-physiological environment for the enzyme of interest.

3. Purification of histone substrates

3.1. Recombinant histones derived from mammalian cell extracts

Zhu et. al. isolated recombinant core histones and oligonucleosomes from HeLa cell nuclear extracts [26]. This method has also been used to isolate H2A/H2B dimers and H3/H4 tetramers [65]. This method was used to isolate both wild type H2A and H2B along with histones with single point mutations at their ubiquitylation sites (H2AK119A and H2BK120A). This assay can also be used to isolate mutant histones that are useful for assessing post-translational modification crosstalk as well. In this assay, 15 grams of protein derived from HeLa cell nuclear extracts was used. The following protocol is adapted from Belotserkovskaya et al. [65]

1. Isolated protein was fractionated by chromatography on a 1.5 L phosphocellulose column (Sigma) equilibrated with buffer C (20 mM TRIS-HCl [pH 7.9], 1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.2 mM PMSF) with 0.1 M KCl.
2. Proteins were then step-eluted with buffer C containing 0.1, 0.3, 0.5 and 1 M KCl, respectively.

3. The histone-containing fraction was determined by western blot analysis and was then dialyzed against buffer C containing 0.1 M KCl and loaded onto a 300 mL DEAE-cellulose column (Whatman).
4. Following thorough washes with buffer C containing 0.1 M KCl, proteins were eluted with buffer C containing 0.35 M KCl.
5. The eluate was then dialyzed against buffer C containing 0.1 M KCl and loaded onto a 100 mL Heparin agarose column (Sigma).
6. After extensive washing with buffer C containing 0.1 M KCl, the proteins were eluted with buffer C containing 0.5 M KCl.
7. The eluate was dialyzed against buffer C containing 0.1 M KCl and loaded onto an 8 mL Mono Q column (Amersham).
8. Proteins were eluted with a 15-column volume linear gradient from 0.1 to 0.7 M KCl in buffer C.

Zhao et.al. used FLAG-tagged H2A and H2B plasmids transfected into HEK293T cells with HA-tagged ubiquitin for their histone isolation and *in vitro* deubiquitylation studies [15]. FLAG-tagged H2A/H2B was also used by Zhu et.al. in HEK293T cells to examine crosstalk between ubH2A, changes in H3K4/K9 methylation, H3K9/K14 acetylation, the association of the linker histone, H1, and the phosphorylated isoform of H1 [16]. They collected whole cell extracts in modified RIPA buffer (50 mM TRIS-HCl [pH 7.9], 150 mM NaCl, 5 mM EDTA, 15 mM MgCl₂, 1% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, 1x protease inhibitor, 1 mM PMSF, and 20 mM NEM). Histones were immunoprecipitated using FLAG M2 beads (Sigma) and competitively eluted using FLAG peptide. Isolated histones can be checked by running a portion on an SDS-PAGE gel and western blotting using specific antibodies against either FLAG (or other affinity tags) or antibodies specific to that histone. These histones can then be used in the enzymatic assays described below.

3.2. Mammalian histones produced as recombinant proteins in *E. coli*

Pavri et al. used mammalian histones ectopically expressed and purified from bacteria to reconstitute the nucleosomal octamer [32]. Detailed methodology for recombinant histone purification from *E. coli* has been published by others [66]. Briefly, constitutively overexpressed His₆-tagged monomeric histones were generated in *E. coli* (BL21(DE3)) cells using the pHCE vector. Histone proteins were purified in one step by metal-chelating chromatography under denaturing conditions.

1. Ten liters of bacteria containing LB culture was harvested and resuspended in 100 mL of TRIS-HCl buffer [pH 8.0] with 500 mM NaCl, 1 mM PMSF and 5% glycerol.
2. Cells were sonicated four times for 1 min each and protein lysate was centrifuged to remove debris (27,000g for 20 min at 4 °C).
3. These pellets were then dissolved in 100 mL of 50 mM TRIS-HCl [pH 8.0] containing 500 mM NaCl, 5% glycerol, and 6 M urea, then centrifuged again (27,000g for 20 min at 4 °C).
4. The insoluble pellets were discarded and His₆-tagged histones were solubilized under denaturing conditions with 6 M urea.
5. This was mixed with 6 mL (50% slurry) of Ni-NTA agarose beads (Qiagen) and rotated at 4 °C for 60 min.

6. The beads were washed with 50 mM TRIS–HCl [pH 8.0] with 500 mM NaCl, 5% glycerol, 6 M urea, and 5 mM imidazole, and were packed into Econo-columns (BioRad).
7. The beads were subsequently washed with 200 mL of the buffer described above and eluted by a 100 mL linear gradient of imidazole from 5 to 300 mM.
8. The histone containing fraction was identified by 15–20% SDS–PAGE and molecular masses were confirmed by mass spectrometry.

3.3. Proteins were purified as monomers and then refolded into H2A/H2B dimers and H3/H4 tetramers

1. This was done by mixing 5 mg of histones in a 1:1 stoichiometry and then dialyzing the mixture overnight at 4 °C in 1L of TRIS–HCl [pH 8.0] with 10 mM DTT, 2 mM EDTA, 6 M urea and 2 M NaCl, after 1 h at room temperature.
2. The mixture was then dialyzed again overnight in the same mixture containing 1 mM PMSF and no urea in order to renature the dimers and tetramers.
3. Impurities and excess histones were precipitated.
4. The salt concentration was reduced by stepwise dialysis against the buffer containing 1 M NaCl for 4 h, 0.5 M NaCl for 4 h and 0.1 M NaCl overnight at 4 °C.
5. The dimers and tetramers were found in the soluble fraction following centrifugation at 27,000g for 20 min at 4 °C.
6. The His₆ tag could then have been removed by treatment with thrombin protease (Amersham: 5 U/mg of histones at RT for 16–20 h) since the tag contains a thrombin protease recognition sequence.
7. Histones could then be subjected to further purification using Superdex 75 gel filtration chromatography (Amersham).

3.4. These were then confirmed to be able to form core nucleosomes with a unit length of DNA

1. Six micrograms of H2A/H2B dimer was added to 6 µg of H3/H4 tetramer and 10µg of PCR-amplified 186 bp segments of DNA at 4 °C in 6 M urea, 2 M NaCl and 0.1 mg/mL BSA.
2. This was dialyzed at room temperature for 4 h against 10 mM TRIS–HCl [pH 8.0] with 1 mM EDTA, 10 mM 2-mercaptoethanol, 2 M NaCl and 5 M urea.
3. Histones were then subjected to a reducing gradient of NaCl by adding the TRIS buffer above without NaCl at 0.8 mL/min.
4. The urea was removed by dialysis against the TRIS–HCl buffer above (without urea) containing 0.6 M NaCl, and then containing 0.1 M NaCl for 16 h at 4 °C.
5. Isolated mono-histones, histone dimers, tetramers and octomers, and mono-nucleosomes can then be used as substrates for enzymatic assays.

3.5. Mammalian histones isolated by chromatography in bulk from animal tissue

UbH2B can be isolated from porcine thymus in DUB assay buffer (100 mM TRIS [pH 8], 1 mM EDTA, 1 mM DTT and 5% glycerol) [17,67]. The original protocol for isolation from both calf and pig thymus by exclusion chromatography in 7 M urea was described by

Thorne et.al. [17,67]. Nakagawa et.al. used a similar protocol to isolate core histones from mouse liver and recapitulated nucleosomes [9].

1. Whole thymus was thawed from liquid nitrogen and homogenized.
2. A solution containing MgCl_2 reduced to 6 mM, 50 mM butyrate and 0.1 mM PMSF was added to the homogenized tissue and the sample was centrifuged to purify thymus from connective tissue, blood vessels and fat.
3. Histones were isolated using 0.4 M HCl to lyse nuclei and then through acetone precipitation.
4. Histone H2B (with ubH2B) was separated using a 1350×40 mm Bio-Gel P-60 column (Bio-rad) equilibrated with 50 mM NaCl, 20 mM HCl, and 0.02% azide using a flow rate of 30 mL/h.
5. UbH2B was separated from H2B by dialyzing the fraction against distilled water before lyophilization.
6. The H2B and ubH2B fractions were then dissolved in 8 M urea, 10% 2-meceptoethanol and 2 mM TRIS for 4 h at 4 °C before reducing the pH to 2.5.
7. The fraction was centrifuged and the solubilized protein was added to a Bio-Gel P60 1440×27 mm column (Bio-rad) equilibrated in 7 M urea, 2nM TRIS [pH 2.2] at a flow rate of 3.7 mL/h at RT.
8. The eluate was pooled in urea and subject to a Sephadex G-75 column (GE: 1500×16 mm, flow rate 20 mL/h) equilibrated with 10 mM HCl and a final purification was made on a Bio-Gel P60 (Bio-rad: 1500×9 mm) equilibrated with 50 mM NaCl, 20 mM HCl, 0.02% sodium azide.
9. Relatively pure histones isolated from mammalian tissues can also be purchased from commercial vendors such as Sigma, which sells histones isolated from calf thymus.

3.6. Isolation of histones from insect cells by acid extraction

Van Der Knaap et.al., isolated ubiquitylated histones from *Drosophila* embryo nuclei and from S2 nuclei by acid extraction [58]. Following an acetone precipitation, histones were renatured by dialysis against multiple changes of HEMG/50.

4. Enzymatic assays

4.1. In vitro assays

In this section, we describe the detailed steps taken to evaluate deubiquitylation events *in vitro*. There are many conditions in which an *in vitro* deubiquitylation assay can be performed. The following are examples of reaction buffers used for *in vitro* enzymatic assays. All buffers are pH 7.4–8.0. This and other aspects of the reaction conditions should be optimized empirically, as shown in Fig. 1.

1. 100 mM TRIS–HCl [pH 7.9], 5% glycerol, 1 mM DTT, and 1 mM EDTA for 2 h at 37 °C was used as a buffer for the reaction with purified FLAG-H2A and –H2B with highly purified TFTC complex [15].
2. This same buffer (except TRIS–HCl was pH 8.0) was used for the reaction with purified USP22 or USP22 as part of the hSAGA complex, and ubH2B at 37 °C for 2 h [17].

3. 125 mM TRIS-HCl [pH 7.4], 50 mM NaCl, 25 mM MgCl₂, 10mM ATP, 1 × ATP regeneration system, and 10% glycerol for 1 h at 37 °C for the reaction with affinity-purified 2A-DUB and purified FLAG-H2A [16].
4. 10 mM TRIS [pH 8.0] 75 mM NaCl, 0.5 mM EDTA, 0.25% NP-40, 5 mM DTT, 5 mM MgCl₂, and 20 mM ATP at 37 °C for 2, 4, and 6h [68].
5. 60 mM HEPES [pH 7.6], 5 mM MgCl₂, and 4% glycerol and reactions were assembled on ice and carried out at 30 °C for 1 h [58].
6. 100 mM TRIS-HCl [pH 8.0], 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 1 ug/mL aprotinin, 1 ug/mL leupeptin and 1 ug/mL pepstatin A at 37 °C for 45 min [12].

The first step for all assays is optimal enzyme and substrate purification. As mentioned previously, there are many methods for enzyme and substrate purification and each method has to be optimized for the experiment at hand. When determining the combination of substrate and enzyme sources to use in the reaction, there are many advantages and disadvantages to consider. Some mentioned in the previous sections include the integrity of post-translational modifications, binding partner/complex presence and origin, amount of purified protein yielded, and how well it mimics *in vivo* enzymatic activity.

Once purification is accomplished, saturating quantities of purified enzyme/enzymatic complex and substrate are added to a reaction buffer, examples of which are described in section 5. The incubation time and temperature should be optimized empirically as well. The function of each enzyme is dependent on the composition of the reaction buffer. In the example shown in Fig. 1, the assay buffer pH is critical for the DUB activity of USP22.

As a typical first attempt, reactions are carried out at 37 °C for 45 min. Reactions are stopped by the addition of SDS-PAGE sample buffer and the products resolved on a gradient gel and analyzed by western blot. In order to analyze the ubiquitylation status of the purified substrates, a pan-ubiquitin antibody, antibodies specific to the histone substrate, or an antibody specific to any epitope tag that is present, can be used. Joo and colleagues also describe an immunodepletion assay in which Ubp-M/USP16 was depleted from an aliquot from their hydroxyapatite column resulting in a decrease in H2A ubiquitylation from that fraction [12].

Methods have also been described in which DUB substrates have been immunoprecipitated and then immunoblotted for ubiquitin to analyze changes in ubiquitylation status. Joo et al. assembled HA-ubH2A containing histone octomers, monomers and oligonucleosomes and then incubated these with HeLa nuclear protein fractions [12]. They observed decreases in ubH2A levels, and noted that Ubp-M/USP16 had substrate specificity for H2A in oligonucleosomes and was unable to deubiquitylate H2A in histone octomers.

4.2. The following protocol is used by our lab for the *in vitro* deubiquitylation of histone H2B by USP22

1. FLAG-USP22 is isolated as described in section 2.4 and ubH2B is isolated as described in section 3.3.
2. FLAG-USP22 and ubH2B are incubated in DUB assay buffer (100 mM Tris [pH 8.0], 1 mM EDTA, 1 mM DTT, and 5% glycerol) at 37 °C for 2 h.
3. Reactions are stopped by the addition of SDS-PAGE sample buffer.
4. Samples are then run on 4%–20% SDS-PAGE gels (Invitrogen) and analyzed by western blot with an anti-H2B antibody (Upstate).

4.3. In vivo assays

There are various ways to analyze *in vivo* deubiquitylation events. A starting point is typically to deplete the DUB of interest using RNAi and then to analyze ubiquitylation of the substrate. Our lab has used lentivirus-containing shRNAs directed towards DUBs such as USP22 to deplete them from cells.

1. Lentivirus is packaged in HEK293T cells via transfection with 3 μ g shRNA construct (Sigma: MISSION), 2 μ g pCMV-R Δ 8.2 and 1 μ g of pCMV-VSVG per 10 cm plate using lipofectamine 2000 reagent (Invitrogen).
2. After 48 h, virus is harvested, filtered, and incubated on target cells for 24 h in the presence of polybrene.
3. Target cells are selected for at least 48 h following infection according to a mock infection plate's survival.
4. As a control, we infect cells with shRNA directed to luciferase (Sigma: MISSION).
5. Protein depletion can be assayed by SDS-PAGE.

Zhu et al. described a global increase in ubH2A in extracts from cells depleted of 2A-DUB [16], and Joo et al. described a change in cell cycle-dependent H2A ubiquitylation when comparing wild type and Ubp-M/USP16 depleted cells [12]. They assayed the ability of Ubp-M/USP16 to deubiquitylate H2A *in vivo* by generating a stable knockdown of Ubp-M/USP16 in HeLa cells using RNAi. The knockdown efficiency was >90% at the Ubp-M/USP16 protein level and an increase in total ubH2A by western blot analysis was noted. Kessler et. al. described changes in ubiquitylation of endogenous proteins upon depletion of HAUSP/USP7 [69]. A 2D-SDS-PAGE was used to analyze the proteomes of cells before and after 48 h of HAUSP depletion. Kessler et.al. found 25 2D gel spots that were altered upon removal of USP7 [69]. These spots were excised and digested with in-gel trypsin before being analyzed by mass spectrometry. This analysis identified changes in 36 proteins, 26 of which were down-regulated and 10 of which were up-regulated upon USP7 depletion. In another example, Nicassio et al. depleted HeLa cells of USP3 and saw increases in the ubiquitylated forms of H2A and H2B by immunoblotting for both ubiquitin and the histones themselves [13].

DUBs can also be overexpressed in a cell culture system and changes in substrate ubiquitylation status then analyzed. Nicassio and colleagues ectopically expressed USP3 in HeLa cells and analyzed the ubiquitylation state of histones H2A and H2B [13]. Nicassio et.al. observed decreased protein levels of the ubiquitylated forms of H2A and H2B but no change in the protein levels of non-ubiquitylated H2A and H2B [13].

Both purified substrate and enzyme can be cotransformed into a culture system like *E. Coli* as described [64]. In this study, *E. coli* cells were co-transformed with a GST-tagged Ubp-M/USP16, and with b-gal tagged ubiquitin. Cultures were grown overnight and subsequently induced with IPTG. Total lysates were then analyzed by SDS-PAGE and immunoblotted with b-gal antisera. As a second substrate, a GFP-ubiquitin fusion protein was expressed from a pET-28a vector (Novagen). Cai et. al. were then able to analyze the deubiquitylase activity of Ubp-M/USP16 by the shift in GFP protein migration by SDS-PAGE, in bacteria cotransformed with Ubp-M/USP16.

Furthermore, histone modifications can be immunoprecipitated in a chromatin immunoprecipitation assay as described below to analyze the reaction responsible for the modification. This was done by Nakagawa et al. to analyze how ubH2A, H3K9me [3] and H3K4 me²/me³ change as genes are up- and down-regulated [9]. Antibodies against the modified histones and qRT-PCR were used to analyze genes that were known to be up- and

down-regulated following a partial hepatectomy. Chromatin double immunoprecipitation has also been used to determine sites of H2A ubiquitylation that are modified by Ubp-M/USP16 [12]. Joo et.al. used anti-FLAG immunoprecipitation followed by anti-HA immunoprecipitation to show colocalization of BMI-1 and ubH2A in cells depleted of Ubp-M/USP16; however, when Ubp-M/USP16 was present there was no ubH2A at these loci [12].

Analyzing enzymatic activity of a deubiquitinating enzyme by these *in vitro* and *in vivo* assays is critical for answering numerous biological questions relative to transcription, chromatin compaction, cell cycle and post-translational cellular regulation. A flow chart showing the stepwise process leading up to these enzymatic assays is shown in Fig. 2. The dynamic ubiquitylation and deubiquitylation of histones has been implicated in these cellular processes and using the tools described here to study them will provide insight into how these cellular processes work, and potentially, how they are altered in diseased states.

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Fig. 1.

pH is critical for *in vitro* enzymatic assays. FLAG-Usp22 (lanes 1–5), FLAG-vector (lane 6), or FLAG-GCN5 (lanes 7–11) was expressed in H1299 cells and affinity purified from H1299 nuclear extracts under nondenaturing conditions. The purification was confirmed by western blotting for the FLAG epitope (top lanes). Purified FLAG-USP22, - GCN5 or - vector was incubated *in vitro* with ubH2B (previously purified from whole thymus) in DUB reaction buffer. Reaction mixtures were then resolved by SDS-PAGE and developed with antisera against H2B (lower lanes). The pH of the reaction buffer was adjusted as shown on top. In the presence of GCN5 or USP22, H2B is deubiquitylated at pH 8.0, but not in control reactions or in more acidic reactions.

Fig. 2.

Flow chart of deubiquitylating assays. Deubiquitinating enzymes are purified from lysed bacteria, drosophila nuclear extracts, yeast and mammalian cell lines. Histones are purified from lysed cell lines, bacteria, homogenized mammalian tissue or insect cells. These purified DUBs and Histones are then used in in vitro assays or co-transformed in an in vivo deubiquitylating assay.

Table 1

Histone targeting DUBs and their orthologs.

Human	Mouse	Fly	Yeast	Substrate
USP3	Usp3	X	X	H2A/H2B
HAUSP/USP 7	Usp7	Ubp64E/USP 7	UBP15	H2B
Ubp-M/USP16	Usp16	X	UBP16	H2A
USP21	Usp21	X	UBP11	H2A
USP22	Usp22	Not/non-stop	UBP8	H2A/H2B
MYSM1/ 2A-DUB	Mysm1	X	X	H2A