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Purification of Unanchored Polyubiquitin Chains from Influenza Virions

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Abstract

Influenza A virus (IAV) is an enveloped virus with a segmented single-stranded negative-strand RNA genome. In general, the role of virally-encapsidated host cell proteins in the viral lifecycle is unclear. The virion contains abundant ubiquitin molecules some of which have been identified as unanchored polyubiquitin chains. These ubiquitin chains have been postulated to play a role in recruiting histone deacetylase 6 (HDAC6) to the cytosolic surface of late endosomes (LEs), promoting IAV uncoating via aggresome processing – a cellular machinery that disposes of protein waste. HDAC6, a class II HDAC, is unusual because it resides mostly in the cytosol instead of the nucleus. It is a unique protein consisting of two catalytic domains (CDs) and a zinc-finger ubiquitin-binding domain (ZnF-UBP) close to its C-terminus. This ZnF-UBP recognizes the unconjugated ubiquitin C-terminus (di-Gly motif) with very high affinity. Biochemical analyses showed that free di-Gly motifs are present in the form of unanchored ubiquitin inside IAV virions. These motifs are exposed following low pH-triggered viral fusion at the LEs, and attract HDAC6 transiently to the cytosolic surface of vesicles. The binding of the two components promotes viral uncoating via HDAC6 interaction with cellular motor proteins dynein and myosin II, and the viral M1 capsid. The cellular mechanism involved is related to aggresome processing, a pathway that promotes degradation of misfolded protein aggregates. K63-linked ubiquitin chains are thought to be the trigger for aggresome processing, though it is still not clear whether such types of chains are prevalent within the IAV capsid. Here, we present methods

using purified ZnF-UBP domain of HDAC6 to immunoprecipitate viral unanchored ubiquitin chains, which can then be used for further biochemical analyses of ubiquitin chain linkage.

Running Title: Unanchored ubiquitin within influenza virions

Key Words: Influenza virus, Ubiquitin, Unanchored ubiquitin, Di-Glycine motif, HDAC6, HDAC6 ZnF-UBP, Aggresome processing

1 Introduction

Dynamic ubiquitin homeostasis is associated with many human diseases including neurodegenerative and infectious disease [1]. Ubiquitination (also known as “ubiquitylation”) is a reversible post-translational modification (PTM) that is involved in protein degradation and regulation of protein localization and function. Ubiquitin is a highly conserved 76-amino acid protein, which itself is modified by ubiquitination, phosphorylation, and acetylation on lysine (K) residues [2,3]. A detailed summary on ubiquitin regulation can be found in many excellent reviews and will not be explained in detail here [1] [2] [3] [4] [5]. Briefly: seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) on ubiquitin can be used for ubiquitination of a large number of proteins, which leads to different outcomes depending on the kind of ubiquitin chain that is attached (K48-linked, K63-linked, mono-ubiquitination, etc.). These PTMs make the ubiquitin functions highly diverse, a fact that has been dubbed the “ubiquitin code” [4]. Ubiquitination on lysines is carried out by specific E3 ligases and ubiquitin chains are removed by deubiquitinases. There are several methods for the identification of ubiquitin molecules using mass spectrometric analyses [6] [7] [8]. By using specific antibodies against different ubiquitin linkages and liquid chromatography tandem-mass spectrometry (LC-MS/MS), the ubiquitin code can be dissected.

Histone deacetylase 6 (HDAC6) is a unique protein that mainly deacetylates K40 on alpha-tubulin in the cytoplasm, as well as heat shock protein 90 (Hsp90) and cortactin [9] [10] [11] [12] [13]. It is known that HDAC6 prefers tubulin dimers over microtubules as an efficient substrate [14] [15]. Structurally, HDAC6 has tandem catalytic domains

(CDs) and a zinc finger ubiquitin-binding domain (ZnF-UBP) which binds to unanchored ubiquitin molecules with extremely high affinity ($K_d=60$ nM) [16] (Fig.1). The affinity is so high that HDAC6 ZnF-UBP is thought to be the strongest binder of ubiquitin among all the known zinc finger domains. The linker region between the two CDs can bind the motor protein dynein (Fig.1)[17]. This dynein-binding region is inactive under normal cellular conditions but is activated under conditions where HDAC6 becomes engaged in response to stress – a mechanism that is not totally understood and involves the binding of unanchored ubiquitin to the ZnF-UBP [17].

During the replicative phase in producer cells, IAV packages C-terminal-free unanchored ubiquitin molecules [18]. Mass spectrometry studies by Hutchinson and colleagues indicate that there are *ca.* 40-50 molecules of ubiquitin encapsidated within a single IAV virion; this number, surprisingly, is similar to the number of neuraminidase molecules [19]. At the late endosomal stage of cell entry, low pH triggers the membrane fusion of influenza virus with the limiting membrane of LEs to form a fusion pore. This likely presents the unanchored ubiquitin chains to the cytosolic surface of the LEs. HDAC6 is then recruited via its ZnF-UBP to the viral fusion site, followed by its interaction with the cytoplasmic motors dynein and myosin II, and most importantly, its interaction with the viral capsid matrix protein (M1) [18]. These interactions lead to a pulling-force that is generated onto the viral capsid in a “tug-of-war” manner that breaks the capsid shell, enabling the release of the viral ribonucleoproteins (vRNPs) into the cytosol [18]. Currently, the main unknown aspects of this model are (i) how the unanchored ubiquitin chains are incorporated into the virion, (ii) the precise linkage of ubiquitin chains that triggers this response and (iii) whether additional proteins are involved.

Ubiquitin chains consist of several types of polyubiquitin linkages. K48-based linkage is mainly used for the proteasome-mediated protein degradation pathway, while K63-based linkage is involved in endocytosis, trafficking, and enzymatic activities [2]. Our recent findings showed that both polyubiquitinated proteins and unanchored ubiquitin are packaged within the IAV virion [18]. Here we will describe a protocol for the purification of unanchored ubiquitin from purified IAV virions. A workflow schematic is represented in Fig.2.

2 Materials (see Note 1)

2.1 Expression and purification of His-tagged HDAC6 (HDAC6 ZnF-UBP domain or HDAC6 Δ ZnF-UBP)

1. cDNA for ZnF-UBP domain of HDAC6 (zebrafish, mouse, human etc.) (see **Note 2**)
2. pOPINF plasmid vector (Oxford Expression Technologies).
3. Gibson Assembly Cloning Kit (NEB).
4. XL10-Gold ultracompetent cells (Agilent Technologies).
5. SOC (super optimal broth with Catabolite repression) medium.
6. LB broth and agar plates with 200 μ g/mL ampicillin.
7. QIAGEN Plasmid Plus Midi Kit (QIAGEN).
8. *E. coli* BL21(DE3) strain for protein expression.
9. 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG) dissolved in ultrapure water, filter sterilized through a 0.22 μ m filter.
10. Avestin EmulsiFlex-C3 cell disruptor.
11. 0.22 μ m syringe filter (Sartorius).
12. Purification buffer:
 - Lysis buffer: 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM, ZnCl₂, 20 mM imidazole, 1 mM TCEP, 0.2 % Tween 20, 1x cOmplete protease inhibitor (Roche), and 3 U/mL Benzonase.
 - Nickel Wash buffer: 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM ZnCl₂, 20 mM imidazole.
 - Nickel Elution buffer: 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM ZnCl₂, 500 mM imidazole.
 - Gelfiltration buffer: 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM TCEP, 0.02 % NaN₃ (see **Notes 3 and 4**).
13. Benzonase nuclease.
14. Ni-NTA Superflow resin (Qiagen).
15. Poly-prep column (BIO-RAD).
16. AKTA Pure **chromatography** system (GE Healthcare).
17. Superdex 75 HiLoad 16/60 gel filtration column (GE Healthcare).
18. Amicon Ultra centrifugal unit, 5 kDa MWCO (Merck Millipore).
19. Bradford Protein Assay kit.

20. Albumin standard ampules of 2 mg/mL.
21. Rotary shaker.
22. Round-bottom 96-well plate.
23. Plate reader.
24. Beckman-Coulter Avanti J25 with JA-17 fixed angle rotor (Beckman coulter)
25. Nalgene Oak Ridge centrifuge tube (50 mL) (Nalgene)

2.2 Expression and purification of His-tagged HDAC6 and HDAC6 Δ ZnF-UBP in insect cells

1. **Adherent-adapted and suspension-adapted Sf9 cells** (Thermo Fisher Scientific).
2. Sf-900TM III SFM (Thermo Fisher Scientific) or EX-CELL[®] 420 Serum-Free Medium for Insect Cells (SIGMA) for suspension culture.
3. Grace's insect medium with 10% fetal bovine serum (FBS) for adherent cell culture.
4. pOPINM plasmid vector (Oxford Expression Technologies).
5. *flash*BAC insect expression system (Oxford Expression Technologies).
6. FuGENE HD transfection reagent (Roche).
7. Amicon Ultra concentration device, 50 kDa molecular weight cut-off (MWCO) (Merck Millipore).
8. Rotary shaker.
9. 6-well plates or 35-mm dishes.
10. 250 mL flask.

2.3 Lysis of IAV X31 virions

1. Purified Influenza A virus X31 strain (an H3N2 reassorted strain derived from the A/Puerto Rico/8/34 (PR8) and A/Hong Kong/1/68 strains) (Virapur Inc., CA, USA) in formulation buffer [40 % sucrose, 0.02 % bovine serum albumin (w/v) (BSA), 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM MgCl₂].
Concentration of the prep: approximately 1.5 mg/mL.

2. Cytoskeleton (CSK) lysis buffer: 10 mM PIPES-HCl pH 6.8, 300 mM sucrose, 100mM NaCl, 3 mM EGTA, 0.1 % (v/v) Triton X-100, 1x cOmplete protease inhibitor (Roche).
3. Eppendorf tubes.

2.4 Unanchored ubiquitin pull-down

1. Wash buffer-1: 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 % Triton X-100, 20 mM imidazole, 1 % (w/v) BSA.
2. Ni-NTA agarose beads equilibrated with Wash buffer-1.
3. Wash buffer-2: 20 mM Tris-HCl pH 7.5, 100 mM NaCl.
4. 2 × Laemmli sample buffer: 125 mM Tris-HCl (pH6.8), 20 % Glycerol, 2 % SDS, 0.02 % Bromophenol blue, 1 mM DTT (see **Note 5**).
5. 4-12 % NuPAGE precast polyacrylamide gel (Invitrogen).

2.5 Linkage analysis of precipitated unanchored ubiquitin chains by Western blotting

1. Semi-dry Western blotting device.
2. PVDF membrane.
3. Transfer buffer: (A); 300 mM Tris, 20 % ethanol, 0.02 % SDS, (B); 25 mM Tris, 20 % ethanol, 0.02 % SDS, (C); 25 mM Tris, 20 % ethanol, 40 mM 6-aminohexanoic acid, 0.02 % SDS.
4. Skim milk powder.
5. 10 × Tris-buffered saline (TBS), adjusted to pH 8.0 with HCl.
6. Plastic hybridization bag.
7. 1 × TBST: 1 × TBS with 0.05 % (v/v) Tween 20.
8. Blocking buffer: 5 % (w/v) skim milk in 1 x TBS.
9. Anti-ubiquitin antibody (P4D1) (1:1,000 dilution) (Santa Cruz Biotechnology).
10. Anti-C-terminal ubiquitin antibody (1:100) (Merck **Millipore**).
11. Anti-ubiquitin antibody Lys48-Specific clone Apu2 (1:100) (Merck **Millipore**).
12. Anti-ubiquitin antibody Lys63-Specific clone Apu3 (1:100) (Merck **Millipore**).
13. Anti-mouse or anti-goat IgG-HRP conjugate (1:1,000) (GE Healthcare).
14. ECL Western Blotting Detection Reagents (GE Healthcare).

Commented [Y1]: Check availability.

15. Poly-ubiquitin K48-linked chains (Boston Biochem).
16. Poly-ubiquitin K63-linked chains (Boston Biochem).
17. InstantBlue protein stain (Expedeon).

3 Methods

3.1 Expression in *E.coli* and purification of His-tagged HDAC6 ZnF-UBP domain

1. Construct a pOPINF vector plasmid containing HDAC6 ZnF-UBP by using standard PCR and Gibson assembly kit.
2. Transform the pOPINF vector plasmid containing HDAC6 ZnF-UBP into *E. coli* BL21 (DE3) cells to express the protein.
 - a. Mix 100 ng of the plasmid vector and 100 μ L of BL21 (DE3) in an Eppendorf tube.
 - b. Place on ice for 30 min.
 - c. Heat at 42 °C for 45 s, then immediately place on ice for 2 min.
 - d. Add 400 μ L of SOC medium (pre-warmed at 37 °C) and incubate at 37 °C for 30 min on the rotary shaker.
 - e. Spread on an LB agar plate containing Ampicillin and incubate overnight at 37 °C.
 - f. Pick up single colonies for protein expression.
3. Pre-culture the single colony in 5 mL of LB medium (Amp+) at 37 °C overnight.
4. Add pre-culture in 1 L of LB medium (Amp+) to induce protein expression with 0.5 mM IPTG, and shake with a rotary shaker at 180 rpm at 20 °C for 20 h.
5. Spin down the *E. coli* BL21 (DE3) cells expressing His-tagged ZnF-UBP protein at 6,500 \times g for 30 min to pellet the cells.
6. Resuspend the cell pellet in 5 mL/g (protein) of Lysis buffer in a 50 mL tube (see **Note 6**).
7. Snap freeze in liquid nitrogen, and transfer to -80 °C (see **Note 7**).
8. Thaw the frozen cell suspension at room temperature and place on ice.
9. All the procedures from here must be performed at on ice or in a cold room. Add 3U/mL of Benzonase nuclease into the cell suspension.
10. To break down the cell wall of *E. coli*, pass through an Avestin EmulsiFlex-C3 cell disruptor (see **Note 8**).

11. Collect the soluble lysate by high-speed centrifugation (JA-17 rotor) at 31,000 x g for 30 min at 4 °C.
12. Transfer supernatant into a new tube and pass through a 0.22-µm filter using a syringe (see **Note 9**).
13. Incubate the soluble lysate supernatant with 1 mL of 50 % slurry of Ni-NTA superflow resin at 4 °C for 30 min in batch mode (see **Note 10**).
14. Transfer the sample into a poly-prep column to remove the unbound fraction.
15. Wash the beads twice with 10 x bed volumes of Nickel Wash buffer (see **Note 11**).
16. Elute with 2 x bed volumes of Nickel Elution buffer (see **Note 12**).
17. To further purify and exchange the buffer, inject the eluted protein into a Superdex 75 HiLoad 16/60 gel filtration column in Gelfiltration buffer (see **Note 13**).
18. Collect the peak fractions, and check the purity of the fractionated protein samples on a 4-12 % NuPAGE gel.
19. Stain the gel with InstantBlue protein stain for more than 30 min.
20. Pool the fractionated protein samples and concentrate with 5 kDa MWCO Amicon ultra filtration device.
21. Measure protein concentration using the Bradford Protein assay.
 - a. Prepare a series of albumin standards of 0 (blank), 125, 250, 500, 750, 1000, 1500, 2000 µg/mL using the Gelfiltration buffer as diluent.
 - b. Pipette 5µL of each standard or the protein concentrate (from step 18) sample into appropriate flat-bottom 96-well plate wells, in duplicate.
 - c. Add 250 µL of the Bradford reagent to each well and mix with a plate shaker for 30 s.
 - d. Remove the plate from shaker and incubate the plate for 10 min at room temperature.
 - e. Measure the absorbance at 595 nm using a plate reader.
 - f. Subtract the average 595 nm measurement for the blank duplicates from the 595nm measurements of all other individual standard and protein sample duplicates.
 - g. Plot the average blank-corrected 595nm measurement for each BSA standard. Use the standard curve to determine the protein concentration (see **Note 14**).

22. If not used immediately, aliquot the protein, snap freeze in liquid nitrogen, and store at $-80\text{ }^{\circ}\text{C}$.

3.2 Expression and purification of His MBP-HDAC6 Δ ZnF in insect cells

1. Transfect the pOPINM vector plasmid containing HDAC6 Δ ZnF-UBP into adherent Sf9 cells to produce the P0 virus supernatant. Plate 1×10^6 cells (in a 6-well dish or 35-mm dish) in 2 mL of Grace's insect medium per well.
2. Incubate at $27\text{ }^{\circ}\text{C}$ for 1 h to allow cell attachment.
3. Prepare 1 mL of transfection mix in serum-free Grace's medium. Mix 500 ng of transfer vector, 100 ng of *flashBAC* DNA, and 5 μL of FuGENE HD reagent in a total 100 μL volume.
4. Incubate the transfection mix at room temperature for 15 min.
5. Add 900 μL of Grace's insect medium to the transfection mix, and immediately continue to the next step.
6. Gently wash the Sf9 cells with serum-free Grace's insect medium.
7. Overlay the transfection mix (1 mL).
8. Incubate at $27\text{ }^{\circ}\text{C}$ in the dark for 24 h.
9. Add 1 mL of Grace's insect medium supplemented with 10 % FBS.
10. Incubate at $27\text{ }^{\circ}\text{C}$ for another 5 days.
11. Collect the viral supernatant by centrifugation at 3,000 rpm (1,200 x g) for 15 min at room temperature.
12. Store the viral supernatant at $4\text{ }^{\circ}\text{C}$ in the dark as P0 virus stock.
13. To amplify the baculovirus, infect 1 mL of P0 virus into 6×10^7 suspension Sf9 cells in 50 mL.
14. Incubate at $27\text{ }^{\circ}\text{C}$ for 1 week shaking on a rotary shaker at 180 rpm.
15. Pellet the cells at 3,000 rpm (1,200 x g) for 15 min.
16. Collect the supernatant as the P1 virus.
17. Infect 1 mL of P1 viral supernatant into 6×10^7 suspension Sf9 cells in 50 mL (see **Note 13**).
18. Incubate at $27\text{ }^{\circ}\text{C}$ for 4 days on a rotary shaker at 180 rpm.
19. Collect the viral supernatant as P2 virus and store at $4\text{ }^{\circ}\text{C}$ in the dark.
20. For protein expression, infect P2 virus (usually 500 μL to 1 mL) into 10^8 suspension Sf9 cells in 50 mL using a 250 mL flask (see **Note 14**).

21. Incubate at 27 °C for 3 days.
22. Collect the Sf9 cell pellet by centrifugation at 3,000 rpm (1,200 x g) for 15 min at room temperature.
23. Snap freeze the cell pellets in liquid nitrogen and transfer to - 80 °C (see **Note 7**).
24. Thaw the frozen cell pellets at room temperature by adding cell Lysis buffer and suspend by pipetting several times (see **Note 8**).
25. Incubate on ice for 30 min.
26. Collect the soluble lysate by high-speed centrifugation (JA-17 rotor) at 31,000 x g for 30 min at 4 °C.
27. Continue to step 12 of section 3.1.
28. Pool the fractionated protein samples and concentrate with a 50 kDa MWCO Amicon ultra filtration device.
29. Aliquot the protein, freeze in liquid nitrogen quickly and store at - 80 °C.

3.3 Lysis of influenza virus

1. Thaw the 100 µL virus aliquots on ice.
2. Add 200 µL of ice cold CSK lysis buffer per 100 µL of virus.
3. Transfer to an Eppendorf tube and suspend gently by pipetting up and down several times.
4. Incubate the tube on ice for 60 min to complete lysis.
5. Make 100 µL aliquots in Eppendorf tubes and proceed to the pull-down assay (section 3.4) or store the lysate at - 80 °C until further use.

3.4 Unanchored ubiquitin pull-down assay

1. All procedures should be performed at 4 °C or in a cold room. Mix 100 µL of the lysed IAV with 1 µg of purified proteins in a buffer containing 20 mM Tris-HCl pH7.5, 100 mM NaCl and 0.1 % Triton X-100 (see **Note 16**).
2. Incubate the mixture at 4 °C overnight with rotation.
3. After overnight incubation, add 20-40 µL of 50 % slurry Ni-NTA agarose beads (equilibrated with Wash buffer-1).
4. Incubate at 4 °C for 30 min with rotation.

5. Spin down the Ni-NTA beads by centrifugation at 1,500 x *g* at 4 °C for 2 min.
6. Wash the precipitated beads twice with Wash buffer-1.
7. Wash the precipitated beads twice with Wash buffer-2.
8. Add 15-20 µL of 2 x Laemmli sample buffer to the beads, and heat at 95 °C for 10 min.
9. Load the samples onto a 4-12% NuPAGE gel.
10. Perform Western blotting analysis (see Fig. 2, Fig. 3).

3.5 Specific linkage analysis for free ubiquitin in the virus particles by Western blotting

1. Activate a PVDF membrane with a 1:1 mixture of 100 % ethanol and transfer buffer (C).
2. Transfer the gel from step 9 of section 3.4 onto the membrane for 2 h (1 cm²/1 mA constant current).
3. Remove the membrane from the blotting machine and wash with 1x TBST for 5 min on a rocker.
4. Block the membrane with 5 % skim milk in 1x TBS on a rocker at room temperature for 1 h.
5. React with primary antibody in Blocking buffer at 4 °C overnight (see **Note 17, 18**).
6. Wash the membrane with 1x TBST for 5 min, three times.
7. React with secondary antibody (1:3000 in Blocking buffer) at room temperature for 1 h.
8. Wash the membrane with 1x TBST for 5 min, three times.
9. React with ECL reagents for 5 min following the manufacturer's instructions.
10. Detect the signal with an X-ray film using a film developer (see **Note 19**).

4 Notes

1. Use ultrapure water and molecular biology grade reagents for all solutions. Follow your local waste disposal regulations.
2. For the negative control for ubiquitin-binding, we use HisMBP [His-tag with maltose binding protein (MBP)] and HisMBP-HDAC6 lacking the ZnF-UBP domain (HisMBP-HDAC6 Δ ZnF).
3. TCEP should be added and pH-adjusted immediately before use, **as long term storage changes pH.**
4. All the buffers must be de-gassed using a filtration device. The gel filtration buffer should be de-gassed as well.
5. Add fresh 1 M DTT immediately before use (1:1000 dilution).
6. **Measure the weight of the pellet by comparison with an empty centrifugation tube.**
7. Freeze the cell pellets at -80 °C for at least 30 min to help cell disruption.
8. Keep the cell lysate at 4 °C on ice.
9. When the syringe filter is easily stuck, use a vacuum filtration device (0.45- μ m).
10. Do not use a polyprep column. Mix the samples in one 50 mL Falcon tube on the rotator.
11. Packed beads are defined as 1x bed volume. **For example, if you use 1 mL of packed bead volume, 10 x bed volume is equal to 10 mL.**
12. If necessary, concentrate the protein materials to 500 μ L-1 mL for the gel filtration injection **by using Amicon Ultra centrifugal units.**
13. If necessary, check the viral titer before protein expression. P0 and P1 virus can be stored at 4 °C in the dark for more than 6 months although the viral titer will decrease gradually.
14. Typically the protein concentration is around 1 mg/mL after Amicon concentration.
15. For protein expression, use a flask that is larger than 5 x volume of the culture, i.e. for 200 mL culture, use a 1L or larger flask. This allows effective air circulation and protein expression in Sf9 cells.
16. Here we use His-MBP and/or HisMBP-HDAC6 Δ ZnF as negative controls.

17. To reduce the volume of primary antibody reaction, use a plastic hybridization bag.
18. Dilute the primary antibodies in Blocking buffer. The dilution is the following: anti-total ubiquitin antibody (1:3000), anti-His antibody (1:1000), anti-C-terminus ubiquitin antibody (1:100).
19. For a positive control of ubiquitin specific linkage, run purified K48- and/or K63-linked polyubiquitin chains on the gel (see Fig.3 A left panel).

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Figures

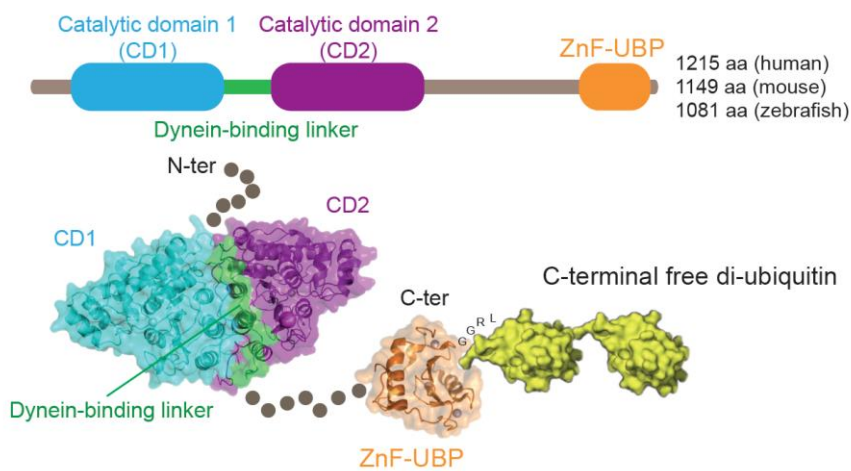


Fig.1 Domain structure of histone deacetylase 6 (HDAC6)

Primary domain structure of HDAC6 conserved in various species. Tandem catalytic domains are shown as CD1 and CD2, respectively. Flexible linker region in between CD1 and CD2 is known as Dynein motor binding linker shown in green. The C-terminal of ZnF-UBP binds to free ubiquitin moiety. 3D crystal structure of HDAC6 is drawn in below. Flexible regions are shown in dotted lines. The surface structure of C-terminal free single di-ubiquitin is shown in yellow.

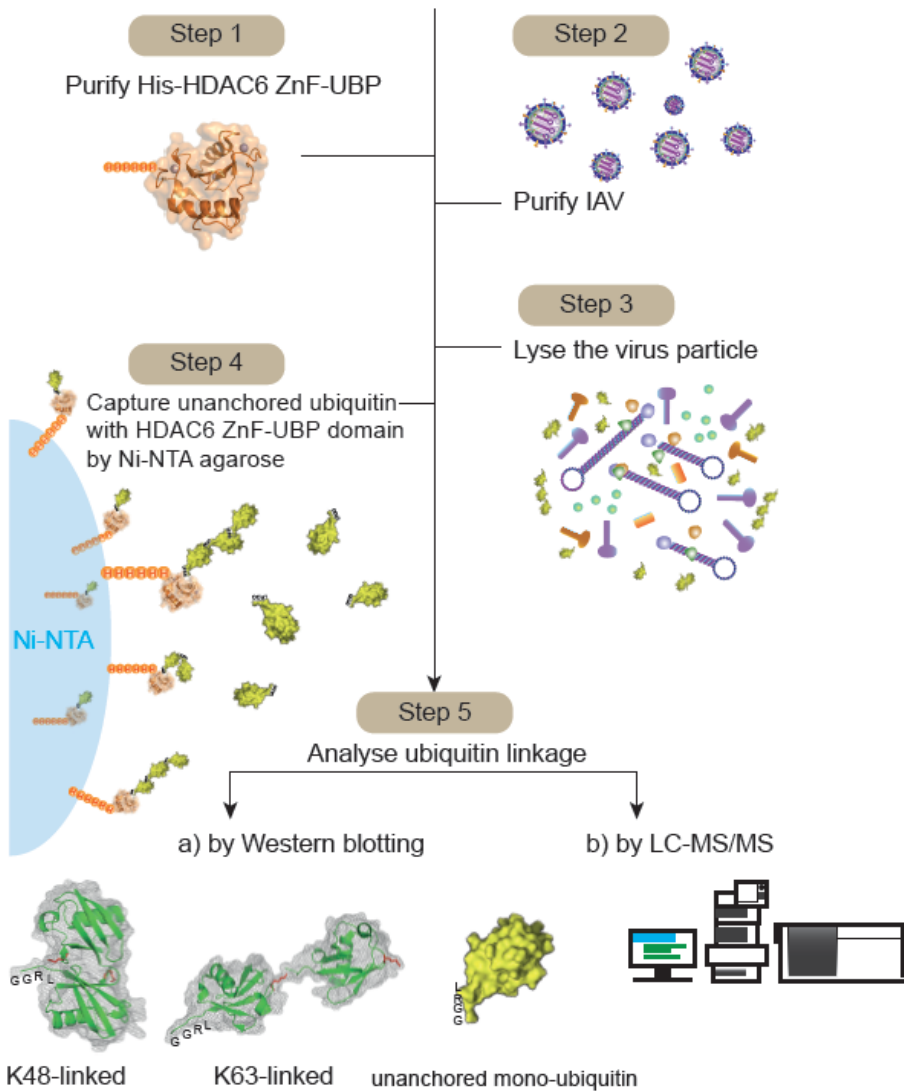


Fig. 2 Flowchart of unanchored ubiquitin chain purification from purified IAV X31 virions.

(Step 1) Purify ZnF-UBP domain of HDAC6 expressed in *E. coli* or Sf9 insect cells.
 (Step 2) Purify IAV. (Step 3) Lyse IAV with cytoskeleton (CSK) buffer containing 0.1 % Triton X-100. (Step 4) Capture and concentrate ubiquitin moieties with free C-termini in

IAV extracts with ZnF-UBP domain of HDAC6. (Step 5) Confirm the ubiquitin linkage by Western blotting (a) and/or LC-MS/MS analysis (b) (not described in this chapter).

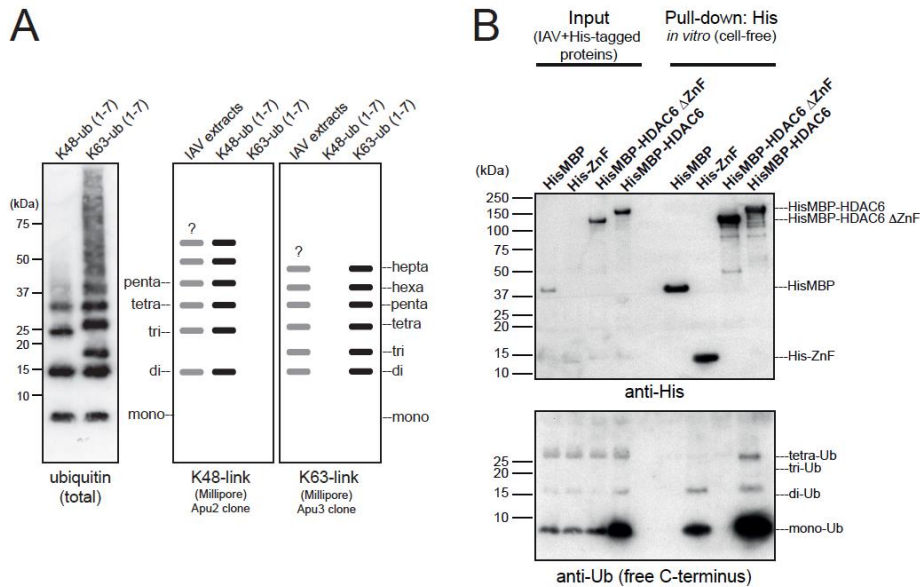


Fig.3 Analysis of ubiquitin-linkage in the IAV particle.

- A) (Left) Purified K48- and/or K63-linked polyubiquitin chains were detected by total ubiquitin antibody. Mobility shift of K48- and K63-linked polyubiquitins are different from each other. (Center) Schematics of expected ubiquitin blot of X31 extracts detected by indicated linkage specific ubiquitin antibodies. K48 specific antibody was used. (Right) K63 specific antibodies are used for detection. K48/K63-linked polyubiquitin (ub1-7) chains are used as positive controls.
- B) Pulled-down ubiquitin in the X31 extracts. After pull-down with ZnF-UBP domain of HDAC6, C-terminal free ubiquitin was detected with specific antibodies (see [18]). Upper panel shows that His-tagged proteins were detected by anti-His antibodies. Viral ubiquitin chains were pulled-down depending on ZnF-UBP domain.