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Purifying Properly Folded Cysteine-rich, Zinc Finger Containing Recombinant Proteins for Structural Drug Targeting Studies: the CH1 Domain of p300 as a Case Example

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[Abstract] The transcription factor Hypoxia-Inducible Factor (HIF) complexes with the coactivator p300, activating the hypoxia response pathway and allowing tumors to grow. The CH1 and CAD domains of each respective protein form the interface between p300 and HIF. Small molecule compounds are in development that target and inhibit HIF/p300 complex formation, with the goal of reducing tumor growth. High resolution NMR spectroscopy is necessary to study ligand interaction with p300-CH1, and purifying high quantities of properly folded p300-CH1 is needed for pursuing structural and biophysical studies. p300-CH1 has 3 zinc fingers and 9 cysteine residues, posing challenges associated with reagent compatibility and protein oxidation. A protocol has been developed to overcome such issues by incorporating zinc during expression and streamlining the purification time, resulting in a high yield of optimally folded protein (120 mg per 4 L expression media) that is suitable for structural NMR studies. The structural integrity of the final recombinant p300-CH1 has been verified to be optimal using one-dimensional ^{1}H NMR spectroscopy and circular dichroism. This protocol is applicable for the purification of other zinc finger containing proteins.
Keywords: p300, CBP, HIF, CH1, Cysteine, Zinc finger, Hypoxia, Recombinant protein purification

[Background] The growth of solid tumors is associated with the development of hypoxic areas due to inappropriate vascular irrigation. In response to a hypoxic microenvironment, tumor cells overexpress Hypoxia Inducible Factors (HIF), a family of heterodimeric transcription factors (Semenza, 2002; Brat and Van Meir, 2004; Kaur *et al.*, 2005). HIFs bind to p300, a transcriptional coactivator, to form a complex that induces HIF target genes, thereby activating the hypoxia response pathway and promoting tumor growth (Kasper and Brindle, 2006; Liu, 2008). The binding domains involved with the HIF/p300 protein-protein interface are the cysteine-histidine-rich region 1 (CH1) domain of p300 and the C-terminal activating domain (CAD) of HIF-1α (Dames *et al.*, 2002; Freedman *et al.*, 2002). The hypoxia response pathway facilitates tumor growth under oxygen limiting conditions. Inhibiting this pathway is a goal for targeted anti-cancer therapy (Post *et al.*, 2004; Belozerov and Van Meir, 2006; Mooring, 2011; Tan *et al.*, 2011; Mun *et al.*, 2012; Wilkins *et al.*, 2016). Small molecule compounds have recently been developed to bind to p300-CH1 and inhibit p300/HIF complexation, inhibiting the hypoxia response pathway and reducing tumor growth (Shi *et al.*, 2012; Yin *et al.*, 2012; Burroughs *et al.*, 2013).

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Furthermore, the p300-CH1 domain has been reported to interact with over 30 additional transcription factors related to cancer and other diseases (Kasper and Brindle, 2006).

This protocol has been developed to purify the p300-CH1 peptide with the purity and structural integrity necessary to conduct structural NMR studies for studying protein-protein and protein-ligand interactions. p300-CH1 poses many challenges for recombinant protein purification, as it contains three zinc fingers and 9 total cysteine residues. p300-CH1 has been reported to be structurally compromised without a 3:1 stoichiometric ratio of zinc (De Guzman *et al*., 2005). Without zinc, cysteine residues typically found at zinc fingers can form unwanted disulfide linkages that are thermodynamically more stable than cysteine-zinc interactions. Proteins without zinc cations occupying their native zinc fingers are prone to oxidation and readily form disulfide linkages, resulting in unwanted protein conformations and protein aggregation.

Introducing zinc (II) to a protein purification protocol is complicated by the fact that zinc (II) interacts with certain buffer salts, reducing agents, and hydroxide ions to form precipitates. Unwanted zinc (II) precipitation can prevent the formation of crucial zinc fingers and even leach out existing zinc fingers to denature the protein. In addition, the reducing agents and buffer salts that precipitate with zinc may be unavailable to serve their respective purposes for the purification. Zinc (II) cations form white ZnOH precipitates with neutral to alkaline buffer conditions (pH 7.0 and above). Zinc (II) also forms precipitates with phosphate buffer and thiol reducing agents (BME and DTT). The buffers and reagents used for the purification procedure must be carefully selected to be compatible with zinc. As a precaution, zinc stocks and zinc containing solutions must be pH adjusted with zinc present to verify that ZnOH precipitates do not form during purification. This is especially important in purifying high yields of properly folded zinc-finger containing proteins.

The purification protocol has been designed with the following strategies to minimize oxidation and maximize yield:

1. Occupy cysteine residues with zinc (II) throughout the protocol
2. Streamline the duration of expression and cleavage
3. Use reducing agents whenever possible

The protocol successfully overcomes issues of reagent compatibility and oxidation, yielding 120 mg of purified recombinant p300-CH1 from a 4 L bacterial culture. Concentration of recombinant p300-CH1 was measured by UV-Vis absorbance at a wavelength of 280 nm. The extinction coefficient of p300-CH1 is calculated to be 5,690 L mol^{-1} cm^{-1} by ExPASy ProtParam tool (Shi *et al*., 2012). According to circular dichroism and 1H NMR, the purified p300-CH1 recombinant protein product is optimally folded without the need for any further modification post-purification. The protocol and its strategies can be applied to other cysteine containing proteins systems to improve yield and purity.

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Materials and Reagents

1. 14 ml polypropylene round-bottom tube (Corning, Falcon®, catalog number: 352057)
2. 1.7 ml polypropylene microcentrifuge tube (Posi-Click Eppendorf) (Denville Scientific, catalog number: C2170)
3. Polypropylene micropipette tips
4. 50 ml polypropylene conical bottom tube (Corning, catalog number: 430291)
5. Two-sided disposable plastic cuvettes 10 mm (VWR, catalog number: 97000-586)
6. 250 ml wide-mouth plastic bottle (Thermo Fisher Scientific, Thermo Scientific™, catalog number: N411-0250)
7. 15 ml polypropylene conical bottom tube (Corning, catalog number: 430052)
8. Chemically competent *E. coli* (One Shot® BL21 Star (DE3)) (Thermo Fisher Scientific, Invitrogen™, catalog number: C601003); store at -80 °C
9. pGEX-6P-1 plasmid expression vector (GE Healthcare, catalog number: 28-9546-48) containing GST-p300-CH1 domain cDNA
   Note: The p300-CH1 insert was isolated from a Thrombin-site containing expression vector (pGEX-2T vector containing p300-CH1 insert) (http://web.expasy.org/protparam/) using BamHI and EcoRI restriction enzymes. The insert was cloned into the pGEX-6P-1 vector.
10. Ice
11. LB Miller broth powdered (Fisher Scientific, catalog number: BP1426-2)
12. Ampicillin sodium salt powdered (Sigma-Aldrich, catalog number: A9518)
   Note: Prepare 100 mM stock (1,000x) in deionized water. Filter sterilize and store at -20 °C.
13. Plasmid DNA Extraction Miniprep Kit (GeneJET) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: K0502)
14. *Bam*HI restriction enzyme FastDigest (800 µl, 1 reaction/1 µl) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: FD0054); store at -20 °C
15. Restriction enzyme buffer FastDigest (10x; 5x 1 ml) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: B64); store at -20 °C
16. *Eco*RI restriction enzyme FastDigest (800 µl, 1 reaction/1 µl) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: FD0274); store at -20 °C
17. Quick-Load 1 kb DNA ladder (New England Biolabs)
18. 5x nucleic acid loading buffer (10 ml, premixed) (Bio-Rad Laboratories, catalog number: 1610767)
19. Deionized water
20. Acrylamide electrophoresis gels (Criterion TGX Precast Gels; 18 well comb, 30 µl loading volume per well, 1.0 mm thickness, 4-20% gel percentage) (Bio-Rad Laboratories, catalog number: 5671094)
21. Agar powdered (Fisher Scientific, catalog number: BP24662)
22. 1x TAE running buffer

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23. Ethidium bromide (10 mg/ml) (Thermo Fisher Scientific, Invitrogen™, catalog number: 15585011)
24. Glycerol 100% (EMD Millipore, catalog number: GX0185)
25. Isopropyl β-D-1-thiogalactopyranoside (IPTG) powdered (Gold Bio, catalog number: I2481C100)
    Note: Prepare 10 ml of 1 M (10,000x) stock in deionized water and store at -20 °C.
26. Zinc chloride ($ZnCl_2$), 99.999% trace metals basis powdered (Sigma-Aldrich, catalog number: 229997)
    Note: Prepare as 100 mM stock in deionized water.
27. Bleach
28. Liquid nitrogen
29. DNase I (2,000 U/ml; 1 ml) (New England Biolabs, catalog number: M0303S); store at -80 °C
30. RNase A (1 ml, 1 mg/ml) (Bioo Scientific, catalog number: 344005)
31. 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (PEFA-BLOC) powdered (Biosynth, catalog number: A-5440)
32. Benzamidine HCl, > 99% powdered (RPI, catalog number: B12000-100.0)
33. Glutathione agarose beads (10 ml 50% slurry in 0.05% sodium azide solution) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 16100)
34. HRV 3C Cysteine Protease PreScission Site (1 mg lyophilized) (AG Scientific, catalog number: H-1192)
    Note: Prepare as 2 mg/ml in Prescission Protease buffer, and store as 50 µl aliquots; store at -80 °C.
35. Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, catalog number: 1610406)
36. Ethylenediaminetetraacetate acid disodium salt (EDTA)
37. Tris base (White Crystals or Crystalline Powder/Molecular Biology) (Fisher Scientific, catalog number: BP152)
38. Sodium chloride (NaCl) (Fisher Scientific, catalog number: BP358-10)
39. Sodium hydroxide (NaOH) (BioUltra, for molecular biology, 10 M in H_2O) (Sigma-Aldrich, catalog number: 72068)
40. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (powder, ≥ 98% purity, 10 g) (Sigma-Aldrich, catalog number: C4706)
41. Tris-d_{11}
42. DTT-d_{10}
43. Acidic resuspension buffer (pH 6.3) (see Recipes)
44. Alkaline resuspension buffer (pH 8.0) (see Recipes)
45. HRV3C PreScission Site Protease buffer (pH 8.0) (see Recipes)
46. 10% (v/v) deuterated Buffer for NMR (pH 8.0) (see Recipes)
47. LB Miller medium (see Recipes)

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Equipment

1. Variable Micropipets (Edvotek, catalog numbers: 591, 5911, 5921)
2. Large silicone non-stick kitchen spatula
3. Orbital shaker (FormaOrbital Shaker, Thermo Electron)
4. Autoclave (STERIS, model: SV-120)
5. Benchtop shaker (Jeio Tech, Lab Companion, model: SK-300)
6. Centrifuge (Beckman Coulter, model: Avanti J-20XP)
7. Centrifuge rotor with 250 ml rotor cavities (16,000 rpm rotor) (Beckman Coulter, model: J-LITE® JLA-16.250)
8. 2 L glass flask (Corning, PYREX®, catalog number: 4980-2L)
9. -80 °C freezer
10. High pressure homogenizer (Avestin, model: EmulsiFlex-C5)
11. Fisher Scientific Isotherm Model 900 cooler (Fisher Scientific, model: Model 900)
12. Ultracentrifuge (Beckman Coulter, model: Optima™ XE-90K)
13. Ultracentrifuge rotor with 50 ml rotor cavities (Beckman Coulter, model: SW 40 Ti)
14. Vertical rotating wheel (Labquake Tube Shaker Rotisserie) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: C415110)
15. Benchtop centrifuge (Thermo Fisher Scientific, model: Sorvall™ ST 16, catalog number: 75004240)
16. Swinging bucket rotor with 400 ml rotor cavities (Thermo Fisher Scientific, Thermo Scientific™, model: TX-400, catalog number: 75003629)
17. Protein electrophoresis apparatus (Bio-Rad Laboratories, model: Criterion™ Cell, catalog number: 1656001)
18. Spectropolarimeter (JASCO, model: J-810; 163-900 nm)
19. Spectrophotometer (Eppendorf, model: BioPhotometer® plus, catalog number: 952000006)
20. 600 MHz NMR spectrometer (Varian, INOVA)

Procedure

Note: Verify all materials and reagents are available and prepare solutions and media (Day 0).

A. Preparing bacterial expression stock (DAYS 1-2)
   1. Transform BL21 (DE3) bacteria with the p300-CH1 expression vector (pGEX-6P-1 plasmid containing the p300-CH1 insert) using the basic protocol outlined for the One Shot® BL21 Star (DE3) Chemically Competent *E. coli*.
   2. Autoclave 4 L of LB Miller broth, and allow the media to cool at room temperature. Once the broth is cool to the touch, add ampicillin to a final concentration of 100 µM.
   3. Pick six colonies, and inoculate each colony in 5 ml of autoclaved LB Miller broth supplemented with 100 µM ampicillin in a 14 ml polypropylene round-bottom tube.

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4. Incubate overnight at 37 °C using the FormaOrbital Shaker at a speed of 242 rpm.
5. Select samples that show bacterial growth, and properly discard any samples without growth.
6. Isolate plasmid from a small sample of the growth media (3 ml) using a GeneJet mini kit (https://tools.thermofisher.com/content/sfs/manuals/MAN0013117_GeneJET_Plasmid_Miniprep_UG.pdf).
7. Verify presence of pGEX-6P-1 vector (4,984 bp) containing insert coding for p300-CH1 (318 bp) by performing restriction digestion with enzymes (*Bam*HI and *Eco*RI). Prepare the following samples:
    a. 10 µl Quick-Load 1 kb DNA ladder
    b. Undigested plasmid: 15 µl of 50 ng/µl DNA, 5 µl of 5x nucleic acid sample loading buffer, 5 µl deionized water
    c. Digested plasmid: 15 µl of 50 ng/µl DNA, 2 µl 10x FastDigest buffer, 1 µl FastDigest *Bam*HI, 1 µl FastDigest *Eco*RI, 1 µl deionized water. Digest at room temperature for 5 min. Add 5 µl of 5x nucleic acid sample loading buffer
8. Perform DNA electrophoresis and verify the presence of pGEX-6P-1 vector and p300-CH1 insert bands.
    a. Cast 75 ml of 4% agarose gel with an 18-well comb.
    b. Run at 125 V for 45 min in 1x TAE running buffer.
    c. Stain the gel in 0.5 mg/ml ethidium bromide solution with water for 15 min.
    d. Check the gel under UV-light to verify presence of bands for pGEX-6P-1 vector and P300-CH1 insert.
9. Prepare 50% glycerol stocks of the bacteria that contain the desired plasmid by mixing 0.5 ml of bacteria with 0.5 ml of autoclaved 100% glycerol in autoclaved 1.7 ml microcentrifuge tubes.
10. Homogenize by gently inverting tube 3 times and store the 50% glycerol BL21 (DE3) stocks at -80 °C.
11. Scratch frozen 50% glycerol plasmid-containing BL21 (DE3) stock with a micropipette tip, and spread onto ampicillin LB-agar plates.
12. Incubate the plate overnight at 37 °C.

B. Overnight culture for bacterial expression (2 h) (DAY 3)
1. Pipette three samples of 10 ml of autoclaved LB Miller broth into a 50 ml conical bottom polypropylene tube, and add ampicillin to a final concentration of 100 µM.
2. Inoculate each 10 ml LB sample with a single plasmid-containing BL21 (DE3) colony.
3. Incubate at 37 °C overnight in an orbital shaker under gentle agitation (242 rpm).

C. Large batch plasmid growth (3-4 h) (DAY 4)
1. Remove flasks containing 4 L LB Miller broth from the cold room, and allow to warm to room temperature.
2. Add ampicillin and $ZnCl_2$ (both used at final concentrations of 100 µM).

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4 ml of 100 mM ampicillin stock

4 ml of 100 mM $ZnCl_2$ stock

Note: Save 1 ml aliquot in spectrophotometer cuvette for OD_{600} Blank.

3. Remove the 20 ml BL21 (DE3) overnight bacterial culture, and evenly distribute to 4 L LB Miller broth.
4. Incubate bacteria at 37 °C at 242 rpm using the orbital shaker until OD_{600} is 0.5-0.7.

   Note: Take ~1 ml aliquots every 30 min starting at 3 h to measure OD_{600}.

D. Inducing protein expression (5 h) (DAY 4)

1. Remove LB bacterial culture flasks from the orbital shaker and add IPTG to a final concentration of 100 µM using the 10,000x IPTG stock.
2. Incubate bacteria at room temperature (22 °C) for 5 h under gentle agitation (242 rpm) in benchtop horizontal shaker (SK-300).
3. Pour 1.2 L bacterial growth media into four 250 ml wide-mouth plastic bottles, and balance the masses of the bottles.

   Note: Leave the remainder of the growth media at room temperature.
4. Pellet bacteria by centrifuging at 4,000 *x g* at 4 °C for 15 min in Beckman Coulter Avanti Centrifuge (J-20XP) using the JLA-16.250 rotor.
5. Discard the supernatant into 2 L Erlenmeyer flasks containing 100 ml of Bleach.
6. Repeat steps D3-D5 until all 4 L of bacteria have been pelleted.
7. Scrape the pellet with a spatula (15-25 g per 4 L bacterial expression) and transfer into a 50 ml conical bottom polypropylene tube.
8. Flash freeze bacterial pellet by plunging tube in a container filled with liquid nitrogen, and keep in -80 °C freezer.
9. Dispose of the bleached supernatant into the sink.

E. Lysing bacterial pellet (5 h) (DAY 5)

1. Thaw the bacterial pellet on ice or in a cold room.
2. Re-suspend pellet in 25 ml acidic resuspension buffer (50 mM Tris, 150 mM NaCl, pH 6.3, see Recipes).
3. Add 15 µl DNAase I (1 mg/ml), 15 µl RNase A, and 3-5 mg of PEFA-BLOC and benzamidine protease inhibitors.
4. Insert a stir bar, and stir in the cold room (4 °C) for 1 h.
5. Homogenize suspended pellet using the high-pressure homogenizer (Emulsiflex C5), cooled with the Fisher Scientific Isotherm Model 900 cooler (setup is pictured in Figure 1).

   Notes:

   a. p300-CH1 is temperature sensitive, and the traditional method of lysing bacteria by alternating between freezing (ice lattice damage; 'freeze burn') and thawing (protein melting) are unsuitable.

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b. *If high-pressure homogenizer is unavailable, sonication at a constant temperature of 4 °C may be a suitable alternative.*

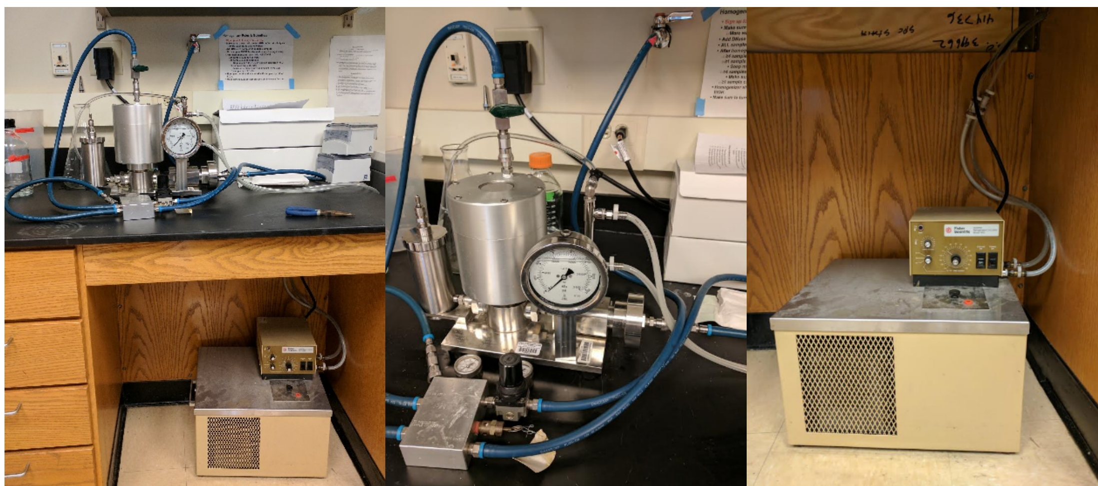

Figure 1. High-pressure homogenizer (Emulsiflex C5), cooled with the Fisher Scientific Isotherm Model 900 cooler

6. Spin down lysate at RCF 205,076 *x g* for 1 h at 4 °C in Optima XE-90 ultracentrifuge (Beckman Coulter) using Ti-40 rotor.
7. Transfer clarified bacterial lysate (containing soluble bacterial components) into 50 ml conical bottom polypropylene tubes.
   Note: Save a 14 µl aliquot for SDS-PAGE to verify that expression has occurred (see sample #1 in Figure 2).

F. Capturing and purifying GST tagged p300-CH1 protein (4 h) (DAY 5)
1. Add 3 ml of GSH-Agarose beads into 50 ml conical-bottom polypropylene tubes.
   Note: Cut the very end of the micropipette tip to aspirate agarose beads.
2. Wash away preservatives and ethanol from beads by filling up to 50 ml with pre-chilled acidic resuspension buffer.
3. Spin the beads at 700 *x g* for 15 min at 4 °C, and decant the supernatant.
4. Repeat steps F2-F3 to further clean the beads, leaving sedimented beads with as little buffer as possible to accommodate for lysate.
5. To lysate, add 3 ml of GSH-Agarose beads, prewashed twice with acidic resuspension buffer to remove ethanol and preservatives.
6. Rotate gently at 4 °C for 3-4 h using the vertical rotating shaker (8 rpm).
7. Spin down beads at 700 *x g* for 15 min at 4 °C to sediment beads using a Benchtop Centrifuge (Sorvall ST16R) with a Swinging Bucket Rotor (TX-400).
   Note: Save a 14 µl aliquot of the supernatant for SDS-PAGE to verify efficiency of GST-tagged recombinant protein capture (see sample #2 in Figure 2).

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8. Wash beads 5 times with 50 ml of prechilled acidic resuspension buffer to eliminate excess $ZnCl_2$.
   Note: Collect 1 ml aliquots of the supernatant after each wash and spin down. Collect UV spectra after each wash and monitor the 260/280 nm absorbance ratios. Wash the beads until the 260/280 nm ratio is below 1.0, which indicates that the sample is free of DNA contamination (https://www.promega.com/-/media/files/resources/application-notes/pathlength/calculating-nucleic-acid-or-protein-concentration-using-the-glomax-multi-microplate-instrument.pdf?la=en).
9. Wash beads 5 times with 50 ml of prechilled alkaline resuspension buffer (50 mM Tris, 150 mM NaCl, pH 8.0, see Recipes) to reestablish alkaline pH for protease activity.
10. After the final alkaline buffer wash, decant the supernatant and transfer the sedimented GSH-Agarose beads into a 15 ml polypropylene tube. The final product of Procedure F is GST-tagged p300-CH1 immobilized to GSH-Agarose beads.
    Note: Save a 10 µl aliquot of beads after final wash for SDS-PAGE to verify purity of captured GST-tagged p300-CH1 after buffer washing (see sample #3 in Figure 2).

G. Inducing cleavage (DAY 5)
1. Add 10 ml of HRV 3C Protease buffer (see Recipes) to the GST-tagged p300-CH1 protein immobilized to GSH-Agarose beads in the 15 ml conical bottom polypropylene tube.
   Note: HRV 3C protease is a cysteine protease that needs reducing agents to maintain its activity. Due to precipitation issues associated with zinc and thiol-based reducing agents, use non-thiol based reducing agents like TCEP.
2. Add 100 µl HRV 3C PreScission site Protease (2 mg/ml).
3. Gently rotate at 4 °C for 16-18 h using a vertical rotating wheel (8 rpm).

H. Purifying cleaved p300-CH1 protein (DAY 6)
1. Remove overnight cleavage mixture from the vertical rotating wheel.
2. Spin down beads at 700 *x g* for 15 min at 4 °C using Benchtop Centrifuge (Sorvall) with a Swinging Bucket Rotor (Thermo Scientific TX-400), and transfer supernatant into a new 15 ml polypropylene tube.
   Notes:
   a. *Save a 10 µl aliquot of beads for SDS-PAGE to verify completion of cleavage (only cleaved GST tag should be present; see sample #4 in Figure 2).*
   b. *Save a 14 µl aliquot of supernatant for SDS-PAGE to verify presence of cleaved recombinant p300-CH1 (see sample #5 in Figure 2).*
3. Wash the GSH-Agarose beads twice with alkaline resuspension buffer, then add 2 ml of GSH-Agarose beads to capture cleaved GST, uncleaved GST-p300-CH1, and HRV 3C Protease.
4. Gently rotate the supernatant at 4 °C for 3-4 h in cold room with the vertical rotating wheel (8 rpm).

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5. Spin down beads at 700 *x g* for 15 min at 4 °C using Sorvall Benchtop Centrifuge with a Swinging Bucket Rotor, and collect supernatant as the final product sample.
   Notes:
   a. *Save a 10 µl aliquot of beads for SDS-PAGE to verify elimination of protease byproducts (see sample #6 in Figure 2).*
   b. *Save a 14 µl aliquot of protein product for SDS-PAGE to verify purity of product (see sample #7 in Figure 2).*
6. The final p300-CH1 recombinant protein sample should be at a concentration of around 800 µM (12 ml total volume).
7. Divide 800 µM recombinant p300-CH1 protein into 500 µl aliquots, flash freeze in liquid nitrogen, and store at -80 °C. Repeated freeze-thaw cycles may degrade the protein concentration by around 10%.

Data analysis

DAY 7

1. Perform SDS-PAGE with collected samples #1-7 and stain with Coomassie (Figure 2) to verify efficacy of purification procedure.
2. Perform Western blot and mass spectrometry analysis to confirm the identity of purified p300-CH1 protein (optional).
3. Perform circular dichroism (CD) analysis of purified protein to verify alpha helical secondary structure (Figure 3).
4. Perform one-dimensional ^{1}H NMR to verify presence of sharp amide proton chemical shifts (Figure 4).

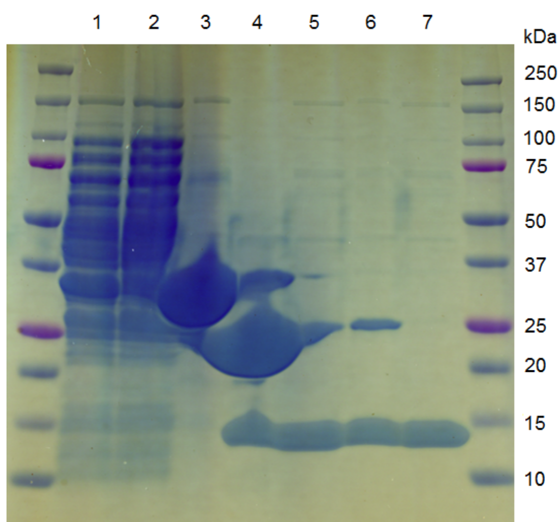

Figure 2. Coomassie-stained SDS-Page of purified p300-CH1 domain expressed from *E. coli*. 1. Bacterial lysate upon expression. 14 µl of bacterial lysate before capture from 50 ml of

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total lysate sample from 4 L bacterial growth medium. 2. Clarified bacterial lysate. 14 μl lysate after capture from 50 ml of total lysate sample from 4 L bacterial growth medium. 3. GST-tagged p300-CH1 immobilized to GSH-Agarose beads. 10 μl beads after capture from 3 ml bed volume of GSH-Agarose beads. 4. Bead-immobilized protease cleavage product: Uncut GST-tagged p300-CH1 (37 kDa) and GST (25 kDa). 10 μl beads after cleavage from 3 ml bed volume of GSH-Agarose beads. 5. Supernatant protease cleavage product: p300-CH1 and free GST impurity. 14 μl supernatant after cleavage from 20 ml total supernatant. 6. Free GST impurities captured with GSH-Agarose beads. 10 μl GSH-Agarose bead capture (GST + GST-p300 + Prescission) from 2 ml bed volume of GSH-Agarose beads. 7. Purified recombinant p300-CH1. 14 μl sample from 20 ml of total recombinant p300-CH1 sample.

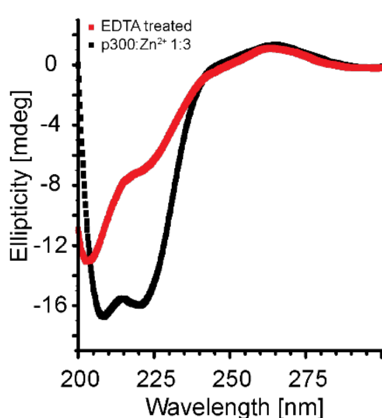

Figure 3. CD spectra of purified p300-CH1 in the absence (red line) or presence (black line) of 3 equivalents of Zn^{2+}. 40 μl of p300-CH1 (700 μM in alkaline resuspension buffer supplemented with reducing agent) were loaded in a 0.1 mm quartz cuvette. EDTA treated sample contained 700 μM p300-CH1 and 2.1 mM EDTA (3 stoichiometric equivalents). Collected 3 scans at normal sensitivity in Jasco J-810 spectropolarimeter.

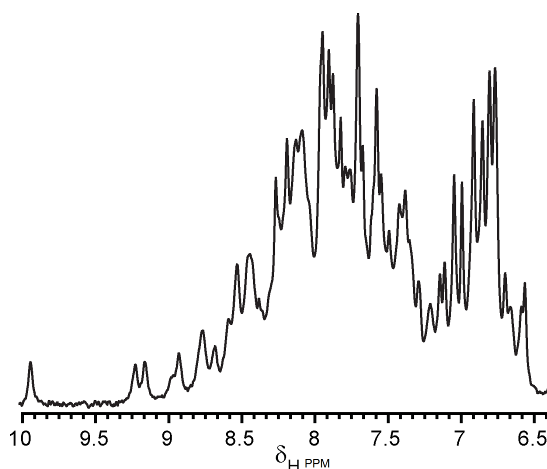

Figure 4. Expansion of 1D ^{1}H NMR spectrum, highlighting amide–NH's, aromatic and NH side chain resonances of purified natural abundance p300-CH1 in the presence of 3 equivalents of Zn^{2+}. 300 μl of purified recombinant p300-CH1 (μM) was filter centrifuged into

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deuterated buffer (10% Deuterium Oxide (v/v) with 50 mM Tris-d_{11}, 150 mM NaCl, and 5 mM DTT-d_{10}, see Recipes) and injected into Varian INOVA 600MHz NMR spectrometer.

Recipes

1. Acidic resuspension buffer (50 mM Tris-HCl, 150 mM NaCl, pH 6.3)
   Add 6.057 g Tris-HCl and 8.766 g NaCl to 1 L deionized water, and the pH should be < 5
   Raise pH with 1 N NaOH
   Autoclave and store in cold room
2. Alkaline resuspension buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0)
   Add 6.057 g Tris-HCl and 8.766 g NaCl to 1 L deionized water, and the pH should be < 5
   Raise pH with 1 N NaOH
   Autoclave and store in cold room
3. HRV3C PreScission Site Protease buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM TCEP, pH 8.0)
   Add 1.433 g TCEP to 100 ml of alkaline resuspension buffer
4. 10% (v/v) deuterated buffer for NMR (pH 8.0)
   10% Deuterium Oxide
   50 mM Tris-d_{11}
   150 mM NaCl
   5 mM DTT-d_{10}
   Adjust pH to 8.0 with 1 N NaOH
5. LB Miller medium
   Prepare four 2 L Erlenmeyer glass flasks with 25 g powdered LB Miller medium and 1 L deionized water each, sterilize by autoclave, cool to room temperature, and store at 4 °C

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