Published in final edited form as:

Methods Mol Biol. 2012 ; 809: 465–472. doi:10.1007/978-1-61779-376-9_30.

Affinity Purification of MLL3/MLL4 Histone H3K4 Methyltransferase Complex

Young-Wook Cho, SunHwa Hong, and Kai Ge

Abstract

Methylation on histone H3 lysine 4 (H3K4) correlates with actively transcribed genes. In mammalian cells, there exist multiple Set1-like histone H3K4 methyltransferase complexes, which have overlapping but distinct subunit compositions. Developing methods to isolate each of these histone H3K4 methyltransferase complexes would help understand the molecular mechanisms by which histone H3K4 methylation regulates mammalian gene expression. In this chapter, we provide a one-step affinity purification protocol on isolation of the MLL3/MLL4 histone H3K4 methyltransferase complex using FLAG-tagged PA1, a unique subunit of the MLL3/MLL4 complex.

Keywords

Histone H3K4 methyltransferase complex; MLL3; MLL4; PA1

1. Introduction

Histone lysine methylation plays essential roles in chromatin dynamics, transcription, and DNA repair. Histone lysine methylation is dynamically regulated by site-specific methyltransferases and demethylases. In yeast, a single Set1 complex, also known as COMPASS, is responsible for all methylations on histone H3K4 (1–3). In mammalian cells, at least six Set1-like histone methyltransferase (HMT) complexes with robust H3K4 methyltransferase activities have been isolated (4). Each of these complexes contains one SET domain-containing homolog of yeast Set1, such as Set1A (also known as Setd1a, KMT2F) (5, 6), Set1B (also known as Setd1b, KMT2G) (7), MLL1 (mixed-lineage leukemia 1, also known as MLL, HRX, ALL1, KMT2A) (8–10), MLL2 (mixed-lineage leukemia 2, also known as TRX2, MLL4, KMT2B) (10, 11), MLL3 (mixed-lineage leukemia 3, also known as KMT2C), and MLL4 (mixed-lineage leukemia 4, also known as ALR, MLL2, KMT2D) (4, 12–14), which carries the enzymatic activity for the associated complex. Based on the homologies in both protein sequences and domain structures, the six Set1-like HMTs fall into three subgroups, Set1A and Set1B, MLL1 and MLL2, and MLL3 and MLL4. ASH2L, RbBP5, WDR5, and DPY30, which are homologs of yeast Set1/COMPASS complex subunits Bre2, Swd1, Swd3, and Sdc1, respectively, form a 4-subunit subcomplex that is not only shared by all mammalian Set1-like HMT complexes, but also critical for the H3K4 methyltransferase activities of these complexes (4, 8). In addition, each of these complexes contains distinct but overlapping subunits (Fig. 1) (10, 11). For example, WDR82 and CXXC1, which are homologs of the Swd2 and Spp1 subunits of yeast Set1/COMPASS complex, selectively associate with Set1A/B complexes (5). Menin, a protein with no homology with any of the yeast Set1/COMPASS complex components, selectively associates with MLL1 and MLL2 complexes (10, 11).

In cells, PTIP and a novel protein PA1 are both unique subunits of the MLL3/MLL4 histone H3K4 methyltransferase complex that contains the enzymatic subunits MLL3 and MLL4, and the histone H3K27 demethylase UTX (4, 15). Methylation on H3K4 is an activating epigenetic mark while methylation on H3K27 is a repressive one. The finding that H3K4 methyltransferases MLL3/MLL4 physically associate with H3K27 demethylase UTX suggests that by adding an activating epigenetic mark and removing a repressive one, the MLL3/MLL4 complex may use two distinct histone-modifying activities to synergistically activate target gene expression. Recent evidence also suggests that MLL3 and MLL4 may exist in the HMT complex in a mutually exclusive manner (16). Here, we describe the one-step isolation of the MLL3/MLL4 histone H3K4 methyltransferase complex from nuclear extracts prepared from a HeLaS cell line stably expressing FLAG-tagged PA1 (4).

2. Materials

2.1. Generation of Stable Cell Lines Using Retrovirus Vector

All cells are routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

1. Phoenix-Ampho retrovirus packaging cell line (ATCC product # SD 3443) and HeLaS cell line.
2. GenJet™ in vitro DNA transfection reagent (SignaGen Laboratories).
3. Bovine serum (for large-scale cell culture only).
4. Retroviral infection medium (RIM) (DMEM + 10% heat-inactivated FBS).
5. Sterile 0.45-μm syringe filter (Millipore).
6. Polybrene (Sigma H9268).
7. G418 (Invitrogen).
8. 1× PBS.
9. Anti-FLAG M2 antibody (Sigma F3165).

2.2. Isolation of MLL3/MLL4 Complex by Anti-FLAG Antibody Immunoprecipitation

1. Retrovirus plasmid pWZLneo-F-PA1 expressing FLAG-tagged PA1 (available from the authors upon request).
2. HeLaS cells expressing FLAG-tagged PA1 (available from the authors upon request).
3. 15- and 50-ml conical tubes.
4. 200-μl and 1-ml wide-orifice tips (see Note 1).
5. Mouse IgG-agarose (Sigma A0919).
6. M2 agarose (anti-FLAG antibody conjugated to agarose, Sigma A2220).
7. 5 mg/ml FLAG Peptide (Sigma F3290).
8. Buffer A: 20 mM HEPES, pH 7.9, 180 mM KCl, 0.2 mM EGTA, 1.5 mM MgCl₂, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P-40.

¹Wide-orifice tips have a larger opening to allow the pipetting of viscous solutions. Wide-orifice tips can be homemade by simply cutting off the end of regulator tips with a razor blade. Use wide-orifice tips to pipette antibody-conjugated agarose.

9. Elution buffer: 20 mM HEPES, pH 7.9, 180 mM KCl, 0.2 mM EGTA, 1.5 mM MgCl₂.
10. Ultrafree-MC centrifugal filter units with microporous membrane 0.45 μm (Millipore).
11. Vivaspin 500 centrifugal filter units, MWCF 10 kDa (Fisher NC9924359).
12. 4–15% SDS-PAGE gel (Bio-Rad).
13. Protease inhibitors: Aprotinin, leupeptin, and pepstatin (Roche).
14. Dithiothreitol (DTT, Bio-Rad, stock solution 1 M).
15. PMSF (Sigma, stock solution 200 mM).

3. Methods

PTIP and PA1 both associate with the MLL3/MLL4 histone H3K4 methyltransferase complex (4). However, ectopically expressed FLAG-tagged PTIP associates not only with the MLL3/MLL4 complex, but also with proteins involved in DNA damage response and repair. In contrast, FLAG-tagged PA1 selectively associates with the MLL3/MLL4 complex (4). Using retrovirus-mediated gene transfer, a HeLaS cell line stably expressing FLAG-tagged, full-length, human PA1 (F-PA1) is established. The MLL3/MLL4 complex can be purified in one step using anti-FLAG antibody immunoprecipitation from nuclear extracts prepared from this cell line.

3.1. Generation of Stable Cell Lines Using Retrovirus Vector

Day 1:

1. In the afternoon, plate 1×10^6 Phoenix-Ampho amphotropic retrovirus packaging cells in 4 ml of culture medium in a 6-cm dish. Incubate cells in a 37°C incubator for 24 h.

Day 2:

2. In the afternoon, dilute 2.5 μg pWZLneo-F-PA1 plasmid in 100 μl of plain DMEM without serum and antibiotics. Dilute 7.5 μl of GenJet™ transfection reagent in 100 μl of plain DMEM. Mix the two solutions and incubate at room temperature for 15 min. Add the 200 μl of mixture to the dish of cells from step 1. Incubate cells in a 37°C incubator for 2 days.

Day 4:

3. In the afternoon, replace the medium with 4 ml of RIM. Plate 3.5×10^5 HeLaS cells in another 6-cm dish.

Day 5:

4. In the morning, collect the 4 ml of retrovirus-containing supernatant, filter through 0.45-μm syringe filter, dilute with an equal volume of fresh RIM, and add 8 μg/ml polybrene.
5. Replace the supernatant of the HeLaS cells with 3–4 ml of the diluted virus-containing supernatant. Incubate cells at 37°C for 2 days.

Day 7:

6. Trypsinize the infected HeLaS cells in the 6-cm dish, transfer 1/5, 1/25, 1/125, and 1/625, respectively, into four 15-cm dishes (i.e., 1:35, 1:175, 1:875, and

1:4,375 dilutions). Add 1 mg/ml G418. Every 3 days, change to fresh medium supplemented with 1 mg/ml G418.

Days 21–30:

- 7 2 weeks since the splitting of the infected HeLaS cells, pick 24 well-isolated single colonies using 200- μ l pipette tips (see Note 2). Transfer each colony into one well of a 24-well plate filled with 1 ml of culture medium containing 0.5 mg/ml G418. Pipette up and down with 1-ml tips to disperse the cells.
- 8 Choose 12–18 fast-growing colonies in the 24-well plate. When cells reach confluency, trypsinize and split cells in one well of the 24-well plate into two wells of 6-well plates (one for freezing down and the other for western blotting). Add 0.5 mg/ml G418.
- 9 After cells reach confluency in 6-well plates, trypsinize cells from one well and freeze down in -80°C freezer (see Note 3). Collect cells from the second well and prepare whole cell extracts for western blot analysis using anti-FLAG M2 antibody. The extracts from the parental HeLaS cells serve as the negative control in western blot. PA1 runs at ~ 42 kDa on SDS-PAGE gels.

3.2. Isolation of MLL3/MLL4 Complex by Anti-FLAG Antibody Immunoprecipitation

After identifying HeLaS cell lines expressing FLAG-tagged PA1 (HeLaS/F-PA1) by western blot, the HeLaS/F-PA1 cells are cultured till confluence in 100×15 -cm dishes in DMEM containing 8% bovine serum, 2% FBS, and 0.1 mg/ml G418. Cells are collected, and nuclear extracts (N.E.) are prepared exactly as described (17).

Next, we describe one-step purification of the MLL3/MLL4 complex by immunoprecipitation with anti-FLAG antibody conjugated to agarose (M2 agarose) from N.E. prepared from HeLaS/F-PA1 cells. All steps described below are performed in cold room or on ice. All buffers are freshly supplemented with 1 mM DTT and protease inhibitors 0.5 mM PMSF, 1 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 0.7 μ g/ml pepstatin.

1. Dilute 100 mg of N.E. protein in buffer A to get a final protein concentration of 2–3 mg/ml. Centrifuge at $25,000 \times g$ for 30 min at 4°C to remove denatured proteins and cell debris. Transfer the supernatant to a 50-ml conical tube.
2. To remove proteins that nonspecifically bind to M2 agarose from the diluted N.E., the supernatant from step 1 is pre-cleared by adding 0.2 ml of mouse IgG–agarose that has been washed twice with 1 ml of buffer A. Rotate the tube for 2 h.
3. Centrifuge at $1,500 \times g$ for 5 min in a swinging bucket rotor, and transfer the supernatant into a new 50-ml conical tube (see Note 4).
4. Repeat steps 2 and 3 once.
5. While doing steps 2–4, equilibrate 0.2 ml of anti-FLAG M2 agarose by washing three times with 1 ml of buffer A for 5 min.
6. Incubate precleared N.E. from step 4 with 0.2 ml of anti-FLAG M2 agarose for overnight immunoprecipitation. Rotate the tube in the cold room.

²To pick single HeLaS cell colonies, first identify well-isolated single colonies under the microscope and draw circles around the single colonies by labeling at the bottom of the 15-cm dishes. Prepare a 24-well cell culture plate filled with 1 ml of culture medium per well. Remove the majority of the culture medium from the 15-cm dishes. In the tissue culture hood, quickly pick single HeLaS cell colonies using 200- μ l pipette tips in a way similar to picking bacteria colonies from agar plates.

³One day before freezing down cells, remove G418-containing medium and change to fresh culture medium without G418.

⁴After centrifugation, the pellet is loose in 50-ml conical tubes. Do not disturb the pellet when transferring the supernatant.

7. The next morning, centrifuge the tube at $1,500 \times g$ for 10 min in a swinging bucket rotor. Transfer the supernatant to a new tube. Keep the agarose beads.
8. Wash the agarose beads by adding 40 ml of buffer A, rotate for 30 min, spin the tube at $1,500 \times g$ for 5 min, and remove the supernatant.
9. Add 10 ml of buffer A to the agarose beads, resuspend, and transfer to a 15-ml conical tube. Rotate for 5 min. Centrifuge the tube at $1,500 \times g$ for 2 min and remove the supernatant. Repeat the wash three more times.
10. Add 1 ml of buffer A to the agarose beads, resuspend, and transfer to a 1.5-ml tube. Rotate for 2 min. Spin at $1,500 \times g$ in a microcentrifuge for 2 min and remove the supernatant. Repeat the wash three more times. After the final wash, spin down the agarose beads and completely remove the supernatant with a 27-G needle.
11. To elute proteins bound to the M2 agarose, dilute 5 mg/ml FLAG peptide solution 20-fold in 1 ml of elution buffer to get a final concentration of 0.25 mg/ml.
12. Elution: Add 0.5 ml of diluted FLAG peptide to the agarose beads after the final wash at step 10. Rotate for 30 min, spin at $1,500 \times g$ in a microcentrifuge for 2 min, and transfer the supernatant to a new 1.5-ml tube. Add 0.5 ml of diluted FLAG peptide to repeat the elution once.
13. Combine the first and second eluates and filter through an Ultrafree-MC centrifugal filter unit by a quick spin to completely remove agarose beads.
14. Concentrate with a Vivaspın 500 (10-kDa cutoff) by centrifuging at $15,000 \times g$ in a microcentrifuge for 4×5 min until the volume is less than 100 μ l.
15. Run the concentrated sample on a 4–15% SDS-PAGE gel and analyze by western blot or mass spectrometry. Alternatively, the purified MLL3/MLL4 complex is subjected to HMT assays.

References

1. Roguev A, Schaft D, Shevchenko A, Pijnappel WW, Wilm M, Aasland R, Stewart AF. The *Saccharomyces cerevisiae* Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. *EMBO J*. 2001; 20:7137–48. [PubMed: 11742990]
2. Briggs SD, Bryk M, Strahl BD, Cheung WL, Davie JK, Dent SY, Winston F, Allis CD. Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. *Genes Dev*. 2001; 15:3286–95. [PubMed: 11751634]
3. Miller T, Krogan NJ, Dover J, Erdjument-Bromage H, Tempst P, Johnston M, Greenblatt JF, Shilatifard A. COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. *Proc Natl Acad Sci USA*. 2001; 98:12902–7. [PubMed: 11687631]
4. Cho Y-W, Hong T, Hong S, Guo H, Yu H, Kim D, Guszczynski T, Dressler GR, Copeland TD, Kalkum M, Ge K. PTIP Associates with MLL3- and MLL4-containing Histone H3 Lysine 4 Methyltransferase Complex. *J. Biol. Chem*. 2007; 282:20395–20406. [PubMed: 17500065]
5. Lee JH, Skalnik DG. CpG-binding protein (CXXC finger protein 1) is a component of the mammalian Set1 histone H3-Lys4 methyltransferase complex, the analogue of the yeast Set1/COMPASS complex. *J Biol Chem*. 2005; 280:41725–31. [PubMed: 16253997]
6. Wysocka J, Myers MP, Laherty CD, Eisenman RN, Herr W. Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. *Genes Dev*. 2003; 17:896–911. [PubMed: 12670868]
7. Lee JH, Tate CM, You JS, Skalnik DG. Identification and characterization of the human Set1B histone H3-Lys4 methyltransferase complex. *J Biol Chem*. 2007; 282:13419–28. [PubMed: 17355966]

8. Dou Y, Milne TA, Ruthenburg AJ, Lee S, Lee JW, Verdine GL, Allis CD, Roeder RG. Regulation of MLL1 H3K4 methyltransferase activity by its core components. *Nat Struct Mol Biol.* 2006; 13:713–9. [PubMed: 16878130]
9. Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD, Hess JL. MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell.* 2002; 10:1107–17. [PubMed: 12453418]
10. Yokoyama A, Wang Z, Wysocka J, Sanyal M, Aufiero DJ, Kitabayashi I, Herr W, Cleary ML. Leukemia proto-onco-protein MLL forms a SET1-like histone methyltransferase complex with menin to regulate Hox gene expression. *Mol Cell Biol.* 2004; 24:5639–49. [PubMed: 15199122]
11. Hughes CM, Rozenblatt-Rosen O, Milne TA, Copeland TD, Levine SS, Lee JC, Hayes DN, Shanmugam KS, Bhattacharjee A, Biondi CA, Kay GF, Hayward NK, Hess JL, Meyerson M. Menin associates with a trithorax family histone methyltransferase complex and with the hoxc8 locus. *Mol Cell.* 2004; 13:587–97. [PubMed: 14992727]
12. Issaeva I, Zonis Y, Rozovskaia T, Orlovsky K, Croce CM, Nakamura T, Mazo A, Eisenbach L, Canaani E. Knockdown of ALR (MLL2) reveals ALR target genes and leads to alterations in cell adhesion and growth. *Mol Cell Biol.* 2007; 27:1889–903. [PubMed: 17178841]
13. Patel SR, Kim D, Levitan I, Dressler GR. The BRCT-Domain Containing Protein PTIP Links PAX2 to a Histone H3, Lysine 4 Methyltransferase Complex. *Developmental Cell.* 2007; 13:580. [PubMed: 17925232]
14. Goo YH, Sohn YC, Kim DH, Kim SW, Kang MJ, Jung DJ, Kwak E, Barlev NA, Berger SL, Chow VT, Roeder RG, Azorsa DO, Meltzer PS, Suh PG, Song EJ, Lee KJ, Lee YC, Lee JW. Activating signal cointegrator 2 belongs to a novel steady-state complex that contains a subset of trithorax group proteins. *Mol Cell Biol.* 2003; 23:140–9. [PubMed: 12482968]
15. Hong S, Cho Y-W, Yu L-R, Yu H, Veenstra TD, Ge K. Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. *Proc Natl Acad Sci USA.* 2007; 104:18439–18444. [PubMed: 18003914]
16. Lee S, Lee D-K, Dou Y, Lee J, Lee B, Kwak E, Kong Y-Y, Lee S-K, Roeder RG, Lee JW. Coactivator as a target gene specificity determinant for histone H3 lysine 4 methyltransferases. *Proc Natl Acad Sci U S A.* 2006; 103:15392–15397. [PubMed: 17021013]
17. Abmayr SM, Yao T, Parmely T, Workman JL. Preparation of nuclear and cytoplasmic extracts from mammalian cells. *Curr Protoc Mol Biol.* 2006 Chapter 12 , Unit 12 1.

Yeast Set1 Complex	Human Set1-like Complexes			
	Set1A/B Complex	MLL1/MLL2 Complex	MLL3/MLL4 Complex	
Set1	Set1A / B	MLL1 / MLL2	MLL3 / MLL4	Enzymatic subunit
Bre2	ASH2L	ASH2L	ASH2L	Common subunits form a subcomplex
Swd1	RbBP5	RbBP5	RbBP5	
Swd3	WDR5	WDR5	WDR5	
Sdc1	DPY30	DPY30	DPY30	
Swd2	WDR82	-	-	Distinct subunits
Spp1	CXXC1	-	-	
		Menin	PTIP & PA1	
		HCF1	NCOA6	
			UTX	

Fig. 1.
Subunit compositions of the yeast Set1 and human Set1-like histone H3K4 methyltransferase complexes.