Purification of Histone Methyltransferases from HeLa Cells

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Although discovered about 40 years ago, histone methylation has remained one of the least understood forms of posttranslational modifications until recently.\(^1,2\) Methylation of histones occurs on both lysine and arginine residues on histone H3 and H4. The known methylation sites include lysines 4, 9, 27, 36, 79, and arginines 17, 26 on histone H3, and lysine 20, and arginine 3 on histone H4. While arginine methylation is catalyzed by members of the protein arginine methyltransferase (PRMT) family, the majority of methylation on lysine residues is catalyzed by proteins containing SET domain with the exception of Dot1.\(^1,3\) Identification and characterization of these histone methyltransferases (HMTases) that target different lysine or arginine residues for methylation is an important step towards understanding the function of histone methylation as well as their combinations with other histone modifications, which is believed to form a “histone code.”\(^4,5\) Dissecting the “histone code” will reveal fundamental regulatory mechanisms in most, if not all, chromatin-templated processes.

Here, we describe several conventional chromatographic strategies aimed at purifying HMTases from HeLa cells. We also describe detailed protocols for preparing histone substrates and for detecting HMTase activities.

Preparation of Histone Substrates and Assay for HMTase Activities

Both core histone octamers and nucleosomes purified from micrococcal nuclease (MNase) digested HeLa cell nuclei can be used for HMTase assays. Because of the variation of concentration and quality of different batches of nuclei or micrococcal nuclease, test digestion is strongly recommended. The following protocol is adapted from a published procedure\(^6\) and can be used for preparation of native oligonucleosomes, mononucleosomes, and histone octamers substrates from HeLa cells for HMTase assays.

Prepare and Digest Nuclei

About 20 l of cultured HeLa cells (5×10⁵ cell/ml) are collected and washed once with PBS. The cell pellet is then resuspended with a type B pestle of a Dounce homogenizer in 100–150 ml buffer N1 (10 mM MES, 0.5 mM sodium metabisulphite, 0.5 mM benzamidine–HCl, 5 mM MgCl₂, 1 mM CaCl₂, 15 mM NaCl, 60 mM KCl, 0.25 M sucrose, 0.5% Triton X-100, 0.1 mM PMSF, 0.5 mM DTT, pH 6.5) to generate nuclei. After washing once with buffer N1, nuclei are collected by spinning at 4000 rpm (Sorvall RTH-750) for 10 min and resuspended in 12 ml buffer N2 (10 mM PIPES, 0.5 mM sodium metabisulphite, 0.5 mM benzamidine–HCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.1 mM PMSF, 0.5 mM DTT, pH6.5). To obtain optimal MNase digestion conditions, 500 μl nuclei suspension is taken out and warmed up to 37°C before adding CaCl₂ to 5 mM final concentration and 5 μl MNase stock (10 U/μl, Worthington Biochemical, Freehold, NJ). Every 5 min for up to 45 min, 50 μl of digested nuclei is transferred to an Eppendorf tube containing 1 μl 0.5 M EDTA to stop the reaction. Add to each tube in order 75 μl H₂O, 30 μl 10% SDS, 25 μl 4 M NaCl, and finally 200 μl phenol–chloroform, vortexing at each step. After centrifugation, 5 μl of the aqueous phases derived from each time point are loaded into a 1% agarose gel to examine the digestion efficiency. Appropriate digestion will yield the majority of products around 2 kb. The bulk of nuclei are then digested using the optimized conditions and stopped by adding 0.5 M EDTA to final concentration of 4 mM. Digested nuclei are centrifuged at 5000 rpm in a Sorvall SS-34 rotor for 5 min to generate the supernatant S1. Pellet is then resuspended in one pellet volume of 1 mM EDTA with 0.2 mM PMSF. The suspension is vortexed and incubated on ice for 15 min before centrifuging at 10,000 rpm (Sorvall SS-34) for 15 min to generate supernatant S2. S1 and S2 are combined and the final salt concentration is brought to 0.75 M by adding 5 M NaCl drop by drop to remove histone H1 (omit this step if H1 is desired). The total concentration of nucleic acid can be determined by measuring A₂₆₀ of 10 μl sample in 1 ml 1 M NaOH.

Sucrose Gradient Purification of Oligonucleosomes

Linear 5–30% sucrose gradients (35 ml) are prepared with buffer NG (10 mM Tris–Cl, 1 mM EDTA, 0.75 M NaCl, 0.3 mM PMSF, pH 7.5) using a binary gradient mixing apparatus (Hoefer SG100, Amersham) and are poured in 40 ml polyallomer tubes (Beckman). About 4–5 ml MNase digested samples are carefully layered on the top of each gradient. Gradients are centrifuged at 26,000 rpm for 16 h using a SW28 rotor (Beckman).
Fractions are then collected manually by siphoning 1 ml fractions from top to bottom of each gradient. Fifty microliters of sample from each fraction is precipitated with 100 µl 25% TCA and the purity of histone proteins is examined by running a 18% SDS-PAGE and viewed by Coomassie staining. The size of nucleosomes in each fraction is examined using the same method as that used in MNase digestion test described earlier. Fractions with good purity of histone and majority of DNA size from 1 to 2.5 kb (7–15 nucleosomes) are pooled and dialyzed against histone storage buffer (10 mM HEPES–KOH, 1 mM EDTA, 10 mM KCl, 10% glycerol, 0.2 mM PMSF, pH 7.5). Purified HeLa nucleosomes are stable for up to 6 months at 4°C and can be stored for longer at −70°C.

Purification of Mononucleosomes

Although top gradient fractions contain some mononucleosomes, we do not recommend them to be used for HMTase activity assay due to impurity. To obtain relatively pure mononucleosomes, fractions containing good purity of histones and majority of DNA size more than 2.5 kb are pooled and dialyzed against Tris–Cl (pH 8.0) buffer with 1 mM PMSF to remove EDTA and sucrose. Materials are concentrated by ultrafiltration (Ultrafree-15, Millipore) and then completely digested with MNase before subjected to sucrose gradient again as discussed earlier.

Purification of Histone Octamers

To purify histone octamers, all other histone containing fractions derived from sucrose gradient are combined and dialyzed to TE buffer with 1 mM PMSF. An appropriate volume hydroxyapatite (RioRad) column is prepared according to the total amount of protein, and equilibrated with buffer NP (40 mM Na2HPO4, 1 mM DTT, 0.2 mM PMSF, pH 6.8) containing 0.3 M NaCl (NP300). The dialyzed samples are loaded onto the column, and the column is washed with 6–10 column volume (cv) buffer NP500. Histone proteins are then eluted with buffer NP2500 and collected at 2 ml per fraction. The purity and the ratio of different core histone proteins are examined by running a 18% SDS-PAGE and viewed by Coomassie staining before fractions are pooled, dialyzed to histone storage buffer and aliquoted.

In addition to cultured HeLa cells, chicken blood is also a good source for preparing substrates for HMTase assay. However, we noticed that the same HMTase has different activity towards histone substrates isolated

from different organisms. In some situations, both chicken and HeLa de-

rived nucleosomes or histones are recommended to be used as substrates
for HMTase assay. Besides native histones and nucleosomes, synthetic
peptides, recombinant histones and reconstituted octamers or nucleosomes
have also been successfully used as substrates for some HMTases although
some HMTases, such as Dot1, prefer native nucleosome substrate.8,9

Histone Methyltransferase Assays

HMTase assays are performed essentially as described.10 Briefly, pro-

tein fractions are incubated with appropriate amount of different sub-

strates (visible in Coomassie) in total of 20–50 µl reactions containing 1/5
volume 5× HMT buffer (100 mM Tris–HCl, 20 mM EDTA, 5 mM PMSF,
2.5 mM DTT, pH 8.0) and 1 µl S-Adenosyl-l-[methyl-3H]methionine
(15 Ci/mM, NEN Life Science Products) for 1 h at 30°C. Reactions are
stopped by addition 1/5 volume of 5× SDS loading buffer (0.25 M Tris–
Cl, 0.5 M DTT, 10% SDS, 0.25% bromphenol blue, 50% glycerol, pH
6.8) and histones are separated on an 18% SDS-PAGE. After Coomassie
staining and destaining, gels are treated with EN3HANCE or ENTENSI-
FY (NEN Life Science Products) for 30–45 min, dried and exposed to
X-ray films (BioMax, Kodak) for an appropriate time. For quantifica-
tion, the gel slices can be excised and counted with liquid scintillation.

Purification of Histone Methyltransferases from Cultured HeLa Cells

Most of HMTases discussed below were purified from HeLa nuclei. The
nuclear proteins of cultured HeLa cells can be divided into nuclear extract
and nuclear pellet fractions based on whether they can be easily extracted
away from bulk of chromatin. Both nuclear extract and nuclear pellet
fractions can be further fractionated on ion-exchange phosphocellulose
P11 (Sigma) or DEAE-52 (Whatman) columns, respectively. Chromatog-
raphy on these two columns is performed conventionally. Chromatography
resins were pretreated following the instruction of the manufacturers
before packing the columns of appropriate size using empty columns from
BioRad.

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Preparation of Starting Materials

HeLa nuclear proteins are separated into nuclear extract (NE) and nuclear pellet (NP) fractions essentially as previously described. All the steps described below are performed in cold room on ice. Briefly, after collection and washing with PBS, cultured HeLa cells are allowed to swell in 5-pellet-volume of buffer A (10 mM Tris–Cl, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, pH 7.9) for 10 min. Cells are then collected by centrifugation and homogenized using a type B pestle in 2 pellet volumes of buffer A. Crude nuclei are pelleted by centrifuging at 2500 rpm (Sorvall RTH-750) for 10 min. Nuclei are resuspended in 3 ml buffer C (20 mM Tris–Cl, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, 25% glycerol, pH 7.9) per 10⁹ cells and homogenized again using a type B pestle. The suspension is then stirred gently using a magnetic stirring bar for 30 min and cleared by centrifuging at 15,000 rpm in a Sorvall SS-34 rotor for 30 min. The supernatant is dialyzed against buffer D (50 mM Tris–Cl, 0.1 mM EDTA, 0.2 mM PMSF, 2 mM DTT, 25% glycerol, pH 7.9) containing 0.1 M KCl and saved as the NE fraction. Nuclear pellet (NP) needs to be solubilized as described before further purification. Briefly, nuclear pellet is resuspended and homogenized in appropriate volume of buffer E (50 mM Tris–Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.2 mM PMSF, 5 mM DTT, 25% glycerol, pH 7.9). After adding 1/10 volume 3 M (NH₄)₂SO₄, the suspension is mixed immediately and DNA should be sheared by sonication until the solution is not viscous anymore. Suspension is then centrifuged at 35,000 rpm in a KAL-40.100 rotor (KOMPSPIN) for 70 min. The supernatant is diluted by addition of 2 volumes of buffer E and cleared of debris again by centrifuging at 40,000 rpm for 1 h. Proteins are precipitated by addition of 0.42 g (NH₄)₂SO₄ per ml supernatant and resuspended in appropriate volume buffer D.

Distribution of HMTase Activities in NE and NP Fractions from HeLa Cells

Proteins in NE fractions (6 g) are further fractionated on a lab-made 700 ml phosphocellulose P11 column equilibrated with buffer D containing 0.1 mM KCl. Proteins bound to the column are step eluted with buffer D containing 0.3, 0.6, and 1.0 M KCl, respectively. Proteins derived from NP fractions (4 g), after ammonium sulfate concentration is adjusted to 20 mM, are loaded onto a lab-made 500 ml DEAE-52 column equilibrated with buffer D containing 20 mM (NH₄)₂SO₄. The bound proteins are step eluted with

0.35 and 0.5 M ammonium sulfate in buffer D. The 0.35 M elution is then dialyzed against buffer D containing 0.1 M KCl and loaded onto a 400 ml phosphocellulose P11 column. Bound proteins are step eluted with buffer D containing 0.3, 0.6, and 1.0 M KCl, respectively. Small aliquots of samples from every P11 fraction are dialyzed against buffer B (40 mM HEPES-KOH, 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 1 μg/ml each of leupeptin, aprotinin, and pepstatin A, 10% glycerol, pH 7.9) containing 50 mM KCl (BC50) and then assayed HMTase activity using both histone octamers and oligonucleosomes as substrates. Results shown in Fig. 1 revealed multiple HMTase activities specific for histones H3 and H4. The fact that different patterns of activities are detected using core histones and nucleosomal histone substrates indicate that HMTases have substrate preferences.

Using the P11 fractions as starting material, by following the HMTase activity, we have purified several HMTases or HMTase complexes. In protocols described below, most chromatographic steps are performed on a ÄKTA fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). In each chromatographic step, every fourth fraction (or portion of a fraction) is dialyzed to BC50 before HMTase activity assay. The same fractions are also analyzed by silver staining of a 6.5–15% linear gradient SDS-PAGE to evaluate the purity of the enzymes.
Purification of Histone H4-specific HMTases

Histone H4 tail can be methylated at arginine 3 (R3) and lysine 20 (K20) by PRMT1\textsuperscript{13,14} and SET8/PR-SET7,\textsuperscript{15,16} respectively. We describe how the two HMTases were purified from HeLa nuclear pellet fractions below.

Purification of Histone H4-R3 Methyltransferase PRMT1

To identify the protein responsible for histone H4 methylation, we focused on the 0.1 M P11 fraction derived from nuclear pellet because of its robust activity towards core histones (see Fig. 1). After dialyzing against buffer D containing 40 mM ammonium sulfate (BD40), the protein samples are loaded onto a high-performance liquid chromatography (HPLC) DEAE-5PW column (TosoHaas, 45 ml) which has been equilibrated with BD40. The bound proteins were eluted with 10 cv linear gradient from BD40 to BD400. Every fourth fraction was dialyzed into BC50 and analyzed for HMTase activity using core histones as substrates. The fractions containing the enzymatic activity (BD120–BD225) were combined and its salt concentration was adjusted to final of 0.5 M ammonium sulfate by addition of saturated \((\text{NH}_4)_2\text{SO}_4\) drop by drop. Samples were then loaded to a 22 ml FPLC Phenyl Sepharose column (Amersham Pharmacia Biotech) and bound proteins were eluted with a 20 cv linear gradient from BD500 to BD0. Fractions between 0.1 and 0.25 M ammonium sulfate were pooled according to the HMTase activity and protein purity analysis. A portion of the pooled material was dialyzed to buffer P (5 mM HEPES-KOH, 40 mM KCl, 0.01% Triton X-100, 0.01 mM CaCl\textsubscript{2}, 0.5 mM PMSF, 1 mM DTT, 2 \(\mu\)g/ml each of leupeptin, aprotinin, and pepstatin A, 10% glycerol, pH 7.5) containing 10 mM K\textsubscript{2}HPO\textsubscript{4}–KH\textsubscript{2}PO\textsubscript{4} (BP10), and loaded onto a 1 ml BP10 equilibrated hydroxyapatite (BioRad) column. Bound proteins were eluted with a 20 cv linear gradient from BP10 to BP600. HMTase activity assay coupled with silver staining of an SDS-PAGE containing the column fractions revealed a single polypeptide of 42 kDa correlates with the HMTase activity (see Fig. 2). Mass spectrometric analysis of the 42 kDa protein identified it to be the human protein arginine


N-methyltransferase 1 (PRMT1). Western blot analysis confirmed that PRMT1 correlated with this H4 HMTase activity in previous columns.

Purification of Histone H4-K20 Methyltransferase SET8

By following the H4-specific HMTase activity, we unexpectedly revealed that H4-R3 can be methylated both in vitro and in vivo. However, our initial goal of purifying an H4-K20 HMTase was not achieved. Since all the P11 fractions capable of methylating free core histone H4 contain PRMT1, which is unable to methylate nucleosomal histone, we used nucleosome substrate to avoid contaminating activity from PRMT1. This analysis revealed 1.0 M P11 fraction from nuclear pellet (see Fig. 1) to be the only fraction capable of methylating nucleosomal H4. Thus, this fraction likely contains the H4-K20 specific methyltransferase(s).

To purify the candidate H4-K20 HMTase(s), the 1.0 M P11 nuclear pellet fraction was fractionated sequentially through four columns (see Fig. 3). The purification was monitored by HMTase assay using oligonucleosome substrates and the purity of proteins was examined by silver staining of 6.5–15% linear gradient SDS-PAGE containing column fractions. The sample was first dialyzed to BD20 and loaded onto a preequilibrated 45 ml HPLC DEAE-5PW column. The bound proteins were eluted with a 12 cv linear gradient from BD20 to BD600. Fractions containing the...
H4-specific HMTase activity were pooled and dialyzed against BP10 before loading to a 5 ml hydroxyapatite (BioRad) column. The bound proteins are eluted with a 12 cv linear gradient from BP10 to BP600. Fractions containing the H4-specific activity were combined and its conductivity was adjusted to BD1000 by adding saturated ammonium sulfate drop by drop with stirring. After cleared of debris by centrifugation, samples were loaded onto a BD1000 preequilibrated 1 ml FPLC Phenyl Sepharose (Amersham Pharmacia Biotech) column. Bound proteins were fractioned with a 20 cv linear gradient from BD1000 to BD0 and the H4 HMTase activity was eluted between 0.75 and 0.56 \( M \) of ammonium sulfate. After dialyzing into BC50, the enzymatic peak fractions were pooled and concentrated by binding to 0.2 ml BC1000. Proteins were then fractionated on a 22 ml Superose 200 (Amersham Pharmacia Biotech) gel-filtration column. Analysis of the fractions derived from the last purification step indicated that the activity eluted with a native size of 50–150 kDa between fractions 50 to 56 (see Fig. 3B). Silver staining of a SDS-PAGE containing the column fractions revealed multiple proteins present in the enzymatically active fractions (see Fig. 3B, top panel). The limited amount of the sample prevented
us from further purification. Since the activity begins with fraction 50 and ends with fraction 56, the candidate protein bands should be present in fraction 50 but absent in fractions 47 and 59. Using these criteria, we identified six protein bands (see Fig. 3B, top panel, indicated by dots and star) that are likely responsible for the enzymatic activity. Mass spectrometry analysis indicated that most masses obtained from the 45 kDa protein, marked by a star (see Fig. 3B, top panel), matched a SET domain-containing protein named PR/SET07 in GenBank (AAF97812). Since all the lysine-specific HMTases identified so far contain a SET domain, the 45 kDa protein is likely responsible for the detected H4-specific HMTase activity.

Because not all masses obtained from the 45 kDa protein can be accounted for by conceptual trypptic digestion of PR/SET07 protein, we obtained and sequenced several human EST clones that are similar or identical to that of the PR/SET07. Conceptual translation of the cDNA from one of the EST clones (BE867579) generated an ORF (open reading frame) of 352 amino acids which contains all the peptides derived from the 45 kDa protein. Thus, we conclude that the 45 kDa protein, named SET8, is distinct from but highly similar to PR/SET07 (84% identical). In an independent study, the PR/SET07 was purified and demonstrated to be an H4-K20-specific HMTase.16

Purification of Histone H3 HMTases

Comparing with histone H4, H3 harbors more sites that can be methylated. The documented sites including lysines 4, 9, 27, 36, 79, and arginines 17 and 26.17 At least one protein responsible for methylation at each of these sites has been identified. Below, we describe procedures used in our lab that lead to the discovery of three different HMTases that target lysine 4, 9, and 27 for methylation, respectively.10,18

**Purification of the Histone H3-K4 Methyltransferase SET7/9**

While pursuing the histone H4-specific methyltransferases described earlier, we also monitored the purification of HMTase activity specific for histone H3. About 7 g of proteins derived from nuclear pellet were dialyzed against buffer D containing 20 mM ammonium sulfate and loaded onto a 900 ml BD20 equilibrated DEAE52 column. Bound proteins were step eluted with 0.1 and 0.6 M ammonium sulfate in buffer D. The salt

concentration of the 0.1 M elution was adjusted to 0.5 M using saturated ammonium sulfate, and loaded onto a 22 ml FPLC Phenyl Sepharose column. Bound proteins were eluted with a 15 cv linear gradient from BD500 to BD0. Fractions containing HMTase activity were combined and dialyzed to BD50 before loading onto a 45 ml DEAE-5PW column. Bound proteins were eluted with a 10 cv linear gradient from BD50 to BD500. Analysis of the fractions derived from this column identified three peaks of HMTase activity towards H3. The third peak was further purified on a 1 ml BP10 equilibrated hydroxyapatite column with a 20 cv linear gradient from BP10 to BP600. The active fractions, eluted between BP30 and BP50, were pooled and concentrated with 0.2 ml DEAE-52 resin, and fractionated on a Sepharose 200 gel-filtration column. HMTase assay coupled with silver staining of a SDS-PAGE containing the column fractions identified that a polypeptide of 45 kDa correlates with the enzymatic activity (see Fig. 4). Mass spectrometry analysis revealed that the protein matched a SET-domain containing protein (KIAA1717) of unknown function in GenBank. Given that this protein does not share any sequence homology with any of the six SET-domain containing proteins in *S. cerevisiae* outside the SET domain, we have named the protein SET7. This same protein has also been independently purified with similar approach and has been named SET9.

Fig. 4. Purification of the H3-K4-specific HMTase SET7. (A) Schematic representation of the purification steps. Numbers represent the salt concentrations (mM) at which the HMTase activity eluted from the column. Three HMTase activity peaks on the DEAE-5PW column are shown in the middle. (B) Analysis of fractions derived from the Superose 200 gel filtration column by silver staining (top panel), HMTase activity assay (middle panel), and Western blotting (bottom panel). The elution profile of the protein markers is indicated on the top of the panel and the protein size marker on SDS-PAGE is indicated to the left of the panel.

Purification of the Histone H3-K9 Methyltransferase SETDB1 Complex

Thus far, at least four proteins have been reported to be capable of methylate histone H3 at lysine 9. These proteins include the Suv39h1,\textsuperscript{20} G9a,\textsuperscript{21} ESET and its human homolog SETDB1,\textsuperscript{22,23} and Eu-HMTase,\textsuperscript{24} a protein similar to G9a. With the exception of Eu-HMTase, information on the native protein complexes of these proteins are currently not available. We describe the purification of the SETDB1 complex from HeLa cells below.\textsuperscript{25}

To purify the native SETDB1 complex, we monitored the purification process with both Western blot and HMTase assays. Western blot analysis revealed that the majority of SETDB1 is in the 0.1 M P11 fraction derived from nuclear extract, which contains strong HMTase activity towards core histone octamer substrates (see Fig. 1). Therefore, the proteins in this fraction were loaded onto a 300 ml DEAE-52 column and step eluted with BC150, BC200, BC350, and BC500, respectively. The SETDB1-containing BC350 fraction was loaded onto a 22 ml Phenyl Sepharose column after its salt concentration was adjusted to 0.5 M with saturated ammonium sulfate. The bound proteins were eluted with a 20 cv linear gradient from BD500 to BD0. SETDB1 containing fractions were combined, dialyzed to BD50, and loaded onto a 45 ml DEAE-5PW column equilibrated with BD50. Bound proteins were eluted with a 5 cv linear gradient from BD50 to BD500. The SETDB1 containing fractions were pooled and concentrated with 200 μl DEAE52 resin and fractionated on a 22 ml Superose 6 (Amerham Pharmacia Biotech) gel-filtration column. Western blot and HMTase assays of the column fractions revealed that SETDB1 coelutes with the enzymatic activity as a complex of 670–2000 kDa. Further purification of the active fractions on a 1 ml BC150 equilibrated Mono Q (Amerham Pharmacia Biotech) column revealed that two protein bands coelute with the HMTase activity (see Fig. 5). Mass spectrometry analysis identified the proteins as SETDB1 and the human homolog of mouse protein mAm.\textsuperscript{26}

Purification of the Histone H3-K27 Methyltransferase EED-EZH2 Complex

As described earlier, the HMTase activity in the 0.5 M P11 nuclear pellet fraction split into three peaks on DEAE-5PW column when core histones were used as substrates. However, only two peaks were detected.

when nucleosomal histone was used as substrates. The second peak fractions, eluted between 0.22 and 0.25 mM ammonium sulfate, were combined and salt concentration adjusted to BD700 using saturated ammonium sulfate column. The bound proteins were eluted with a 10 cv linear gradient from BD700 to BD0. Active fractions were pooled and dialyzed to BP10 before loading to a 1 ml hydroxyapatite column and eluted with 20 cv linear gradient from BP10 to BP600. The enzymatic peak fractions that were eluted between BP80 and BP130 were pooled and concentrated by ammonium sulfate precipitation, and further fractionated on a 22 ml Superose-6 gel-filtration column. HMTase assay coupled with silver staining of an SDS-PAGE containing the column fraction revealed that six proteins coelute with the HMTase activity between 440 and 670 kDa (see Fig. 6B). Coimmunoprecipitation confirmed that five of the six proteins exist as a protein complex (see Fig. 6C). Mass spectrometry identified these proteins as EZH2, SUZ12, AEBP2, EED, and RbAp48. Using a slightly different purification scheme, a similar complex containing EZH2, SUZ12, EED, and RbAp48 was also purified from HeLa cells. In addition, equivalent protein complexes were also purified from Drosophila embryo extracts.

Fig. 5. Purification of the H3-K9-specific HMTase SETDB1 complex. (A) Schematic representation of the purification steps. Numbers represent the salt concentrations (mM) at which the HMTase activity eluted from the column. (B) Analysis of fractions derived from the Mono Q column by silver staining (top panel), HMTase activity assay (middle panel), and Western blotting (bottom panel). SETDB1 is indicated by a "*" and its associated protein is indicated by a "**".

Figure 6. Purification of an H3-K27-specific HMTase complex. (A) Schematic representation of the purification steps. Numbers represent the salt concentrations (mM) at which the HMTase activity eluted from the column. Two HMTase activity peaks on the DEAE-5PW column are shown in the middle. (B) Analysis of fractions derived from the Superose 6 gel-filtration column by silver staining (top panel), HMTase activity assay (second panel), and Western blotting (bottom two panels). Each of the five copurifying proteins is indicated by a “*”. The elution profile of the protein markers is indicated on the top of the panel and the protein size marker on SDS-PAGE is indicated to the left of the panel. (C) Silver stained SDS-polyacrylamide gel demonstrating coimmunoprecipitation of the five components using anti-SUZ12 antibody. “In” and “Fl” represent input and flow-through, respectively. The protein size marker on SDS-PAGE is indicated to the left of the panel.

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