Multiple enzymes and enzymatic complexes coordinately regulate the addition and removal of post-translational modifications on histone proteins. The oncoprotein Ash2L is a component of the mixed lineage leukemia (MLL) family members 1–4, Setd1A, and Setd1B mammalian histone H3K4 methyltransferase complexes and is essential to maintain global trimethylation of histone H3K4. However, regulation of these complexes at the level of expression and activity remains poorly understood. In this report, we demonstrate that Ash2L is methylated on arginine residues both in vitro and in cells. We found that both protein-arginine methyltransferases 1 and 5 methylate Arg-296 within Ash2L. These findings are the first to demonstrate that post-translational modifications occur on the Ash2L protein and provide a novel example of cross-talk between chromatin-modifying enzyme complexes.

Changes in gene expression are brought about in part by dynamic regulation of chromatin structure and govern numerous cellular processes, including differentiation. In this manner, transcription of the developmental Hox gene cluster is temporally and spatially regulated by the opposing action of chromatin modifiers belonging to the Trithorax (Trx) and Polycomb group (PcG) families (1, 2). Several Trx and PcG genes encode lysine methyltransferases that contain a suppressor of variegation, enhancer of zeste, and Trithorax (SET) domain and target histone proteins to activate or repress transcription, respectively. Genetic alterations of several Trx and PcG genes, including members of the mixed lineage leukemia (MLL) family, have been linked to oncogenesis in mammals and are especially prevalent in hematological malignancies (3–8).

MLL, Setd1A, and Setd1B methyltransferase complexes catalyze mono-, di-, and trimethylation of histone H3K4 (9–15). The absent, small, homeotic discs 2-like (Ash2L) protein functions at the molecular level along with WDR5 and RbBP5 to form a submodule of the Setd1A, Setd1B, and MLL methyltransferase complexes. Interestingly, the Ash2L, RbBP5, WDR5, DPY30 submodule was shown to possess an intrinsic methyltransferase activity toward histone H3K4 in vitro that is independent of MLL, although none of these proteins contain a catalytic SET domain (16). Furthermore, deletion of Ash2L in cell lines leads to decreased global levels of histone H3K4me3, whereas global levels of histone H3K4 methylation are unchanged in the absence of MLL (9, 17, 18). Likewise, MLL complexes reconstituted in vitro that lack Ash2L exhibit reduced di- and trimethyltransferase activity toward recombinant histone H3 (16, 17, 19). These results demonstrate that Ash2L is necessary to maintain histone H3K4 methylation levels in vivo, although the molecular mechanism whereby Ash2L promotes lysine di- and trimethylation remains unclear.

Ash2 is a member of the Trx family and was identified in a screen for Drosophila mutants exhibiting imaginal disc abnormalities and late larval/early pupal lethality (20). Ash2L is ubiquitously expressed in humans with the highest level of expression detected in the fetal liver and testis (21, 22). Mice lacking Ash2L die prior to embryonic implantation (23), emphasizing the importance of appropriate Ash2L expression during mammalian development. It is unclear whether the critical role for Ash2L during embryonic development is linked to maintenance of histone lysine methylation patterns.

Histones and other cellular proteins are also methylated on arginine residues. Arginine methylation influences protein nuclear localization and intermolecular interactions, thereby affecting various cellular processes, including mRNA splicing and gene transcription. Currently, eight genes have been identified that encode active protein-arginine methyltransferases (PRMTs) in mammals (PRMTs 1, 2, 3, 4, 5, 6, 7, and 8) (24, 25). These enzymes mediate mono- and dimethylation of arginine residues and are classified as Type I (PRMTs 1, 2, 3, 4, 5, 6, 7, and 8) or Type II (PRMT 5) enzymes based on their ability to cata-

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2 The abbreviations used are: Trx, Trithorax; H3K4me3, histone H3 lysine 4 trimethylation; SET, suppressor or variegation, enhancer of zeste, and Trithorax; AdoMet, S-adenosyl-L-methionine; OHT, 4-hydroxytamoxifen; MLL, mixed lineage leukemia; PcG, Polycomb group; Ash2L, absent, small, homeotic discs 2-like; PRMT, protein-arginine methyltransferase; MEF, murine embryonic fibroblast; EEN, extra eleven nineteen; CREB, cAMP-response element-binding protein.
lyze asymmetric (asymmetric \(\omega-N^2,N^6\)-dimethylarginine) or symmetric (symmetric \(\omega-N^2,N^6\)-dimethylarginine) dimethylation, respectively (24, 25). The characterization of PRMT7 remains controversial (26, 27).

PRMT1 catalyzes ~85% of all asymmetric dimethylation events that occur on arginine residues in mammalian cells (28). Therefore, it is not surprising that PRMT1 is required for mammalian development and survival as PRMT1\(^{-/-}\) mice die around embryonic day 6.5 (29). Furthermore, murine embryonic fibroblasts (MEFs) lacking PRMT1 display genomic instability and fail to divide, indicating that PRMT1 is required for cell proliferation and survival (30). PRMT1 has been functionally linked to MLL-mediated transformation by cooperating with the leukemogenic MLL fusion protein MLL-EEN to promote self-renewal and the colony-forming ability of primary hematopoietic progenitors (3).

Cross-regulation of histone modifications is an intense area of investigation (31), although little is known regarding the mechanisms of genomic targeting or the interplay of catalytic activities between various histone-modifying enzymes or complexes. Biochemical studies have been used to dissect the mechanisms regulating mono-, di-, and trimethylation of lysine residues by the MLL methyltransferase, but these have relied on in vitro reactions using recombinant proteins expressed and purified from either insect cells or *Escherichia coli* (16, 17, 19), which may lack post-translational modifications. Within this study, we investigated whether Ash2L is a target for post-translational modifications. Here, we demonstrate that Ash2L is methylated in vitro and in cells. PRMT1 asymmetrically dimethylates Ash2L at Arg-296 in vitro and in cells, and PRMT5 also methylates this site in vitro. In determining that Ash2L is a substrate for PRMT activity, we highlight the potential for a novel example of cross-talk between PRMTs and lysine methyltransferase complexes.

**EXPERIMENTAL PROCEDURES**

**Generation of Plasmids and Cell Lines**—A human Ash2L/pcDNA3 expression construct was purchased from AddGene. Oligonucleotide primers with restriction enzyme linkers were designed to amplify various Ash2L deletion mutants from the human cDNA construct (supplemental Table S1). PCR amplification was carried out with mutants from the human cDNA construct (supplemental Table S1) to generate Ash2L proteins containing an sequence at BamHI and XhoI within the multiple cloning site of the pcDNA3 vector to generate Ash2L proteins containing an MYC epitope tag and HindIII and BamHI restriction enzyme linkers (supplemental Table S1) for expression of glutathione-S-transferase complexes.

Single strand oligonucleotides encoding the c-MYC epitope tag and HindIII and BamHI restriction enzyme linkers were annealed, digested, and ligated to the multiple cloning site of the pEGFP-N1 vector (Clontech) at XhoI and EcoRI within the multiple cloning site for expression of GFP fusion proteins.

**PRMT-mediated Methylation of Ash2L**

A 2-kb fragment corresponding to nucleotides 10134–11930 of the human MLL mRNA sequence (GenBank™ accession NM_005933) was isolated by KpnI digestion of the MLL/pCXN2 plasmid and then ligated to the pKSII plasmid to serve as a PCR template. Oligonucleotide primers with XhoI and BamHI restriction enzyme linkers (supplemental Table S1) were used to amplify the DNA fragment encoding MLL amino acids 3793–3969. The PCR product was digested and ligated to the pcDNA3.1 vector containing an HA epitope sequence.

The RbBP5 and WDR5 expression constructs were generously provided by Dr. David Skalnik (Indiana University, Indianapolis, IN), and Dr. Jay Hess (University of Michigan, Ann Arbor, MI) kindly provided the MLL/pCXN2 plasmid. All plasmids were verified by DNA sequencing provided by the DNA Analysis Core Facility at the University of Texas M. D. Anderson Cancer Center.

**Cell Culture**—Human embryonic kidney (HEK-293T and HEK-293a), human cervical carcinoma (HeLa), and MEF cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% l-glutamine. The human acute promyelocytic leukemia (HL-60) cell line was cultured in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% l-glutamine. HEK-293T cells stably expressing FLAG-Ash2L were generously provided by Dr. Danny Reinberg (New York University, New York, NY) (32), and PRMT1 conditional MEF cells were kindly provided by Dr. Stéphane Richard (McGill University, Montreal, Quebec, Canada) (30).

**shRNA Knockdown**—Ash2L stable knockdown cell lines were generated using pGIPZ or pLKO.1 lentiviral vectors purchased from Open Biosystems. HEK-293T cells were co-transfected with pGIPZ or pLKO.1 shRNA expression vectors targeting human Ash2L, PRMT1, PRMT3, PRMT5, or scrambled control with the viral envelope and packaging plasmids pMD2.G and psPAX2. Viral supernatants were collected 48 h post-transfection and added to HeLa cells in the presence of 8 μg/ml Polybrene (Sigma) for transduction. Clones stably expressing the shRNAs of interest were selected with 5 μg/ml puromycin for at least 72 h and were used for subsequent biochemical analysis.

**Recombinant Protein Expression and Purification**—BL21 DE3 competent *E. coli* cells were transformed with various Ash2L/pGEX4T-1 expression constructs. Cultures were grown to log phase and then treated with 0.4 mM isopropl-1-thio-β-D-galactopyranoside for 4 h at 23 °C to induce protein expression. Cells were resuspended in 1× PBS containing 1% Triton X-100, 0.1% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and then lysed by sonication. The extracts were cleared by centrifugation at 10,000 rpm for 30 min at 4 °C. The clarified lysate was incubated with glutathione-agarose (Amersham Biosciences) for at least 1 h at 4 °C. The beads were washed four times with lysis buffer and transferred to a chromatography column, and bound proteins were eluted with elution buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% β-mercaptoethanol, 10 mM glutathione).
In Vitro Methyltransferase Reactions—Recombinant proteins were incubated with 1.5 μCi of [S-3H]adenosyl-L-methionine ([3H]AdoMet) (PerkinElmer Life Sciences, NET155; 10 Ci/mmol) in methyltransferase reaction buffer (25 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 8% glycerol) for 1 h at 30 °C as described previously (33). Reactions were stopped by the addition of 4× protein sample buffer, and the products were resolved on 4–12% NuPAGE gels (Invitrogen). The gels were stained with Coomassie Blue, treated with En3Hance solution (Eldex Laboratories, Napa, CA) with an additional 200 μCi of purified recombinant Ash2L proteins were used as substrates for the reactions, and 5–10 μg of purified recombinant PRMT enzymes were used as the enzyme source. Alternatively, FLAG-immunoprecipitated material prepared from FLAG-Ash2L stable HEK-293T cells or 50 μg of nuclear extract prepared from HEK-293T or HL-60 cells were used as the source of methyltransferase activity.

For amino acid analysis, Coomassie-stained gel slices corresponding to the GST-Ash2L(1–322) and GST-Ash2L(314–628) bands were acid-hydrolyzed with 6 N HCl (w/v). Briefly, the dried gel slices were weighed and placed inside a 6×10.5-cm column f6N PBS; rinsed with ice-cold methanol; and then permeabilized with methanol at −20 °C for 5 min. The cells were incubated for 5 min with a 1:1 methanol:PBS solution followed by a PBS wash. The coverslips were mounted onto slides with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) and imaged on an Olympus FV1000 confocal system.

PRMT1+/+ and PRMT1−/− MEFs were treated with 2 μg/ml 4-hydroxytamoxifen (OHT) for 3 days as described previously (30). The cells were then seeded onto glass coverslips and cultured in fresh medium lacking OHT for 3 additional days. The cells were rinsed, fixed, and permeabilized as above. The cells were first blocked for 20 min at room temperature (0.5% gelatin, 1× PBS) and then incubated with antisera against Ash2L (Cell Signaling Technology), Ash2L (Abcam) followed by washes (0.1% gelatin, 1% normal goat serum, 1× PBS) and incubation with Alexa Fluor 488- or 594-conjugated rabbit secondary antibodies (Invitrogen) for detection of Ash2L and PRMT1, respectively. Following several additional washes, the coverslips were mounted and imaged as described above.

Metabolic Labeling—Cells were transiently transfected with Ash2L-GFP expression constructs using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s recommendations. Twenty-four hours post-transfection, cells were metabolically labeled with [3H]methylmethionine (PerkinElmer Life Sciences, NET061X) as described previously (35). Ash2L proteins were immunoprecipitated overnight with antibodies against GFP (Invitrogen).

Co-immunoprecipitation and Western Blotting—Ash2L expression constructs encoding N-terminal MYC epitope tags were transiently co-transfected along with FLAG/HA-RbBP5, FLAG/HA-WDR5, or HA-MLL expression constructs into HEK-293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Cells were harvested 24 h post-transfection, and whole cell extracts were prepared in lysis buffer (250 mM NaCl, 5 mM EDTA, 50 mM HEPES (pH 7.5), 0.1% Nonidet P-40, 0.5 mM DTT, 0.1% protease inhibitor mixture (Sigma)). MYC-conjugated agarose (Sigma) was added to the lysates for immunoprecipitation. The beads were washed four times with wash buffer (20 mM Tris (pH 7.4), 300 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, 5 mM EDTA, 25% glycerol), and bound proteins were eluted with protein sample buffer. Western blotting was carried out using standard techniques, and the antibodies used to probe the membranes were as follows: c-MYC (Santa Cruz Biotechnology), HA (Roche Applied Science), FLAG (Sigma), Ash2L (Cell Signaling Technology), PRMT1 (Cell Signaling Technology), β-actin (Santa Cruz Biotechnology), and GFP (Covance).

RESULTS

Ash2L Is Methylated in Vitro—Because Ash2L is an essential component of multiple lysine methyltransferase complexes, we tested whether it is a target for methylation. FLAG-Ash2L was immunoprecipitated from HEK-293T stable cell lines and used as a source of enzyme activity for an in vitro methyltransferase assay with [3H]AdoMet as the methyl donor. Several proteins were labeled following the reaction, including recombinant his-
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FIGURE 1. Ash2L is methylated in vitro. A, Ash2L was immunoprecipitated from HEK-293T cells stably expressing FLAG-Ash2L. The FLAG-immunoprecipitated proteins were eluted from beads and incubated with [3H]AdoMet in the presence and absence of recombinant histone H3 for in vitro methyltransferase assays. Reactions lacking eluate (Buffer) and FLAG immunoprecipitation reactions carried out in untagged HEK-293T cells (293T) serve as negative controls for the assay. MLL, Ash2L, and PRMT5 were detected by Western blot in the various FLAG eluates used for [3H]AdoMet methyltransferase reactions (bottom panel). MLL-C, C-terminal protein fragment of MLL. B, recombinant histone H3, GST, or GST-Ash2L were incubated as substrates in the presence of [3H]AdoMet and HEK-293T nuclear extract for in vitro methyltransferase assays. The methylated substrates are indicated with arrows. Recombinant histone H3 serves as a positive control, whereas GST serves as a negative control for the assay. IB, immunoblot.

Ash2L Is Methylated on Arginine Residues

To determine whether methylation of Ash2L occurs independently of the FLAG epitope tag, recombinant GST-tagged Ash2L was expressed and purified from E. coli and used as a substrate in an in vitro methyltransferase assay with nuclear extract prepared from HEK-293T cells as the source of methyltransferase activity (Fig. 1B). Full-length Ash2L was methylated in vitro following incubation with nuclear extract and [3H]AdoMet, indicating that enzymes responsible for methylating Ash2L are present in the nuclear fraction. Furthermore, labeling of Ash2L occurred in the absence of UV cross-linking, thereby suggesting a stable methylation event rather than transient AdoMet binding by Ash2L.

Ash2L contains an N-terminal plant homeodomain finger (21) and a C-terminal SP1a and ryanodine receptor domain (Fig. 2A). We next determined whether methylation of Ash2L occurs within these conserved regions. Various GST-Ash2L truncation mutants were purified from E. coli and used as substrates for in vitro methyltransferase assays (Fig. 2A). Following incubation with [3H]AdoMet and HEK-293T nuclear extract as the source of methyltransferase activity, full-length Ash2L as well as Ash2L(1–322) and Ash2L(233–322) were methylated in vitro, whereas Ash2L(1–240) and Ash2L(314–628) were not methylated (Fig. 2B). These results indicate that the region encoded by amino acids 233–322, which lies outside of conserved domains identified within Ash2L, is both necessary and sufficient for methylation of Ash2L in vitro.

To determine whether Ash2L is methylated on lysine or arginine residues following an in vitro methyltransferase assay, 3H-labeled GST-Ash2L(1–322) and GST-Ash2L(314–628) were acid-hydrolyzed and analyzed by high resolution cation exchange chromatography for the presence of methylated arginine or lysine species (38). The amino acid analysis revealed the presence of methylated arginine residues within the first 322 amino acids of Ash2L, but very little methylation was detected in the GST-Ash2L(314–628) fragment (Fig. 2C). GST alone was analyzed in parallel as a negative control (Fig. 2C). The major radiolabeled methylated species in the hydrolysate of the 1–322 polypeptide was ω-monomethylarginine. However, small amounts of radioactivity were detected at positions corresponding to asymmetric and symmetric ω-dimethylarginine.

Ash2L Is Methylated in Vivo and in Cells—Given the results of the methyltransferase assays using Ash2L truncation mutants and the amino acid analysis (Fig. 2, B and C), the sequence between amino acids 233 and 322 was analyzed for the presence of conserved arginine residues. Three arginine residues, Arg-296, Arg-300, and Arg-315, occur in the human Ash2L amino acid sequence (Fig. 3A). Site-directed mutagenesis was carried out to change Arg-296, Arg-300, and Arg-315 to alanine residues to test whether loss of these sites results in loss of methylation. In vitro methyltransferase assays performed with recombinant full-length GST-Ash2L encoding the wild-type Ash2L protein or Ash2L point mutants revealed that methylation of Ash2L is abolished when Arg-296 was mutated, indicating that this is the primary site of arginine methylation (Fig. 3B). Methylation of Ash2L still occurred when either the Arg-300 or Arg-315 sites were mutated (Fig. 3B), although the signal decreased noticeably when Arg-300 was mutated, suggesting that the integrity of this site may be required for full methylation of Ash2L.

Despite several attempts, we were unable to detect arginine-methylated Ash2L using mass spectrometry analysis (data not shown). We instead turned our attention to an established metabolic labeling protocol to determine whether Ash2L is meth-
PRMT-mediated Methylation of Ash2L

A

| Full length | PHD | SPRY |
|-------------|-----|------|
| 1-240       |     |      |
| 1-322       |     |      |
| 233-322     |     |      |
| 314-628     |     |      |

Methylation:

+  -  +  +  -

B

Figure showing GST-Ash2L purification and methylation assay. Substrate: H3, GST, Full length, 1-240, 1-322, 233-322, 314-628.

C

Coomassie stain and autoradiograph showing methylation of GST-Ash2L (1–322) and (314–628).}

FIGURE 2. Ash2L is methylated on arginine residues in vitro. A, the recombinant GST-Ash2L truncation mutants used as substrates for in vitro methyltransferase assays are illustrated and accompanied by a summary of the methylation status of each protein. B, Ash2L proteins purified from E. coli were incubated with HEK-293T nuclear extract in the presence of [3H]AdoMet. Reaction products were separated by SDS-PAGE, and then the gel was stained with Coomassie Blue (left panel) and treated for fluorography (right panel). Recombinant histone H3 serves as a positive control, whereas GST serves as a negative control for the assay. Methylated Ash2L proteins are indicated with arrows. Asterisks mark the GST-Ash2L fusion proteins. C, GST-Ash2L(1–322) was methylated in vitro as in B and then acid-hydrolyzed into single amino acids. The individual amino acids were separated by high resolution cation exchange chromatography along with methylated lysine and arginine standards. The radioactive fractions (solid trace) were analyzed for the presence of tritium and compared with the elution profiles of the known standards (dashed trace). Here, the tritiated methylated derivatives would be expected to elute slightly before the non-isotopically labeled derivatives (34, 68). DMLys, dimethyllysine; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; MMA, monomethylarginine; PHD, plant homeodomain finger; SPRY, SPIa and ryanodine receptor domain.
Metabolic labeling of exogenously expressed Ash2L-GFP fusion proteins in HeLa cells revealed that Ash2L is methylated on Arg-296 in cells as well (Fig. 3C). Methylation of Ash2L was reduced when Arg-300 was mutated as was observed in the in vitro methyltransferase assays. Taken together, these results confirm that Arg-296 is the primary site of methylation in Ash2L and that the Arg-300 residue plays a role in facilitating Arg-296 methylation both in vitro and in cells.

**PRMT1 and PRMT5 Methylate Arg-296 in Vitro**—We next determined the PRMT(s) responsible for methylating Ash2L. Various recombinant PRMT proteins that are known to be active in mammals (24) were expressed and purified from E. coli (GST-PRMT1, -3, -4, and -6) or from mammalian cells (MYC-PRMT5). The PRMT enzymes were incubated with [3H]AdoMet and GST-Ash2L(233–322) proteins as substrates for in vitro methyltransferase reactions. The results revealed that PRMT1, PRMT3, and PRMT5 methylate Ash2L in vitro, whereas PRMT4 and PRMT6 do not (supplemental Fig. S1). PRMT3 is localized exclusively in the cytoplasm (39), but Ash2L has been shown to localize primarily in the nucleus (23). Therefore, further experiments will be required to determine whether Ash2L is a bona fide substrate of PRMT3 in vivo.

Given that PRMT1 and PRMT5 methylate cytoplasmic and nuclear substrates (24, 40), we focused on whether these enzymes directly methylate Ash2L Arg-296 in vitro. Full-length recombinant GST-Ash2L was labeled by [3H]AdoMet following incubation with recombinant PRMT1 (Fig. 4A, first panel, top), but a catalytically dead mutant version of PRMT1 (G80R) failed to methylate Ash2L (Fig. 4A, first panel, top). PRMT1-mediated methylation of Ash2L was abolished when Arg-296 was mutated (R296A or R296A,R300A lanes) and was decreased substantially when Arg-300 was mutated in agreement with our results above (Fig. 3). These findings indicate that PRMT1 directly methylates Ash2L at Arg-296 in vitro.

PRMT5-mediated methylation of Ash2L was strikingly similar to the pattern observed for PRMT1 as it also methylated Ash2L at Arg-296 (Fig. 4A, second panel, top). Consistent with the result shown in supplemental Fig. S1, PRMT6 failed to methylate Ash2L in vitro (Fig. 4A, third panel, top), indicating that methylation of Ash2L is carried out specifically by certain PRMTs.

**PRMT1 Asymmetrically Dimethylates Ash2L Arg-296 in Cultured Cells**—PRMT1 activity is often directed toward but not restricted to arginine residues bordered by glycines (41–43). Given that Arg-296 lies within a “GRG” site and given a previ-
ous link between PRMT1- and MLL-EEN-mediated transformation (3), we focused our attention on PRMT1-mediated methylation of Ash2L.

Because PRMT1 is a Type I PRMT (44), we generated an antibody that specifically recognizes Ash2L peptides asymmetrically dimethylated at Arg-296 (R296me2a). The Ash2L R296me2a antibody recognized exogenous Ash2L expressed in HEK-293T cells, but mutating Arg-296 to lysine abolished the signal (supplemental Fig. S2A). In addition, the antibody failed to recognize recombinant Ash2L expressed and purified from E. coli, which lacks PRMT activity (35, 45), further indicating that the antibody specifically recognizes Ash2L methylated at Arg-296 and not the unmodified Arg-296 residue (supplemental Fig. S2A).

### FIGURE 4. PRMT1 asymmetrically dimethylates Ash2L in vitro and in cells.

**A.** recombinant GST-PRMT1 or GST-PRMT6 fusion proteins purified from E. coli or MYC-PRMT5 purified from HEK-293T cells was incubated with [³H]AdoMet for in vitro methyltransferase assays. Substrates are indicated above the panels, and the source of enzyme activity is given below the panels. The GST-Ash2L fusion proteins used as substrates are indicated above each panel as well. Methylated Ash2L and histone H4 are indicated with arrows on the autoradiograph (top panel). GST serves as a negative control, whereas histone H4 serves as a positive control for the assays. B, whole cell extracts prepared from HEK-293 cell lines stably depleted for endogenous Ash2L were analyzed by Western blot with an antibody raised against asymmetric dimethylated Ash2L Arg-296 (Ash2L R296me2a). Methylated Ash2L is indicated with an arrow. Detection of a nonspecific band is marked by an asterisk (*NS). The blot was reprobed for total Ash2L and β-actin as loading controls. Recombinant Ash2L purified from E. coli serves as a negative control for antibody specificity. C, HEK-293 cells were treated with the global methylation inhibitor adenosine-2',3'-dialdehyde (AdOx) for 24 h. Whole cell extracts were analyzed by Western blot with the Ash2L R296me2a antibody. Methylated Ash2L is indicated with an arrow. Detection of a nonspecific band is marked by an asterisk (*NS). HEK-293 cells stably depleted for Ash2L and recombinant Ash2L purified from E. coli serve as negative controls for antibody specificity. The blot was stained with Ponceau S as a loading control. D, PRMT1 flox MEF cells were treated with OHT to deplete endogenous PRMT1. Whole cell extracts were analyzed by Western blot with antibodies against methylated Ash2L (Ash2L R296me2a), Ash2L, PRMT1, and β-actin as a loading control. Methylated Ash2L is indicated with an arrow (top panel), shRNA depletion of Ash2L in HEK-293T cells serves as a negative control. The asterisk denotes a nonspecific band detected by the Ash2L R296me2a antibody (*NS). IB, immunoblot.
296 Ash2L peptide (asymmetric \(\omega-N^G,N^G\text{-dimethylarginine}\)) but not unmethylated Ash2L peptide effectively competes for antibody binding (supplemental Fig. S2C).

The Ash2L R296me2a antibody recognized endogenous Ash2L in HEK-293T whole cell extracts, and the signal was appropriately decreased upon stable shRNA depletion of endogenous Ash2L (Fig. 4B). A higher molecular weight band also appeared, but it was not depleted following Ash2L shRNA treatment and therefore is denoted by an asterisk as nonspecific. To further confirm the specificity of the Ash2L R296me2a antibody for methylated Ash2L, HEK-293T cells were treated with the global AdoMet inhibitor adenosine-2',3'-dialdehyde. The level of methylated Ash2L decreased, whereas the total Ash2L protein level was unaffected following treatment with a low dose of adenosine-2',3'-dialdehyde, indicating that depletion of the intracellular pool of AdoMet inhibits methylation of Ash2L in cells (Fig. 4C). Again, a higher molecular weight band appeared that was not decreased following Ash2L shRNA treatment and therefore is marked as nonspecific. HEK-293T cells treated with scrambled or Ash2L-specific shRNAs as well as recombinant Ash2L purified from \(E.\ coli\) served as controls for antibody specificity (Fig. 4C).

To determine whether PRMT1 methylates Ash2L in cells, PRMT1 conditional-null MEFs (PRMT1\(^{-/-}\)) were treated with OHT to deplete endogenous PRMT1 (PRMT1\(^{-/-}\)) as described previously (30). Western blot analysis using the Ash2L R296me2a antibody showed that methylation of Ash2L decreased compared with wild-type (PRMT1\(^{+/+}\)) following depletion of PRMT1 (Fig. 4D), indicating that PRMT1 mediates asymmetric dimethylation of Ash2L Arg-296 in cells. A lower molecular weight band appeared in the PRMT1\(^{+/+}\) MEFs but not the PRMT1\(^{-/-}\) or PRMT1\(^{-/-}\) MEFs treated with OHT on the Ash2L R296me2a Western blot. Because this band was not detected by the Ash2L antibody, which recognizes all known Ash2L isoforms, or in HEK-293T cell extracts, the band was marked as nonspecific. Depletion of endogenous PRMT1 did not have an effect on Ash2L total protein levels as measured by Western blot (Fig. 4D), suggesting that methylation of Ash2L by PRMT1 does not affect its stability. However, Ash2L is a very stable protein with a half-life of greater than 24 h (46), so we cannot completely rule out the possibility that methylation affects Ash2L protein turnover.

**PRMT1-mediated Methylation Is Not Required for Ash2L Nuclear Localization**—The Arg-296 residue within Ash2L resides within a putative bipartite nuclear localization signal (21), and Ash2L protein expression is observed mainly in the nucleus (23). To determine whether methylation of Arg-296 affects Ash2L nuclear localization, immunofluorescent staining of endogenous Ash2L was carried out in PRMT1\(^{+/+}\) and PRMT1\(^{-/-}\) MEFs following treatment with OHT. Fluorescence microscopy imaging revealed that Ash2L retained nuclear localization and was excluded from DAPI-bright heterochromatic regions in PRMT1\(^{-/-}\) MEFs (Fig. 5), suggesting that PRMT1-mediated methylation is not required for appropriate Ash2L subnuclear localization. Immunofluorescence imaging analysis of wild-type Ash2L- and R296A-GFP fusion proteins further demonstrated that methylation of the Arg-296 residue is not required for appropriate nuclear localization of Ash2L (supplemental Fig. S3, rows 2 and 3). The basic charge of the Arg-300 residue is necessary for nuclear localization as Ash2L R300A- and R296A/R300A-GFP fusion proteins displayed aberrant cytoplasmic localization (supplemental Fig. S3, rows 4 and 6), whereas Ash2L R300K- and R296K/R300K-GFP fusion proteins retained appropriate nuclear localization (supplemental Fig. S3, rows 5 and 7). Additional subcellular localization experiments performed in HeLa cells stably depleted of PRMT5 or in PRMT3\(^{+/+}\) and PRMT3\(^{-/-}\) MEFs (47) confirmed that methylation of Ash2L by PRMT5 (supplemental Fig. S4) or PRMT3 (supplemental Fig. S5) is not required for Ash2L nuclear localization. To further define the role of Arg-296 methylation in Ash2L nuclear localization, the Ash2L R296me2a was used to analyze Ash2L R296me2a nuclear localization in PRMT1\(^{+/+}\) and PRMT1\(^{-/-}\) MEFs treated with OHT. In wild-type MEFs, methylated Ash2L shows a nuclear staining pattern similar to the signal observed for total Ash2L, which overlaps with nuclear localized PRMT1 (supplemental Fig. S6, compare upper and lower panels). Furthermore, the signal for methylated Ash2L is substantially reduced in the absence of PRMT1 (supplemental Fig. S6, lower panels). These data indicate that methylated Ash2L is localized in the nucleus and that PRMT1 mediates methylation of Arg-296 in cells. Of note, because of the nonspecific protein bands recognized by the Ash2L R296me2a antibody for Western blot analysis, immunofluorescence may include other nuclear proteins in addition to methylated Ash2L.

**Methylation of Ash2L Arg-296 Is Not Required for MLL Complex Integrity or Maintaining Global Histone H3K4me3 Levels**—Determining how protein interactions within the MLL complex regulate mono-, di-, and trimethylation of histone H3K4 is an active area of investigation. The core subcomplex of WDR5, RbBP5, and Ash2L interacts with the catalytic SET domain of MLL to mediate enzymatic activity toward histone H3K4 (16–19) (Fig. 6A). To investigate whether methylation of Ash2L affects its association with the MLL complex, co-immunoprecipitation experiments were performed with epitope-tagged complex members. Mutation of Ash2L at Arg-296, Arg-300, or Arg-315 did not disrupt the interaction between Ash2L and the MLL C-terminal SET domain (HA-MLL(3793–3969)) (Fig. 6B). Likewise, mutation of Ash2L Arg-296, Arg-300, or Arg-315 did not disrupt interactions between Ash2L and RbBP5 (Fig. 6C) or
Ash2L and WDR5 (Fig. 6D). These results indicate that methylation of Ash2L Arg-296 is not required for MLL complex integrity. This result is somewhat expected given that recombinant MLL-C, Ash2L, WDR5, and RbBP5 purified from E. coli interact and form a complex with mono- and dimethyltransferase activity toward histone H3K4 (16).

It is possible that methylation of Ash2L regulators molecular interactions within the complex that direct different degrees of lysine methylation. To this end, we analyzed global histone H3K4me3 levels in cells depleted for endogenous Ash2L and stably expressing wild-type or Arg-296 mutant Ash2L using the FlpIn recombinase system (Invitrogen). Depletion of endogenous Ash2L resulted in an expected decrease in global histone H3K4me3 levels (17, 18), and reintroduction of wild-type Ash2L restored global histone H3K4me3 levels compared with cells treated with scrambled shRNA (Fig. 6E). However, introduction of Ash2L Arg-296 mutant proteins also restored global histone H3K4me3 levels, suggesting that methylation of Arg-296 is not required to maintain global histone H3K4me3 (Fig. 6E). Further Western blot analysis revealed that global histone H3K4me2 levels were also unaffected in cells stably expressing Ash2L Arg-296 mutants (data not shown).

**DISCUSSION**

We describe here the novel finding that Ash2L, a component of mammalian histone H3K4 methyltransferase complexes, is arginine-methylated in cells. To our knowledge, this is the first post-translational modification reported for Ash2L. The methylated Arg-296 residue lies within a conserved basic stretch of amino acids between the plant homeodomain finger and the SPIa and ryanodine receptor domain (Figs. 2 and 3). The Arg-300 residue is required for efficient methylation of Arg-296 both in vitro and in cells (Figs. 3 and 4) and is critical for Ash2L nuclear localization (supplemental Fig. S3).
We determined that Ash2L Arg-296 is asymmetrically dimethylated by PRMT1 in vitro and in cells and also methylated by PRMT5 in vitro. PRMT1 and PRMT5 share several common substrates, including histone H4R3 (48, 49) and transcriptional regulatory proteins SPT5 (50) and MBD2 (51). Asymmetric dimethylation of histone H4R3 by PRMT5 is associated with transcriptional repression (53, 54). Symmetric dimethylation of histone H4R3 mediated by PRMT5 recruits the DNMT3a DNA methyltransferase, which binds to H4R3me2s via a plant homeodomain finger, to promote transcriptional repression (55). It is evident that different effector molecules are recruited to the same histone residue by the presence of either a symmetric or asymmetric dimethylation state and therefore likely that differential methylation of Ash2L by either PRMT1 or PRMT5 leads to distinct functional outcomes as well.

The biological function of Ash2L remains elusive. Ash2L, PRMT1, and MLL1 are ubiquitously expressed in the developing embryo, whereas lethality occurs earlier in Ash2L-null mice (E3.5) compared with mice lacking PRMT1 (E6.5) or MLL1 (~E9.5) (23, 29, 56, 57). Furthermore, mouse ES cells lacking Ash2L are not viable (23), suggesting that Ash2L is essential for cell survival and proliferation during early embryogenesis. Although it does not appear that methylation of Ash2L at Arg-296 is required for maintenance of global histone H3K4me3 levels using a cell culture model system, we cannot rule out the possibility that methylation of Ash2L is important for the regulation of global H3K4 methylation patterns in a temporal manner, such as during development. It may also be that methylation of Ash2L regulates histone H3K4 methylation in a gene-specific manner rather than on a global level. For example, acetylation of the GATA1 transcription factor is required for in vivo chromatin binding of specific target genes, although acetylation mutants do not show nuclear localization or in vitro DNA binding defects (58). In addition, it is likely that only a fraction of the Ash2L molecules in the cell are methylated at any given time and that methylation elicits specific downstream effects on gene transcription or DNA repair. Future studies will define the genomic localization of Ash2L and associated methyltransferase complexes to determine whether methylation affects binding to specific target genes in vivo.

Mechanisms that govern the targeting of MLL and Setd1 lysine methyltransferase complexes to specific genomic targets are not defined, although these enzymes are known to methylate histone H3K4 at distinct sites of genes (59). Ash2L interacts with the phosphorylated form of the transcription factor, MeF2d, and directs MLL2, MLL3, and Set1 methyltransferase activity to muscle-specific genes to promote myogenesis (60). Ash2L also interacts with the Ap2δ transcription factor and recruits the MLL2 complex to activate transcription of HoxC8 during development (61). Therefore, these results suggest that Ash2L may serve as a targeting molecule for lysine methyltransferase activity by interacting with transcription factors in a post-translational modification or temporally dependent manner, respectively. Methylation of Arg-296 may affect protein-protein interactions or chromatin interactions that recruit Ash2L and associated lysine methyltransferase activity to specific genomic loci to regulate chromatin-templated processes, such as gene transcription and DNA repair. Additionally, methylation of Ash2L Arg-296 may affect recruitment of lysine methyltransferase activity to non-histone substrates that play a role in various cellular processes. Determining the protein-protein interactions affected by asymmetric $\omega_{-N^G,N^G}$-dimethylarginine or symmetric $\omega_{-N^G,N^G}$-dimethylarginine of Ash2L Arg-296 will provide insight into the biological significance of this post-translational modification. Likely candidates include proteins that contain methylarginine binding domains, such as Tudor domains (62, 63).

Ash2L was recently identified as an oncoprotein, although the mechanism by which Ash2L promotes transformation has not been clearly defined (46). In human tumors, Ash2L is not mutated or overexpressed; however, increased protein expression is observed, suggesting that post-translational regulation of Ash2L is associated with transformation (46). In addition, knockdown of Ash2L inhibits tumor cell proliferation and colony formation in a RAS-dependent transformation model (46). The steady state level of Ash2L is not altered in PRMT1-deficient MEFs (Fig. 4), suggesting that asymmetric dimethylation of Arg-296 does not affect Ash2L protein stability in untransformed cells. These results suggest that although mutations of Ash2L may not cause cancer per se post-translational regulation of Ash2L may promote transformation.

PRMT1 interacts with the oncogenic MLL-EEN fusion protein to promote transformation of hematopoietic progenitors but does not associate with wild-type MLL in untransformed cells (3). Direct fusion of PRMT1 to MLL results in increased histone H4R3 methylation and transcriptional activation of HoxA9 (3). Although overexpression of HoxA9 is necessary to sustain MLL-mediated leukemogenesis, it is not required for initiation (64–67), suggesting that additional mechanisms may exist through which PRMT1 activity promotes transformation. Our results clearly demonstrate Ash2L as a substrate of PRMT1, which provides a unique link between these proteins with transforming potential. Future studies will determine whether PRMT-mediated methylation of Ash2L also plays a role in oncogenesis.

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