Ribosomal Protein rpS2 Is Hypomethylated in PRMT3-deficient Mice*

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Rafal Swiercz, Donghang Cheng, Daehoon Kim, and Mark T. Bedford

From the University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas 78957

PRMT3 is a type I arginine methyltransferase that resides in the cytoplasm. A large proportion of this cytosolic PRMT3 is found associated with ribosomes. It is tethered to the ribosomes through its interaction with rpS2, which is also its substrate. Here we show that mouse embryos with a targeted disruption of PRMT3 are small in size but survive after birth and attain a normal size in adulthood, thus displaying Minute-like characteristics. The ribosome protein rpS2 is hypomethylated in the absence of PRMT3, demonstrating that it is a bona fide, in vivo PRMT3 substrate that cannot be modified by other PRMTs. Finally, the levels 40 S, 60 S, and 80 S monosomes and polyribosomes are unaffected by the loss of PRMT3, but there are additional as yet unidentified proteins that co-fractionate with ribosomes that are also dedicated PRMT3 substrates.

Arginine methylation is a common posttranslational modification that occurs in both the nucleus and the cytoplasm (1). The methylation of arginine residues is catalyzed by at least two different classes of protein arginine methyltransferase (PRMT) enzymes. The Type I enzymes catalyze the formation of asymmetric $N^G,N^G$-dimethylarginine residues and the Type II enzyme catalyzes the formation of symmetric $N^G,N^G$-dimethylarginine residues. In mammals, there are five Type I enzymes (PRMT1, 3, 4, 6 and 8).

Proteins that are substrates for the PRMTs usually contain glycine and arginine-rich patches, GAR motifs, that are the sites of methylation. The major pools of protein that are arginine methylated are the heterogeneous nuclear ribonucleoproteins, histones, and ribosomal proteins. Thirty years ago, HeLa cell ribosomal proteins were shown to be heavily lysine- and arginine-methylated (2), and further two-dimensional gel electrophoresis studies of purified ribosomes demonstrated that at least six prominent proteins are arginine methylated (3). PRMT3 is a ribosome-associated protein (4, 5) and may be responsible for much of the arginine methylation that occurs in this molecular machine.

PRMT3 was identified as a PRMT1 binding protein in a yeast two-hybrid screen (6). However, gel filtration analysis of RAT1 cells demonstrated that PRMT3 occurs as a monomer and is not complexed in vivo with PRMT1 (6). PRMT3 also interacts with the tumor suppressor DAL-1 (7). This interaction inhibits PRMT3 enzymatic activity, both in in vitro reactions and in cell lines. At the organ level, PRMT3 is ubiquitously expressed (6), but in the brain it displays higher expression in neurons than in glial cells (8). PRMT3 is the only type I arginine methyltransferase that is localized exclusively to the cytoplasm (6, 9). Another unique property of PRMT3 is that it harbors a zinc finger domain at its N terminus. It has been proposed that this domain may play a role in the regulation of PRMT3 activity or in the recognition of PRMT3 substrates (6, 10). Deletion analysis studies demonstrated that PRMT3 lacking the zinc finger domain is still active in vitro, however the zinc finger minus enzyme loses its ability to methylate substrates when presented with a complex mix of hypomethylated proteins isolated from RAT1 cells (10). This suggests that the zinc finger is the substrate recognition module of PRMT3. Indeed, it was recently found that the 40 S ribosomal protein S2 (rpS2) is a zinc finger-dependent substrate of PRMT3 (5). Importantly, in fission yeast this same enzyme/substrate pair (PRMT3/rpS2) exists (4), and the disruption of the rmt3 gene in this organism results in an imbalance in the 40 S:60 S free subunit ratio. Furthermore, rmt3-null cells compensate for the loss of RMT3 by increasing the production of 40 S ribosomal proteins (11). Taken together, these findings demonstrate a highly conserved role for arginine methylation in ribosomal assembly and protein biosynthesis.

To establish the biological role of PRMT3 in mice, we generated a knock-out model system. We investigated the effects of PRMT3 loss on mouse embryo development. In addition, we used cell lines derived from these mice to investigate the methylation status of ribosome-associated proteins.

**EXPERIMENTAL PROCEDURES**

Generation of PRMT3-targeted Mice—The ES cell line (XC265, strain 129/Ola) was kindly provided by BayGenomics. These ES cells were transferred to the M.D. Anderson Cancer Center’s genetically engineered mouse facility for blastocyst injection and chimeric mice production. Southern analysis with an “external” probe confirmed three XC265 chimeras successfully transmitted the targeted allele through the germine. The Prmt3+/- mice are fertile and healthy, with no obvious abnormalities. These lines are being maintained on a mixed 129/B6 background and an inbred 129 background. The external probe for Prmt3 knock-out genotyping is 540 bp long and includes exon 14 and surrounding 5’ and 3’ intron sequences. It was
Antibodies and Plasmids—All GST fusion proteins were subcloned in pGEX6p1 (Amersham Biosciences). pGEX6p1-PRMT3 and pGEX6p1-rpS2 were generated by PCR using FirstChoice™ PCR-ready mouse kidney cDNA (Ambion, Austin, TX) as described previously (5). The truncated form of GST-PRMT3 (GST-PRMT3334) was generated by PCR using 5’-TCA GGT ACC CTC GAG CCG CCG CAT GTG GAC-3’ and 5’-ACG GGT ACC TCA TTA AAC CAG GAA AGG TTC TTC-3’ oligos, pGEX6p1-PRMT3 as a template, and the ExSite™ site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to a manufacturer’s protocol. The FLAG-tagged PRMT3 was subcloned into a pTRACER vector (Invitrogen). The FLAG-entrapped PRMT3 was subcloned into a pTRACER vector (Invitrogen) as described previously (5). The αPRMT3 antibody was raised in rabbits against GST-PRMT3(εf), which encodes amino acids 1 to 183 of mouse PRMT3. The αrpS2 antibody was raised in rabbits against GST-rpS2, which encodes amino acids 1–293 of mouse rpS2. The αS6 antibody was obtained from Dr. Stephane Richard (University of McGill, Montréal, Canada). αrpS6 antibody was obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-βGal was obtained from Cortex Biochem (San Leandro, CA).

In Vitro Methylation Assays Using GST-PRMT3—The GST, GST-GAR, GST-rpS2, GST-PRMT3, and GST-PRMT3334 were expressed and purified as described previously (5). In vitro methylation reactions were performed in a final volume of 30 μl of PBS (pH = 7.4). The reaction contained 0.5–1.0 μg of sub-strate and 1 μg of recombinant GST-PRMT3 of GST-PRMT3334. All methylation reactions were carried out in the presence of 0.42 μM [3H]AdoMet (S-adenosyl-L-[methyl-3H]methionine) (79 Ci/mmol from a 7.5 μM stock solution; PerkinElmer Life Sciences). The reaction was incubated at 30 °C for 1 h, then subjected to fluorography by separation on SDS-PAGE, transferred to a PVDF membrane, treated with EnHANCE™ (PerkinElmer Life Sciences) and exposed to film overnight.

In Vitro Methylation of Cell Extracts Using GST-PRMT3—Individual embryos from Prmt3+/−/− intercrosses at E13.5 were placed into culture as described (12). Cells were maintained on a 3T3-culture protocol in which 106 cells were passed onto a gelatinized 10-cm dish every 3 days. MEF extracts were prepared using mild lysis buffer (10 mM Tris-Cl (pH = 7.50), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA). Cell debris was spun down for 10 min at 14,000 rpm at 4 °C. The resulting extract was dialyzed overnight at 4 °C against PBS. The protein concentration was determined by the Bradford method. Dialyzed MEF extracts were stored at −80 °C until further analysis. For the in vitro methylation assays shown in Fig. 2 the reactions were performed in a final volume of 30 μl of PBS. Briefly, 50 μg of total protein MEF extract was mixed with 1 μg of GST-PRMT3 and 2 μl of [3H]AdoMet (79 Ci/mmol from a 7.5 μM stock solution) and incubated for 90 min at 30 °C. Methylated proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and visualized by fluorography. For the immunodepletion experiment total cell lysates were incubated with the immobilized antibodies four successive times to remove all traces of rpS2.

In Vitro Methylation Assay with Ribosomal Fractions—The in vitro methylation assays shown in Fig. 4 used FLAG-PRMT3, because we wanted to carry out Western analysis of rpS2 after fluorography to confirm equal loading, and the αrpS2 antibody that we have was raised against a GST fusion of rpS2. For this purpose, HeLa cells were transfected with 10 μg of FLAG-PRMT3 using 30 μl of Lipofectamine™ 2000 (Invitrogen). At 24 h after transfection, cells were washed with ice-cold PBS, and cell extracts were prepared using mild lysis buffer. FLAG-tagged PRMT3 was immunoprecipitated using anti-FLAG™-M2 beads (Sigma-Aldrich). After overnight incubation at 4 °C, FLAG-tagged PRMT3 bound to anti-FLAG™-M2 beads was washed with PBS and resuspended in PBS to a final volume of 60 μl. Ribosomal fractions corresponding to cytoplasm (fractions 1–5), 405 (fractions 6 and 7), 60 S (fractions 8 and 9), and 80 S (fractions 10 and 11) ribosomal subunits, and polysomes (fractions 12–18) were pulled together. For the in vitro methylation assay, 10 μl of concentrated proteins were mixed with 10 μl FLAG-PRMT3 bound to anti-FLAG™-M2 beads and 2 μl of [3H]AdoMet (79 Ci/mmol from a 7.5 μM stock solution). The final volume of the reaction was adjusted to 30 μl with PBS. After 90 min of incubation at 30 °C, methylated proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and visualized by fluorography.

Polysome Profiling—MEF cells (passage 2) from Prmt3+/+ and Prmt3−/− E13.5 embryos, at 90% confluency were plated in a 15 cm 3 Dulbecco’s modified Eagle’s medium supplemented with 10% serum and 0.1 mg/ml cycloheximide. After that, cells were washed with ice-cold PBS (containing 0.1 mg/ml cycloheximide). Next, cell extract was prepared using 500 μl of polysome lysis buffer (1.0% Triton X-100, 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 0.5 unit/μl RNase inhibitor, 0.1 mg/ml cycloheximide, 0.2 mg/ml heparin, protease inhibitors). Following a 10-min incubation on ice, cellular debris was sedimented by centrifugation at 14,000 rpm for 15 min at 4 °C. A total of 20 A260 units of extracts were layered onto 5–45% sucrose gradients prepared in polysome lysis buffer and centrifuged for 3 h at 39,000 rpm at 4 °C in a Beckman SW41Ti rotor. The gradients were then fractionated by upward displacement with Fluorinert FC-40 (Isco Inc., Lincoln, NE), using a gradient fractionator (model 640; Isco Inc.) connected to a UV monitor for continuous measurement of the absorbance at 254 nm. Eighteen 0.6-ml fractions were collected, and proteins were concentrated to a final volume of 100 μl using Ultrafree-™-MC centrifugal filter devices (5,000 Da molecular weight cutoff; Millipore Corp., Bedford, MA). The sucrose was replaced by four changes of PBS buffer, 300 μl each. The final volume was adjusted to 100 μl with PBS. Concentrated proteins were separated by SDS-PAGE and transferred to a PVDF membrane.

β-Galactosidase Staining and Histological Analysis—E10.5 embryos were fixed in 2% glutaraldehyde and stained for β-galactosidase expression as described previously (13). E12.5 embryos were fixed in formalin and embedded in paraffin wax. Embryos were sectioned at 3 mm and subjected to immunohis-
tochemical localization of β-galactosidase using a specific antibody (Cortex Biochem, San Leandro, CA). Staining was performed using the En-Vision system (Dako, Carpinteria, CA), and the counterstain was hematoxylin.

RESULTS AND DISCUSSION

Generating Mice Deficient in PRMT3—The Prmt3 gene has been targeted in mouse embryonic stem cells using gene trapping technology developed by Skarnes et al. (14). The targeted ES cell clone (XC265) was obtained from the BayGenomics research consortium. The mutant ES cell line contains an insertional mutation in intron 14 of Prmt3 (Fig. 1A). This insertion is predicted to leave PRMT3 largely intact and only removed the last 34 amino acids of this enzyme, unless the insertion destabilizes the transcript. Structural data reveals that the C-terminal portion of PRMT3 is an elongated nine-stranded β-barrel (15), and the truncation in the XC265 cells will eliminate the last three of these β-strands (Fig. 1C). The removal of the last β-strand (number 15) of yeast Hmt1 is sufficient to prevent substrate binding and almost completely disrupt enzymatic activity (16). Thus, it is likely that the truncation generated by the trapping mutant in the XC265 ES cell line will fully incapacitate PRMT3.

To test this, we generated a truncated GST-PRMT3Δ34 and compared it to wild-type PRMT3 in an in vitro methylation assay on two known substrates, rpS2 and GAR (Fig. 1B). GST-PRMT3Δ34 displayed no enzymatic activity. Thus, even if the truncated transcript is stable in the XC265 ES cells, it will likely be non-functional. To characterize the biological function of PRMT3, we proceeded to generate chimeric mice using XC265 ES cells that were then bred to establish heterozygous (Prmt3+/−) progeny. A probe for Southern analysis was developed for genotyping these mice. The gene-trapping vector introduces a new BamHI site, which results in a 6-kb down-shift of the Southern band for mice carrying the mutant allele (Fig. 1D).

The Effects of PRMT3 Loss on rpS2 Methylation—Prmt3−/− mice were normal and fertile and were intercrossed, producing Prmt3−/− mice and embryos. To facilitate additional studies of PRMT3 cellular functions, we cultured MEFs from E13.5 embryos. Four Prmt3−/+ MEF lines (1, 5, 9, and 12) and four Prmt3−/− MEF lines (3, 6, 10, and 13) were established. The integrity of PRMT3 expression in the established MEF lines was confirmed by Western blot analysis (Fig. 2A). A longer exposure of this Western reveals that there is a faint band at the position of the endogenous PRMT3 protein. It also exposes the existence of a large molecular weight band that is unique to the Prmt3−/− MEF line and may represent the PRMT3-βgeo fusion protein. To confirm that this unique band is indeed the PRMT3-βgeo fusion protein, we immunoprecipitated PRMT3 from a Prmt3−/+ MEF line (12) and a Prmt3−/− MEF line (3) and performed Western blot analysis with an anti-βGal antibody. The same blot was stripped and re-probed with an αPRMT3 antibody. Both antibodies recognize the same band at roughly 170 kDa (Fig. 2B). Again in this experiment we see a faint band at the position of the endogenous PRMT3 protein, and densitometric quantitation was performed on the endogenous band from the wild-type and knock-out cell lines. We conclude that the insertional mutation results in a hypomorphic Prmt3 allele, with 5–7% wild-type PRMT3 expression levels in Prmt3−/− cells. This is reminiscent of Prmt1−/− cells that were generated in a similar fashion through insertional mutagenesis (17).

We have previously shown that the ribosomal protein rpS2 interacts with the zinc finger of PRMT3 and that this ribosomal protein is an in vitro substrate for this enzyme (5). To determine
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whether rpS2 is an in vivo substrate for PRMT3, we methylated total cell lysates from wild-type (5 and 12) and knock-out (3 and 10) cells with GST-PRMT3 and tritium labeled AdoMet. We see that a protein the size of rpS2 (30 kDa) is strongly methylated in knock-out cells but not in wild-type cell extracts (Fig. 2C, lanes 13–16). This indicates that rpS2 is hypomethylated in Prmt3 knock-out cells, thus making it a good in vitro substrate. However, in Prmt3 wild-type cells rpS2 is likely heavily methylated, with most methylation sites already fully occupied, thus making it a poor substrate for recombinant PRMT3. The same amount of rpS2 is present in both wild-type and knock-out cells. To unequivocally confirm that this 30-kDa labeled band is indeed rpS2, we immunodepleted rpS2 from total cell lysates and detected no methylated band following GST-PRMT3 methylation of these preparations (Fig. 2C, lanes 5–8). The 30-kDa labeled band is still present after immunodepletion with pre-immune sera (Fig. 2C, lanes 9–12). Furthermore, immunoprecipitated rpS2 from knock-out cells is a good substrate for GST-PRMT3 (Fig. 2C, lanes 1–4), and migrates at the same position as the methylated band in the total cell lysate. Taken together, these data support the finding that rpS2 is the major PRMT3 substrate.

We can also show that rpS2 is hypomethylated in Prmt3 knock-out cells in a more direct fashion. The rpS2 protein contains two potential arginine methylation motifs at its N-terminal end, two copies of an RGGEF motif and eight direct RG repeats. Deletion analysis has shown that the primary site of rpS2 methylation by PRMT3, in vitro, is the RG repeat region (5). A methyl-specific antibody (ASYM24) has been raised to methylate RG repeats (18). Using this ASYM24 antibody, we have shown that rpS2 is hypomethylated in PRMT3 knock-out cells (Fig. 2D). A small amount of immunoreactivity is seen with rpS2 from knock-out cells, which can be eliminated with adenosine dialdehyde treatment. The residual amounts of PRMT3 in these cells (Fig. 2, A and B) may account for this methylation.

Prmt3-deficient Embryos Are Small—At weaning, Prmt3−/− mice occur at roughly the expected mendelian ratios indicating that the reduced levels of PRMT3 found in these mice are not embryonically lethal (Table 1). However, when we generated the MEF lines from E13.5 embryos we noticed that the mutant embryos were consistently smaller than their wild-type littermates (Fig. 3A). It should be noted here that the pigmentless eye of the Prmt3−/− is not part of the PRMT3 null phenotype. Rather, it is due to the fact the 129/Ola ES cells, which were used

[open arrowhead] are depicted. Densitometric quantitation was performed on the endogenous PRMT3 band from the wild-type and knock-out cell lines and represented graphically. C, in vitro methylation assays reveal that rpS2 is a major PRMT3 substrate. Total cell lysates were methylated in vitro with GST-PRMT3 and [3H]AdoMet (lanes 13–16). Immunodepletion of total cell lysates was performed with either preimmune sera (lanes 9–12) or the anti-rpS2 antibody (lanes 5–8), followed by an in vitro methylation reaction. In lanes 1–4 IPed rpS2 was subjected to the same in vitro methylation reaction. Duplicate blots were prepared and subjected to Western analysis with anti-GST, anti-β-actin, and anti-rpS2 antibodies (lower panels). D, total cell lysates from wild-type (lane 12) and Prmt3−/− (lane 10) MEFs were subjected to Western analysis with the methyl-specific Asym24 antibody. A duplicate blot was probed with the rpS2 antibody to demonstrate equal loading. The 105 lane represents Prmt3−/− cells that have been treated for 7 days with 20 μM adenosine dialdehyde. Densitometric quantitation was performed on the immunoreactive Asym24 band from the wild-type and knock-out cell lines and represented graphically. IP, immunoprecipitation.

FIGURE 2. The ribosomal protein rpS2 is hypomethylated in Prmt3 knock-out cell lines. A, MEF lines were generated from E13.5 embryos. Four wild-type (lanes 1, 5, 9, and 12) and four knock-out (lanes 3, 6, 10, and 13) lines were derived. Immunoprecipitation/Western analysis of PRMT3 revealed almost total absence of endogenous PRMT3 levels (black arrowhead). When the Western is subjected to a long exposure, a weak band is seen in knock-out cells, which may be due to alternative splicing around the trapping vector. In addition, a new band is seen in knock-out cells (open arrowhead) that is likely due to the fusion of Prmt3 to the βgeo gene of the trapping vector. B, detection of the PRMT3-βgeo fusion and quantitation of the residual PRMT3 expression in knock-out cells. PRMT3 was IPed from wild-type (lane 12) and knock-out (lane 3) cells and Western analysis was performed with an anti-βGal antibody. The same blot was stripped and re-probed with an anti-PRMT3 antibody. Endogenous PRMT3 (black arrowhead) and the PRMT3-βgeo fusion (open arrowhead) are depicted. Densitometric quantitation was performed on the endogenous PRMT3 band from the wild-type and knock-out cell lines and represented graphically. C, in vitro methylation assays reveal that rpS2 is a major PRMT3 substrate. Total cell lysates were methylated in vitro with GST-PRMT3 and [3H]AdoMet (lanes 13–16). Immunodepletion of total cell lysates was performed with either preimmune sera (lanes 9–12) or the anti-rpS2 antibody (lanes 5–8), followed by an in vitro methylation reaction. In lanes 1–4 IPed rpS2 was subjected to the same in vitro methylation reaction. Duplicate blots were prepared and subjected to Western analysis with anti-GST, anti-β-actin, and anti-rpS2 antibodies (lower panels). D, total cell lysates from wild-type (lane 12) and Prmt3−/− (lane 10) MEFs were subjected to Western analysis with the methyl-specific Asym24 antibody. A duplicate blot was probed with the rpS2 antibody to demonstrate equal loading. The 105 lane represents Prmt3−/− cells that have been treated for 7 days with 20 μM adenosine dialdehyde. Densitometric quantitation was performed on the immunoreactive Asym24 band from the wild-type and knock-out cell lines and represented graphically. IP, immunoprecipitation.
by BayGenomics to generate their gene-trap library, carry a mutation in the pink-eyed dilution (p) gene on mouse chromosome 7, and Prmt3 maps very closely to this gene. Thus, these two mutations usually segregate together.

To further investigate this developmental phenotype, we established time pregnancies and investigated embryonic weights at E13.5 and E18.5. We found that mutant embryos at E13.5 are on average 75% the size of their wild-type littermates (Fig. 3B). By E18.5, this size difference is not as striking, with mutants on average 82% the size of their wild-type littermates (Fig. 3C), and by weaning age (P21) there is no size difference between mutants and wild-types.

Like PRMT1, PRMT3 is ubiquitously expressed in adults (6). The mutant PRMT3 allele is expressed as a PRMT3-mutants on average 82% the size of their wild-type littermates (Fig. 3). By E18.5, this size difference is not as striking, with mutants on average 82% the size of their wild-type littermates (Fig. 3C), and by weaning age (P21) there is no size difference between mutants and wild-types.

Like PRMT1, PRMT3 is ubiquitously expressed in adults (6). The mutant PRMT3 allele is expressed as a PRMT3-βgeo fusion, which facilitates X-gal staining of Prmt3−/− embryos to reflect the endogenous PRMT3 expression pattern. X-gal staining of Prmt3−/− E10.5 embryos reveals expression at the base of the neural tube, in elements of the hepatic/biliary primordial and in the olfactory placode (Fig. 3D). By E12.5 PRMT3 expression becomes broader, as detected by anti-β-galactosidase antibody staining (Fig. 3E).

The 40 S:60 S Ribosomal Subunit Ratio Remains Unperturbed in Prmt3−/− Mouse Cells—In fission yeast, the loss of Prmt3 (the PRMT3 orthologue) results in a striking ribosomal subunit imbalance, with the reduction of 40 S and accumulation of 60 S ribosomal subunits (11). To determine whether the same phenomenon occurs in mammalian cells, we purified polysomes from primary wild-type and Prmt3−/− MEFs by sucrose gradient fractionation. The polysome profile of these two cell types was undistinguishable (Fig. 4, A and B). In addition, Western blot analysis shows that both rpS2 and rpS6 remain associated with the ribosomal fractions. Thus, the arginine methylation of rpS2 by PRMT3 is likely not required for either its incorporation into ribosomes or for the maintenance of ribosomal structural integrity. However, it should be noted that rpS2 methylation is decreased by 80% and is not fully absent in the Prmt3−/− MEFs (Fig. 2D), thus the integrity of the ribosome may be maintained by this residual methylation. We also investigated the efficiency of overall protein synthesis in primary MEFs by 35S incorporation into nascent proteins and found that the protein synthesis rate was unperturbed by the loss of PRMT3 in MEFs (data not shown).

Ribosomal-associated Proteins Other than rpS2 Are PRMT3-specific Substrates—To investigate whether any ribosomal proteins other than rpS2 are methylated by PRMT3, we pooled the sucrose gradient fractionation and methylated them with FLAG-PRMT3 in vitro. No methylated substrates are detected in the ribosomal fractions from wild-type cells, indicating that the methylatable sites are fully occupied in the Prmt3−/− substrates. These substrates are hypomethylated in the Prmt3−/− fractions and are thus good targets for the in vitro methylation reaction (Fig. 4C). Again, we clearly detect rpS2 as the predominant PRMT3 substrate. However, we also see a protein of 40 kDa that is methylated in knock-out fractions but not in the wild-type fractions. Like rpS2, this protein is most abundant in the 40 S fraction. Data base searches of ribosomal proteins of this size that also harbor a GAR-motif were performed, but no candidate substrate was identified. Clearly, the use of hypomethylated Prmt3−/− cellular extracts will aid in the identification of new PRMT3 substrates in the future.

Mammalian PRMT3 and its primary substrate, rpS2, are both ribosome-associated proteins (5). The disruption of the Prmt3 gene results in reduced embryo body size during organogenesis and the fetal growth period. E13.5 embryos are undergoing rapid growth, and if gross protein synthesis or even the translation of a few select transcripts are attenuated due to the loss of rpS2 (and other protein) methylation, this may manifest as a reduction in size. This size phenotype is not as striking at E18.5 but is still significant, and by birth we cannot identify mutants based solely on their size. This phenotype is reminiscent of mutations in Drosophila ribosomal protein genes, collectively known as Minutes (19). Indeed, a P element

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**TABLE 1**

| Age  | Total offspring | Genotype  | +/+ | +/− | −/− |
|------|----------------|-----------|-----|-----|-----|
| E13.5 | 181           | +/+       | 63  | 79  | 39  |
| E18.5 | 189           | +/−       | 45  | 99  | 45  |
| Weaning | 586         | −/−       | 161 | 305 | 120 |

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**FIGURE 3.** Embryos lacking a functional Prmt3 gene exhibit a small size phenotype. A, embryonic (E13.5) size difference between wild-type (+/+ ) and Prmt3 mutants (−/−). Body weights of embryos at E13.5 (B) and E18.5 (C) were measured and plotted as an average with the standard deviation. The number of individuals analyzed is shown on Table 1. Independent two sample t tests reveal highly significant differences (p value < 0.001) between wild-type and mutant embryos at both E13.5 and E18.5. D, X-gal staining of E10.5 wild-type (+/+ ) and Prmt3 mutant (−/−) embryos. E, immunohistochemical localization of β-galactosidase in a Prmt3 mutant embryo at day E12.5 viewed at ×40 magnification. Selected views include the spinal cord, trigeminal ganglion, and the liver. Brown stain represents β-galactosidase expression.
insertion into the Drosophila rpS2 gene, referred to as string of pearls, has Minute-like characteristics (20).

Although the 40 S:60 S ribosomal subunit ratio remains unperturbed in Prmt3-deficient mouse cells, it is possible that significant changes in the translational activities of selected mRNAs may occur in these cells to compensate for the loss of PRMT3, as is the case in the rmt3-null cells (11). Of particular interest would be the 40 S ribosomal protein-encoding mRNAs that are redistributed to active larger polysomes in fission yeast mutant cells.

It has long been known that ribosomal proteins are heavily methylated on both arginine and lysine residues (2). Subsequently, amino acid analysis of two-dimensional separated ribosomal proteins identified six different protein spots that were arginine-methylated and one lysine-methylated protein spot (3). More recently, using methylarginine-specific antibodies a number of ribosomal proteins were affinity-purified as methylated proteins (18). The physiological role of rpS2 methylation has so far remained obscure. To some extent, this enzyme/substrate pair of PRMT3/rpS2 is evocative of the S6 kinase/rpS6 pairing. Both enzyme substrate pairs have been conserved over an extended evolutionary period and are found from yeast to man (4, 5, 21, 22). The phosphorylation of rpS6 occurs in response to numerous growth stimulatory agents and temporally correlates with the initiation of protein synthesis (23). There is as yet no evidence that the methylation of rpS2 can be regulated by dynamic signal transduction events. Indeed, all experiments point to the fact that rpS2 is constitutively methylated in cells.

The region of rpS2 that is methylated by PRMT3 has been mapped to a repeat of eight RG residues at its N-terminal end (5). This is a classic GAR motif. In vitro this motif can be meth-

FIGURE 4. In addition to rpS2, another protein that co-fractionates with ribosomes is also methylated by PRMT3, but the general ribosomal profile is unperturbed by the loss of PRMT3. A and B, sucrose gradient fractionation of ribosomes isolated from primary wild-type (WT) (A) and knock-out (KO) (B) MEFs. The polysome profile is depicted graphically. The position of each fraction is marked on the trace. The positions of free 40 S and 60 S ribosomal subunits, monosomes (80 S), and polysomes are shown. "Top" denotes the 5% end of the sucrose gradient and "Bottom" the 45% end. Collected fractions were concentrated to a final volume of 100 µl, and sucrose was replaced with PBS buffer and subjected to Western blot analysis with rpS2 and rpS6 antibodies. C, wild-type and Prmt3−/− primary MEFs were subjected to sucrose gradient fractionation and the following fractions were pooled: 1–5 (cytoplasm), 6 and 7 (40 S), 8 and 9 (60 S), 10 and 11 (80 S), and 12–18 (polysomes). The pooled fractions were subjected to an in vitro methylation reaction with immunoprecipitated FLAG-PRMT3 and [3H]AdoMet. After fluorography (upper two panels), the membranes were subjected to Western blot analysis with the rpS2 antibody (lower two panels). A black circle marks the position of an unknown PRMT3 substrate.
ylated by PRMT1, PRMT3, PRMT5, PRMT6, and PRMT8 (24, 25). However, in cells derived from Prmt3 knock-out embryos, rpS2 is hypomethylated, which indicates that in vivo no other PRMT can cover for the loss of PRMT3. This is likely due to the fact that the zinc finger of PRMT3 interacts strongly with rpS2. Indeed, rpS2 was identified as a PRMT3 substrate by using the rpS2 zinc finger as an affinity reagent (5). Thus, in certain cases it may be important for PRMTs and their substrates to be tightly associated.

Of the nine identified PRMTs, four have been genetically targeted in the mouse. Prmt1 nulls are early embryonic lethal and die shortly after implantation (17). Carm1 knock-out embryos survive to birth and die perinatally (12). In addition to their small size, Carm1 null embryos have a T cell developmental defect (26). Prmt2 has recently been knocked out in mice and display no gross abnormalities (27). Prmt3 is now the latest member of the arginine methyltransferase family to be knocked out in mice, with null embryos displaying a clear size difference during gestation and null cells providing genetic evidence that the ribosomal protein rpS2 is a dedicated PRMT3 substrate.

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