Methods

Constructs and antibodies. All RCC1 constructs were as previously described[3]. Wild type and K4Q mutant SET vectors were designed as for the RCC1 vectors but using human SET cDNA. The human RB1 ORF (Open Biosystems, Huntsville, AL) was amplified to introduce a 5' *HindIII* restriction site, C-terminal Flag tag, and 3' *BamHI* site, and subcloned into the mammalian expression vector pRK7. The human NRMT ORF (Open Biosystems) was amplified to introduce a 5' *XbaI* restriction site, C-terminal Flag tag, and 3' *EcoRI* site, and subcloned into pRK7. The Flag tag was alternatively included in the 5' primer to create the N-terminally tagged NRMT. NRMT was also cloned into the *XbaI* and *BamHI* sites of pKGFP2, and into the *NdeI* and *XhoI* sites of pET30a to produce NRMT-His_6. All His_6 proteins were purified as previously[24].

Rabbit anti-me2-SPK and anti-me3-SPK antibodies were as previously described[3]. They were used in immunoblots at 1:5,000 and 1:10,000 dilutions, respectively. Rabbit anti-me2-PPK antibody was produced against 2me-P-P-K-R-I-A-K-R-R-S-C synthesized by American Peptide (Sunnyvale, CA). Rabbit anti-NRMT was produced against the C-terminal C-R-Q-E-N-L-P-D-E-I-Y-H-V-Y-S peptide synthesized by GenScript Corporation (Piscataway, NJ). This peptide sequence is common to human, mouse, and dog. Both peptides were coupled to activated keyhole limpet hemocyanin, and antisera were produced by Cocalico Biologicals, Inc. (Reamstown, PA). Anti-me2-PPK was purified as described for the anti-me2-SPK and anti-me3-SPK antisera, to remove antibodies against non-methylated peptide[3]. Both antibodies were diluted 1:1000 for immunoblots. The following antibodies were also used for immunoblots: mouse anti-RB1 (1:2000, Cell Signaling Technology, Beverly, MA), mouse anti-FLAG M2-HRP (1:1000 Sigma-Aldrich, St. Louis, MO), mouse anti-β-catenin (1:3000 BD Biosciences, San Jose, CA), and rabbit anti-GFP (1:1000 Invitrogen, Carlsbad, CA).

***In vitro* methylation assays.** To assay column fractions, 50 µl samples were buffer-exchanged into MTase buffer (50mM Tris, 50mM K Acetate, pH 8.0) using Centri Spin columns (Princeton Separations, Adelphia, NJ). 1 µg of-His_6 RCC1 was added as substrate and 100 µM SAM was added as the methyl donor. For assays on immunoprecipitates, FLAG-NRMT was eluted from M2 agarose (Sigma) in MTase buffer with 0.1 mg/ml FLAG peptide, then His_6-RCC1 and SAM were added as above. For testing recombinant NRMT activity, 0.3 µg His_6-NRMT was mixed with same amounts of RCC1-His_6 and SAM and brought to 50 µl with MTase buffer. All reactions were incubated 1 h at 30°C and analyzed for methylation by immunoblot.

For ELISA methylation assays, HEK 293LT cells were calcium phosphate-transfected with 6 µg pKVenus, NRMT-pCMV-SPORT6, or FLAG-NRMT. 24 h post-transfection, cells were harvested and used to make nuclear extract[25]. 10 µg nuclear extract was mixed with 1 µg RCC1-His_6 and 0.55 µCi ^{3}H-SAM and brought to 50 µl with MTase buffer. Reactions were incubated 1h at 30°C and transferred to Pierce Protein A-coated ELISA plates (Thermo Scientific, Waltham, MA) pretreated with 1:200 anti-me2-SPK. After 1 h, the reaction was removed and wells were treated with 1% SDS for 30 min. This solution was then quantified for incorporated ^{3}H-methyl by scintillation counting.

Expression, purification and Factor Xa digestion of XPK-EGFP mutant substrates
XPK-EGFP substrate proteins were expressed from a modified pET15b (Novagen) expression vector in BL21 *E.coli* and purified on Ni-NTA beads, then cleaved using Factor X (Sigma-Aldrich). 50 µl reactions were performed in MTase buffer, using 3.0 pmol of recombinant NRMT plus 120 pmol of XPK-EGFP. The reaction was started by addition of 1ul of [^{3}H]-SAM (0.55µCi/ul), and incubated for 2 h at 30^{O}C. Reactions were filtered through nitrocellulose, washed with 50mM sodium bicarbonate and subjected to scintillation counting.

Mass Spectrometry. For in-solution enzymatic digestion, active and adjacent non-active HAP fractions were dried, and reconstituted in 100 mM ammonium bicarbonate (SigmaAldrich). The sample was reduced, alkylated and digested with Trypsin (Promega) at an enzyme:protein ratio of 1:20 at 37°C for 6 h[26]. For in-gel enzymatic digestion, coomassie-stained SETα-FLAG bands were excised and subjected to in-gel digestion as described previously[27], with slight modification. Gel pieces were incubated with endoproteinase GluC (Roche) overnight at room temperature followed by peptide extraction. Extracted peptides were dried by using a SpeedVac concentrator (Savant Instruments), resuspended in 0.1% glacial acetic acid, and stored at -35°C until analysis. For both methods, an aliquot of peptide solution was loaded onto a capillary precolumn (360-µm outer diameter´ 75-µm inner diameter) packed with irregular C18 packing material (~520 µm)[26,28]. The precolumn was washed with 0.1% acetic acid and then connected with Teflon tubing to an analytical column packed with regular C18 packing material (5 µm) and a 5-µm emitter tip[26,28]. Samples were analyzed by nanoflow HPLC-microelectrospray ionization on a linear quadrupole ion trap-Fourier transform mass spectrometer (LTQ-FT; Thermo Electron)[29]. GluC digested SETα-FLAG was also analyzed using a Thermo LTQ instrument modified for electron transfer dissociation (ETD) (90ms ETD reaction) for adequate tandem mass spectrometry (MS/MS)[30,31]. All spectra were interpreted manually.

For analysis of the tissue immunoprecipitations, while still on beads, the purified proteins were reduced and carbamidomethylated on Cys residues and half of each was proteolytically digested in 100 mM ammonium bicarbonate (pH 8) using either endoproteinase Asp-N or Glu-C (Roche) for 6 h at room temperature as similarly described[27]. Following digestion, the generated peptides were removed from the N-terminal peptides still associated with the anti-me antibody on the agarose beads and later acidified to pH 3.5 using glacial acetic acid. An aliquot of the endoproteinase Asp-N and Glu-C generated peptides were pressure loaded onto an irregular C18 (6 cm in length, 5-20 µm diameter, 120 Å pore size, YMC Co., Ltd. Kyoto, Japan) capillary precolumn (360 o.d. x 75 i.d.) and washed with 0.1% acetic acid (Sigma Aldrich) prior to connecting the precolumn to a C18 (8 cm in length, 5 µm diameter, 120 Å pore size, YMC Co., Ltd.) analytical capillary column (360 o.d. x 50 i.d.) equipped with an electrospray emitter tip as described [26,28]. Digested samples were gradient eluted for mass analysis at a flow rate of 60 nL/min using nanoflow HPLC and electrospray ionization into a linear ion trap and Fourier transform hybrid mass spectrometer (LTQ-FTMS or LTQ-Orbitrap, Thermo Scientific, Bremen, Germany) as described above. Mass spectrometers used were front end ETD (FETD)-enabled to allow both CAD and ETD analyses for each digested sample in addition to high resolution precursor mass measurements. Mass analyses were

completed with one high resolution (60,000 resolution at 400 m/*z*) MS1 scan followed by 8-10 CAD or ETD data dependent MS2 scans. For ETD experiments, MS^2 parameters were set as follows: 35 ms reaction time, 3 m/*z* precursor isolation window, charge state rejection "on" for +1 and +2 charge state precursor ions, 2 x 10^5 FTMS full AGC target, 1 x 10^4 $ITMS^n$ AGC target, 2 x 10^5 reagent target with azulene as the electron transfer reagent. All data were searched using the Open Mass Spectrometry Search Algorithm (OMSSA) against the entire mouse RefSeq database (downloaded 06/2009) followed by manual confirmation.

To reduce the number of sample analyses, the separated bead aliquots from each digest were recombined for N-terminally modified peptide analyses. Peptides resulting from the Asp-N/Glu-C digests yet still retained on agarose beads were placed on a spin column (Thermo Scientific) washed with two 20 uL aliquots of Elution Buffer pH 2.8 (Pierce Crosslink Immunoprecipitation Kit, Thermo Scientific) and centrifuged at 3000 x g for 2 min to elute the newly released N-terminal peptides through the molecular weight cutoff filter. N-terminal peptides were reconstituted in 0.1% acetic acid, loaded and mass analyzed as described above. However, a top 5 data dependent CAD/ETD MS2 toggle instrument method was implemented. All data were search using OMSSA and all N-terminally modified peptides were verified by manual confirmation.

siRNA and lentiviral knockdowns. HeLa and HEK 293LT cells were grown in DMEM supplemented with 5% calf serum and 5% FCS, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin (GIBCO, Gaitherberg, MD). HCT116 cells were grown in McCoy's 5A Medium supplemented with 5% calf serum and 5% FCS, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin (Quality Biologicals, Gaithersburg, MD). Control, human RCC1, and human NRMT siRNA SMARTpools were obtained from Thermo Scientific. The sequences of the RCC1 SMARTpool were described previously[11]. Sequences of the NRMT SMARTpool are: GCAAGAGGGUGAGGAACUA, GGAAGUUUCUGCAGAGGUU, GCCAAGACCUACUGGAAAC, and UAGAAGACGAGAAGCAAUU. Hela cells were transferred to antibiotic-free medium for 24 h, then transfected with siRNA SMARTpools using the Oligofectamine protocol from Invitrogen, with 180 pmol of siRNA per 35 mm plate. Cells were harvested after 72 h and analyzed by immunoblot.

The human and mouse NRMT lentiviral shRNAmir pGIPZ constructs were obtained from Open Biosystems and grown and purified according to their protocol. The targeting sequence of the shRNA against NRMT is AGAGAAGCAATTCTATTCCAAG; and the control sequence is CCCTGCCAGACAGTACCAATTA. To make virus, 2.5 x 10^6 293LT cells were calcium phosphate transfected with 20 μg of the NRMT pGIPZ plasmid, 6 μg of the VSVG coat protein plasmid (pMD2G), and 15 μg of packaging plasmid (psPAX2). Viral supernatants were collected after 48 h, concentrated with 100K ultrafilters (Millipore, Billerica, MA) and titered in 293LT cells. 2 x 10^4 293LT or HCT116 cells were then infected with virus to an MOI of 1 or 5. After 3 days, 2 μg puromycin/ml was added, to select for transduced cells, and surviving cells were maintained in the same medium. For the rescue, 10,000 293LT cells were infected with lentivirus (control, NRMT shRNAmir, or NRMT shRNAmir coexpressing murine NRMT-FLAG) to an MOI of 3. The cells were grown for 2 days and lentivector expressing cells were selected by addition of 2 μg

puromycin/ml. The cells were grown 2 additional days and lysed (500mM NaCl; 50mM Tris pH, 8.0; 5mM $MgCl_2$; 1mM EDTA; 1mM EGTA; 0.1% NP-40; BME and protease inhibitors). 50ug of each lysate was analyzed by Western blot.

SPK peptide docking analysis
ICM-PRO software (Molsoft LLC) was used for docking of the model substrate peptide SPKRIA to NRMT[32]. A rigid ICM model of the protein was prepared from the PDB coordinates (PDB ID: 2EX4) using the ICM conversion procedure that includes the addition and local minimization of hydrogen atoms in the internal coordinate space and the selection of energetically favorable side chains for His, Asn, and Gln. All water molecules were removed from the structure and only one protein molecule from the asymmetric unit was taken for the calculations. ICM-PRO was then used for the identification of possible binding sites (pockets) in the receptor structure from maps calculated with a grid size of 0.5 Å. The hexapeptide SPKRIA model was generated using the Monte Carlo conformational procedures in Macromodel 7.1 (Schrodinger Inc.). An optimized peptide model was placed in the vicinity of the identified pocket and the docking was performed applying the ICM-PRO template docking approach.

Isothermal Titration Calorimetry
Isothermal titration calorimetry (ITC) experiments were performed using an ITC200 microcalorimeter (MicroCal). Recombinant NRMT was dialyzed overnight at $4^{O}C$ against methyltransferase buffer. Peptides were purchased from Genscript Corp. in lyophilized form, and dissolved in the same buffer. Exothermic heats of reaction (μcal/s) were measured at $25^{O}C$ by repeated, automated injection of the SPK or SPQ peptides (40 μl, 610 μM), spaced at 2-min intervals, into 280 μl of NRMT (61.12 μM). Binding curves were analyzed by non-linear least squares fitting of the data using the Origin (MicroCal) software package.

Immunofluorescence and Live Cell Imaging. 293LT cells grown on Lab-Tek II chambers (Nunc, Naperville, IL) were fixed in 1:1 methanol/acetone then blocked in 1% gelatin. Endogenous RCC1 was detected with the RCC1(N-19) antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) at a dilution of 1:200. Alexa-Fluor 488-conjugated donkey anti-goat secondary antibody was used at 1:1000 (Invitrogen). Cells were counterstained with 1:1000 Draq5 (Alexis Corp., San Diego, CA). HeLa cells expressing NRMT-GFP were fixed in 4% paraformaldehyde (PFA), permeabilized in 0.5% Triton X-100, and counterstained with 1:1000 Draq 5. Imaging was performed on a Zeiss LSM510 Meta confocal microscope, using a 100x oil immersion lens (na 1.3), at 512x512 pixel resolution, and a zoom of 2.0. Images were processed and quantified using ImageJ 1.41b software.

Endogenous NRMT staining was performed on HeLa cells fixed in 4% PFA, permeabilized in 0.5% Triton X-100, and blocked in 1% gelatin. Anti-NRMT was purified using the C-terminal NRMT peptide and diluted 1:200. Alexa-Fluor 594-conjugated goat anti-rabbit secondary was used at 1:1000 (Invitrogen). The cells were conterstained with DAPI (1 ng ml^{-1}). For mitotic spindle staining, stably transduced 293LT cells were fixed as the HeLas. Tubulin was detected with mouse anti-α-tubulin at 1:500 (Sigma). Alexa-Fluor 594-conjugated goat anti-mouse secondary was used at

1:1000 (Invitrogen). Cells were counterstained with DAPI. Both experiments were visualized using a wide field microscope (Eclipse T200; Nikon, Tokoyo, Japan) equipped with a 60x n.a. 1.2 Plan-achromatic water immersion lens and a charge-coupled device camera (Orca C472-95-12NRB; Hamamatsu Phototonics, Tsukuba, Japan). Images were collected at a 12-bit depth and 1,024 x 1,280 pixel resolution with 1 x 1 binning using Openlab 4.0 software (Improvision, Coventry, UK). Images were processed using ImageJ 1.41b software.

For live cell imaging, stably transduced 293LT cells were plated in 2-well Lab-Tek II coverglass (Nunc) in DMEM/F12 50/50 without phenol red (Cellgro, Manassas, VA) supplemented with 10% FBS (Gibco) and transiently transfected with 1.6 μg pK-RCC1-RFP using lipofectamine 2000 (Invitrogen). After 24 hrs, cells were counterstained with Hoechst dye (1 μg ml^{-1}), visualized on a Nikon (Eclipse TE2000-E) with a Yokogawa spinning disk confocal system (Solamere Technologies) using a 60× (NA = 1.40) objective lense (Solamere Technology). Images were processed and quantified using ImageJ 1.41b software.

Tissue Immunoprecipitations

10 μg of normal rabbit IgG (Santa Cruz Biotechnology, Inc.) and 5 μl (approximately 10 μg) of purified anti-me3-SPK serum were covalently crosslinked to Protein A/G resin using the Pierce Crosslink Immunoprecipitation Kit (Thermo Scientific). Whole mouse spleen or cardiac tissue were lysed in tissue lysis buffer (50 mM Tris-HCL, pH 8.0; 150 mM NaCl; 1 mM EDTA; 10 mM $MgCl_2$; 0.1% NP40 and protease inhibitors) and homogenized. The resuspension was spun at 13,200 rpm at 4°C for 10 minutes. The supernatant was then passed through an empty Handee Spin Column (Thermo Scientific) to remove remaining debris and added to the appropriate columns. The immunoprecipitation was performed as recommended by the manufacturer (Thermo Scientific). Samples dedicated for mass spec were washed and analyzed on-bead. Sample used for Coomassie and Western analysis were eluted according to the protocol.

Supplementary References

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