Supplementary information

Methylation and phosphorylation switch between adjacent lysine and serine determines human DNMT1 stability

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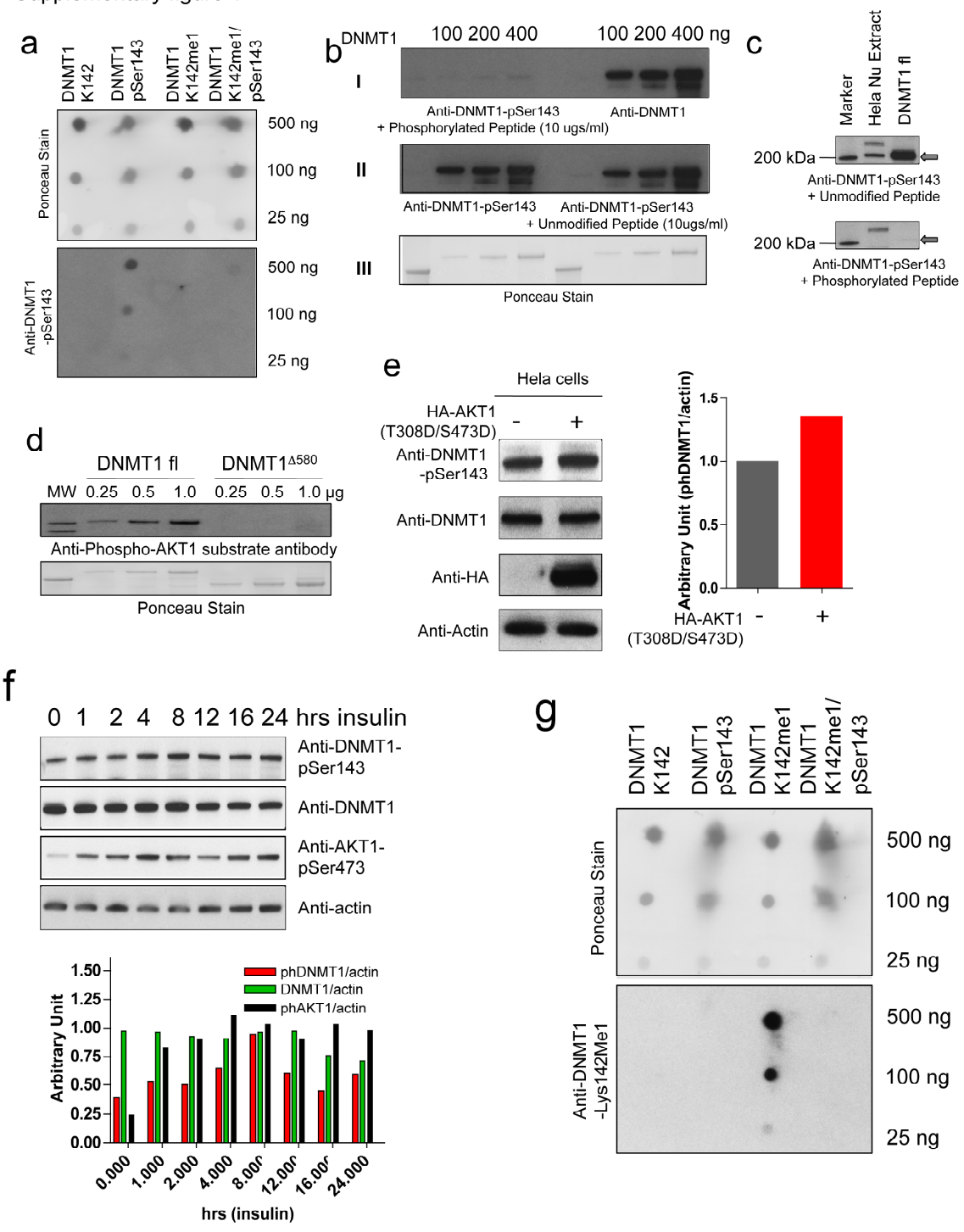
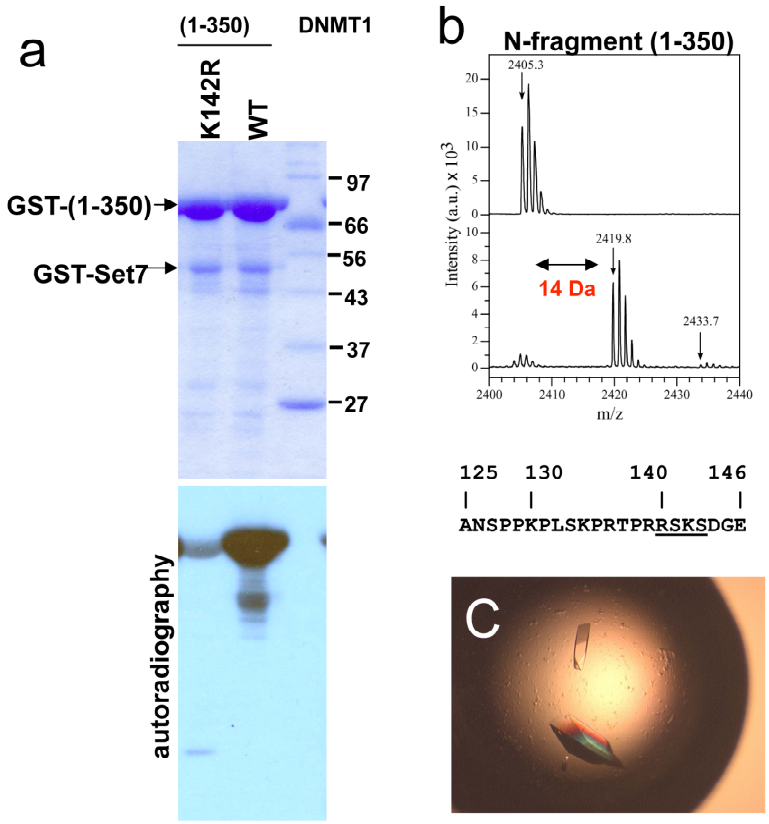
Supplementary figure 1



Specificity of DNMT1-pSer143 and DNMT1-Lys142me1 antibodies (**a**) Varying amount of peptides representing unmodified and modified fragments of DNMT1; DNMT1K142, DNMT1pS143, DNMT1K142me1, DNMT1K142me1pS143 were probed with anti-DNMT1-pSer143 in a dot blot assay. Both ponceau stain and western blot are

shown. (**b**) Varying amount of phosphorylated recombinant DNMT1 expressed in Sf9 insect cells using baculovirus expression system was Western blotted. The Western blots were probed with anti-DNMT1-pSer143 antibody in the presence of the competitor S143 phosphorylated peptide (panel I, left) or an identical unmodified peptide (panel II, right). Note in the absence of Ser143 phosphorylated peptide, but in the presence of identical unmodified peptide the antibody recognized phosphorylated DNMT1 (panel II). Competition with phosphorylated peptide abolished antibody binding in the top panel. Control western blot with anti-DNMT1 reveals equal loading. Ponceau staining reveals equal amount of proteins being blotted onto the membrane (panel III). All the competitor peptide concentration was 10 μg/ml. (**c**) Hela nuclear extract and recombinant DNMT1 were western blotted and probed with anti-DNMT1-pSer143 antibody in the presence of unmodified (DNMT1K142) peptide, upper panel; and modified (DNMT1pSer143) peptide, lower panel. Note that in the presence of DNMT1pSer143 (10 μgs/ml) the phosphorylation specific DNMT1 signals are lost. A non-specific higher molecular weight band is visible. (**d**) Another western blot assay demonstrating the amino terminus serine as the phosphorylated species. DNMT1 fl or a deletion mutant lacking the first 580 amino acids is probed with anti-phospho AKT1 substrate antibody. Ponceau stain of the transferred protein is shown at the bottom. (**e**) Increased phosphorylation at Ser143 of endogenous DNMT1 in the presence of constitutively active AKT1 kinase in Hela cells. Antibodies are indicated at the left. Upregulation of DNMT1pSer143 (phDNMT1) is shown as a ratio between phDNMT1 and actin at the right panel. (**f**) Endogenous DNMT1 is phosphorylated at Ser 143 in response to stimulation of AKT1 pathway by insulin (100 ng/ml) in HeLa cells. Samples taken after the indicated hrs was western blotted and probed with antibodies as shown. Up-regulation of DNMT1-pSer143 (phDNMT1) and phosphorylated AKT1 (phAKT1) are shown as a ratio between DNMT1 and actin. (**g**) Same as in panel a, except the antibody probe was anti-DNMT1-Lys142me1.

Supplementary figure 2

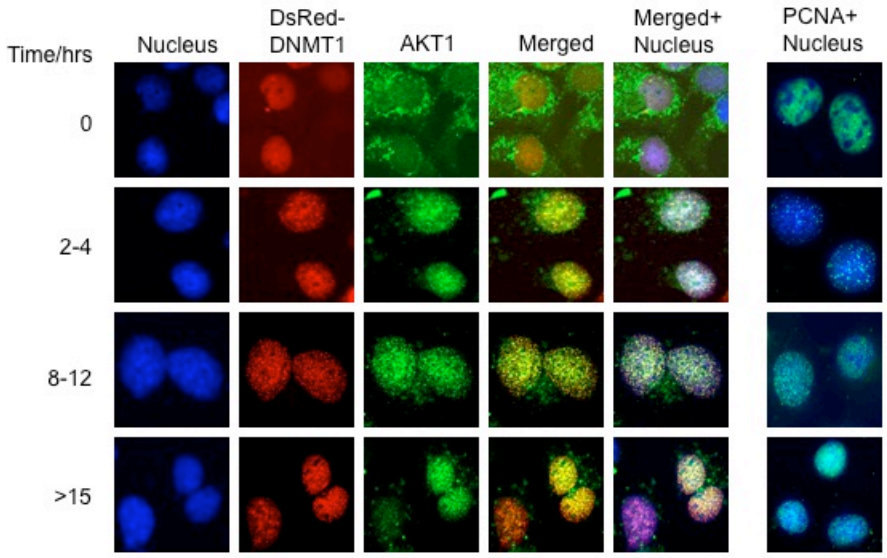


Lysine methylation site analysis on DNMT1 and crystallization of SET7/9 (**a**) The human DNMT1 N-terminal fragment (residues 1-350) was methylated *in vitro* by SET7/9. Point mutation of K142R abolished methylation. Reactions were carried out in a 20 µl mixture for 8 min at 30°C containing 1.75 µM SET7/9, 4.6 µM GST-DNMT1, 2.75 µM [methyl-^{3}H]AdoMet, in the buffer of 20 mM Glycine pH 9.8 and 5 mM DTT. The reaction products were separated by 13% SDS-PAGE and analyzed by fluorography. (**b**) Mass spectrometry analysis of the methylated peptide of DNMT1 isolated by in-gel digestion from its N-terminal domain (1-350) confirms Lys142 methylation site. Methylation of the fragment was performed by incubating 5 µM DNMT1 (1-350), 1 µM SET7/9, 100 µM AdoMet at 30˚C for 10 minutes in a 20 µl reaction mixture containing 20 mM Glycine 9.8 and 5 mM DTT. Reaction mixtures were run on a 13% poly-acrylamide gel and stained with commassie blue. The band corresponding to the DNMT1

fragment was excised from the gel, cut into small cubes and distained using 50% acetonitrile (AcCN)-water. Distained gel cubes were dried under vacuum and rehydrated in 20 to 30 μL of 10 mM ammonium-bicarbonate buffer containing 0.1 μg of V8 protease for 16 hr at 37˚C. Peptide fragments were extracted from the gel cubes using 60% AcCN-water containing 0.1% TFA and analyzed by MALDI-TOF mass spectrometry on a Bruker Ultra FlexII TOF/TOF instrument.

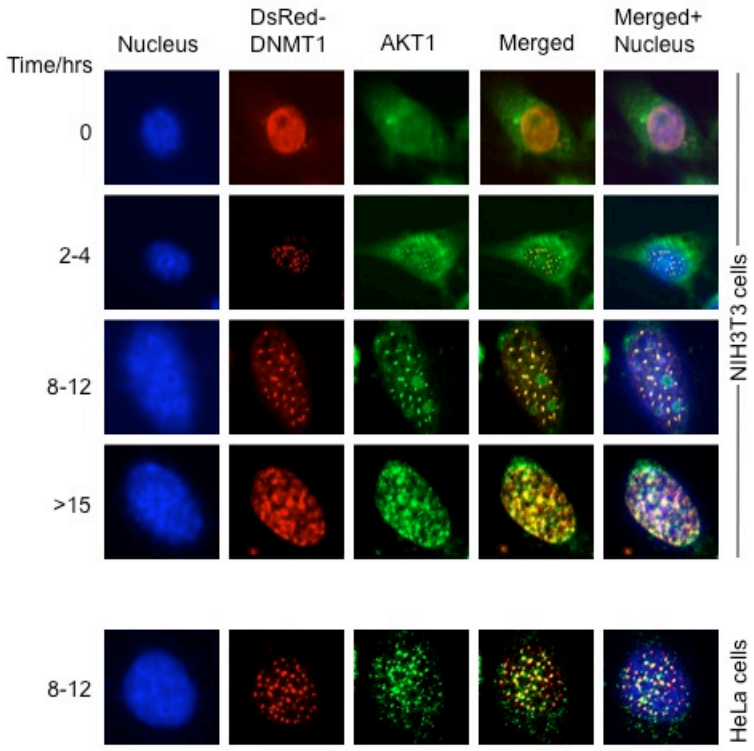
In the bicarbonate buffer, V8 primarily digest at the carboxyl side of Glu, but it also can digest after Asp, albeit at a 300 fold slower rate[41]. V8 digestion map of the DNMT1 fragment (1-350) using PeptideMass software (Expasy Server) showed a peptide fragment from residues Ala117-Glu146 with a theoretical mass of 3261.7345 Da (or 3277.7294 Da if Met122 is oxidized). This fragment contains Asp124. Additional cleavage at the carboxyl side of Asp124 generates a second fragment (Ala125-Glu146) with a theoretical mass of 2405.3007 Da ($M+H^+$). In the MALDI mass spectrometry analysis we detected both Ala125-Glu146 (2405.3 Da) as well as Ala117-Glu146 (3262.6/3276.9 Da) fragments. Both of these fragments show a 14 Da shift upon treatment with SET7/9 (only mass peaks corresponding to Ala125-Glu146 fragment are shown) indicating a mono-methylation. (**c**) Crystals of SET7-DNMT1 peptide in the presence of AdoMet.

Supplementary figure 3



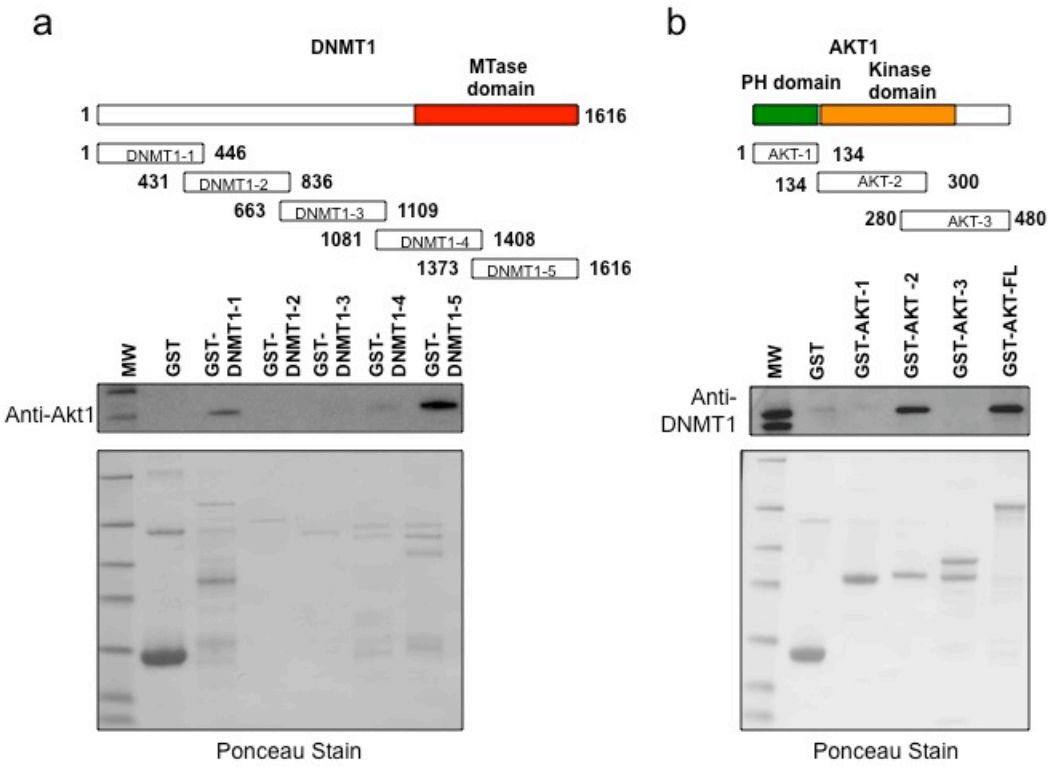
Field of cells demonstrating colocalization of DsRed-DNMT1 and AKT1 in COS-7 cells. Transiently expressed DsRed-DNMT1 (red) in COS-7 cells and endogenous AKT1 kinase (green) using an anti-AKT1 antibody. Cells were released from G1/S arrest and followed for the time shown through S and G2 phase. Nuclear staining was performed with Hoechst stain. To monitor cell cycle stage anti-PCNA (green) and Hoechst nuclear staining was also performed on the same batch of synchronized cells side by side.

Supplementary figure 4



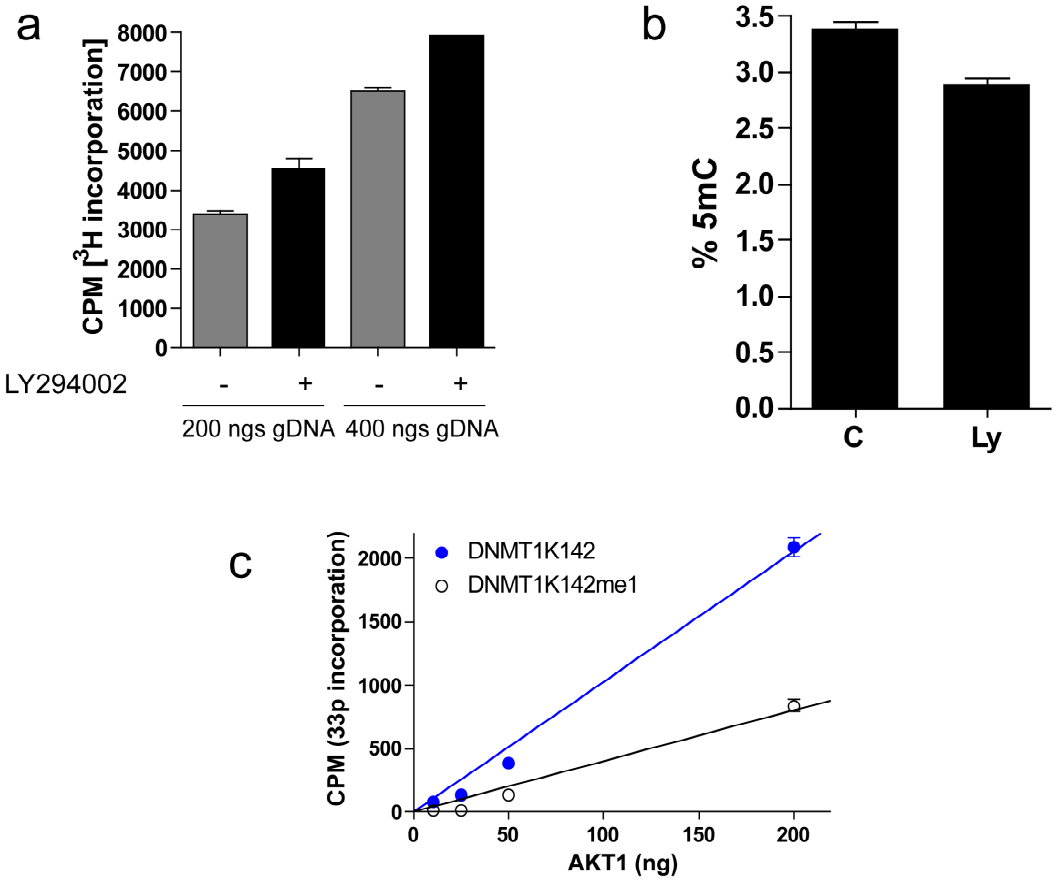
Transiently expressed DsRed-DNMT1 (red) in mouse NIH3T3 and Hela cells and endogenous AKT1 kinase (green) using an anti-AKT1 antibody. NIH 3T3 cells were released from G1/S arrest and followed for the time shown through S and G2 phase. Nuclear staining was performed with Hoechst stain. The bottom panel represents colocalization studies in HeLa cells. A repetitive time point is shown.

Supplementary figure 5



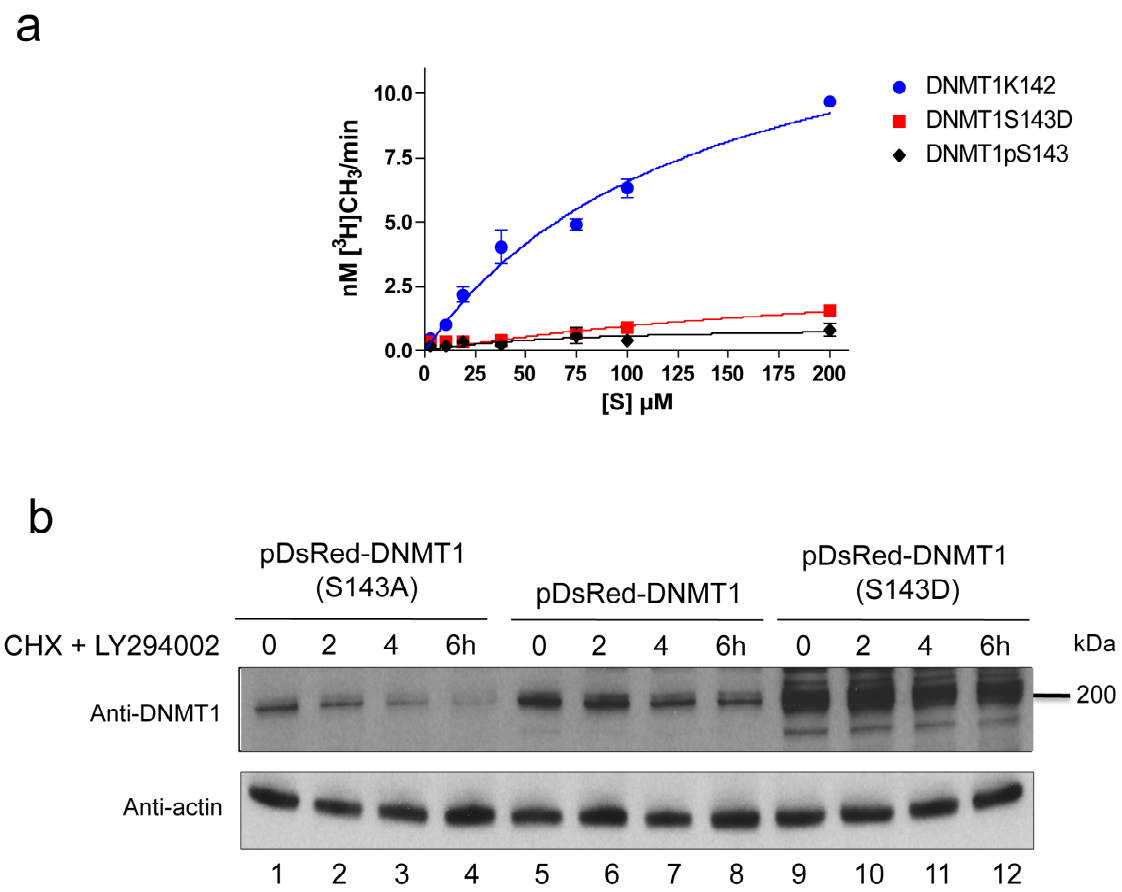
Direct interaction between AKT1 and DNMT1 (a) Interacting domain mapping between DNMT1 and AKT1. Top panel shows GST fusion fragments schematically below the full-length enzyme along with amino acids numbers. The bottom panel is the western blot analysis of the interacting fragments of DNMT1 and full-length AKT1 using anti-AKT1 antibody. **(b)** Interacting domain mapping between AKT1 and DNMT1. Experiments similar to (a) were performed using GST fusions of AKT1 using full-length DNMT1. Western blot was performed using anti-DNMT1.

Supplementary figure 6



AKT1 inhibition leads to reduce DNA methylation in HeLa genome (**a**) LY294002 treated genomic DNA is hypomethylated. Extracted genomic DNA was methylated with M.SssI methyltransferase in the presence of tritiated AdoMet. Each sample was assayed in duplicate with varying substrate DNA. (**b**) HPLC analysis of 5-methylcytosine (5-mC) in the genomic DNA of HeLa cells treated with AKT1 inhibitor LY294002 and control genomic DNA. The genomic DNA was isolated after 20 hrs of LY294002 treatment and analyzed by HPLC. The quantitative data was measured from three independent measurements. The content of 5mC for control LY294002 treated cells were 3.4±0.06 and 2.9±0.05 ($p < 0.05$) respectively. (**c**) AKT1 kinase assay on DNMT1K142 and DNMT1K142me1 peptide. The background counts were subtracted and data represented show end point reads in a triplicate assay. The trend remained same in repeats.

Supplementary figure 7



Role of peptide mimicking phosphorylation on lysine methylation and DNMT1 stability (a) *In vitro* SET7/9 methylation reaction in presence of various concentrations of DNMT1 peptides representing either native DNMT1K142 (blue filled circle), peptide-mimicking phosphorylation DNMT1S143D (filled red squares) or phosphorylated DNMT1pSer143 (black filled diamond). (**b**) Half life assay of DsRed-full-length DNMT1 Ser143 mutant (S143A) (lanes 1-4), DNMT1 wild type (lanes 5-8), phospho mimicking mutant S143D (lanes 9-12). Lane numbers are marked at the bottom, and relative molecular weight is shown at the right. The transfected cells were treated with CHX (cycloheximide) plus LY294002 and the extracts (normalized in equal amounts of lysate indicated by actin) were western blotted and probed with indicated antibodies (DNMT1 monoclonal antibody from Abcam, ab13537).