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Full Length Research Paper

pH uniquely modulates protein arginine methylation

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Protein arginine methyltransferases (PRMTs) function in the alkaline milieu of the nucleus and at neutral pH of the cytosol. Accordingly, several PRMTs are broadly active over a range of pHs. We investigated the effect altering pH had on protein arginine methylation using a variety of defined substrates, recombinant PRMTs and cell extracts.We demonstrate that pH-induced alterations in the extent of methylation and the methyl-product formed depend both on the particular substrate assayed and the PRMT that modifies it. We also find that transient intracellular alkalinization of mouse embryonic P19 neurons by NH_4Cl results in sustained changes in substrate methylation. Altogether our results are consistent with a hypothesis in which altered substrate methylation resulting from pH-induced changes of PRMT activities coupled with low levels of demethylation may provide the long-term tag(s) necessary for the formation and maintenance of "molecular memory".

Key words: Arginine methylation, protein arginine methyltransferase, pH, P19 cells, insect cell lysate, SmD1 peptide.

INTRODUCTION

pH homeostasis is crucial to life (Garcia-Moreno, 2009). Because of this organisms have evolved complex molecular systems that both sense pH e.g. carbonic anhydrase (CA) (Seksek and Bolard, 1996; Tresguerres et al., 2010) and the capsacin receptor (TRPV1) (Dhaka et al., 2009) aswell asmaintain intracellular and intraorganellar pH; the latter include: the Na^+/H^+antiporter (Murer et al., 1976), the acid pumping VATPase (Tabares and Betz, 2010), a group of Cl^-/HCO_3^-exchangers (Chesler, 2003) and Na^+ coupled bicarbonate transporters (NCBTs) (Majumdar and Bevensee, 2010).

Changes in intracellular or intraorganellar pH affect the charge state of many amino acid side chains and thus can profoundly alter the activities of enzymes. Indeed, the fact that every enzyme has an optimal pH at which it functions is a basic principle of biochemistry (Fersht, 1977). The pH of the nucleus is among the most basic of all of the organelles (Seksek and Bolard, 1996). Thus, it is not surprising that enzymes operating in this milieu would tend to have pH optima that are more basic. Protein methyltransferases (PMTs) methylate a variety of nuclear proteins including histones (Izzo and Schneider, 2010), splicing factors (Chen et al., 2010; Deng et al., 2010), spliceosomal components (Miranda et al., 2004), transcription factors (Kowenz-Leutz et al., 2010), and mRNA export complex components (Hung et al., 2010). In addition, several protein methyltransferases modulate their own activities via auto methylation (Chin et al., 2007; Frankel et al., 2002; Kuhn et al., 2010). In keeping with this, Zhang et al have shown that protein lysine

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methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs) are quite active at alkaline pH (Cheng et al., 2005; Zhang and Cheng, 2003). This feature assists in catalysis as the substrate amino lysine and amino arginine groups of these enzymes must be deprotonated in order to initiate a nucleophilic attack on the Ado-Met methyl group. Nevertheless, PRMTs are also found in the cytoplasm, which is pH-neutral (Garcia-Moreno, 2009), where they methylate RNA binding proteins (Dolzhanskaya et al., 2006; Stetler et al., 2006; Xie and Denman, 2011), spliceosomal components (Friesen et al., 2001; Meister et al., 2001), and receptors (Chen et al., 2004; Infantino et al., 2010). In this case it is likely that hydrophobic groups surrounding the methyltransferase active site play a role in lowering the pKa of the target amino substituent group so that it can stay in the deprotonated state required for its methylation.

To date, the effect pH has on protein arginine methyltransferase activity has not been systematically evaluated. Here we have begun to address this problem by assessing *in vitro* protein arginine methylation at neutral and basic pH, approximating the cytosol/-peroxisome and the mitochondria/nucleus environments, respectively (Abad et al., 2004; Garcia-Moreno, 2009; Jankowski et al., 2001; Seksek and Bolard, 1996). We demonstrate that the pH at which *in vitro* methylation reactions occur significantly impacts the type and extent of methylation in a substrate-specific manner. We also find that intracellular alkalinization results in the modification of protein arginine methyltransferases activity ex vivo, implying that protein arginine methylation is functionally coupled to local pH changes.

MATERIALS AND METHODS

Buffers and chemicals

HMTase Buffer is 50 mM Tris-HCl pH 9.0, 1 mM PMSF and 0.5 mM DTT as previously described (Denman, 2006; Dolzhanskaya et al., 2006). PBS and SNARF-1AM were purchased from Invitrogen. Protease inhibitors were purchased from Roche. ω-N^G-Monomethylarginine (MMA), ω-N^G, N^G-asymmetric dimethylarginine (aDMA), ω-N^G, $N^{G'}$-symmetric dimethylarginine (sDMA) and S-adenosyl-homocysteine (SAH) were obtained from Sigma. [^{3}H]-S-Adenosyl-L-methionine 15 Ci/mmol was purchased from MP Biomedical, Inc.

Proteins, peptides and antibodies

Recombinant PRMT1 and recombinant CARM1/PRMT4 were purchased from Millipore. Recombinant PRMT5 and PRMT7 were obtained from Origene. Histone H3 and H4 were purchased from Roche Applied Biosciences. Myelin basic protein (mouse) was obtained from Sigma. CIRP overexpression lysate and NOLA1 protein were purchased from GenWay. *FMR1* exon 15-17 truncation mutants, $FMRP_{Ex15a}$ and $FMRP_{Ex15b}$ were prepared as previously described (Dolzhanskaya et al., 2006; 2008). Biotinylated-SmD1peptide, biotin-KREAVAGRGRGRGRGRGRGRGRGGPRR, was synthesized by CPC Scientific.Antibodies to PRMT1, PRMT4, PRMT5 and PRMT7 were purchased from Millipore. Anti-Hsp70c mAb was obtained from Stressgen. Anti-dimethylarginine antibody, ASYM24, was purchased from Millipore.

In vitro translation systems

TNT rabbit reticulocyte lysate (RRL) *in vitro* translation lysate was purchased from Promega; the insect cell *in vitro* translation kit (ICL) was purchased from Qiagen.

Protein preparation and Western blotting

Proteins were prepared from cultured cells as previously described (Sung et al., 2003). Western blotting was performed as described (Sung et al., 2003). Rabbit polyclonal antibodies to ASYM24, PRMT1, PRMT4, PRMT5 and PRMT7 were used at a 1:300, 1:1000, 1:1000, 1:500 and 1:500 dilution of primary antibody, respectively. HSP70c was detected using a 1:5000 dilution. A 1:5,000 dilution of HRP-conjugated goat anti-rabbit secondary antibody or HRP-conjugated goat anti-mouse secondary antibody (Pierce) was used for detection. Blots were blocked for 1 h at room temperature in PBS supplemented with 3% non-fat dry milk and probed overnight in fresh buffer with the corresponding primary antibody at 4°C. Blots were developed using the PicoTag system (Pierce).

In vitro methylation assays

In vitro methylation by the endogenous methyltransferases (MTs) of the proteins in cultured cell lysates (30 µg), or *in vitro* translation lysates (30-50 µg) or various substrate proteins (1 µg) was performed in either HMTase-Buffer (50 mM Tris-HCl pH 9, or pH 7.5, 0.5 mM DTT) or PBS supplemented with 1 µCi of ^{3}H-SAM in a total volume of 20 µl. *Because ^{3}H-SAM is reconstituted in weak acid the actual pH of these reactions is 8.3 and 7.0, respectively.In vitro* methylation by recombinant PRMTs were performed by denaturing the cell lysates (30 µg), or *in vitro* translation lysates (30-50 µg) for 3 min at 75°C and then incubating them in either HMTase-Buffer (50 mMTris-HCl pH 9, 0.5 mM DTT) or PBS supplemented with 1 µCi of ^{3}H-SAM and the indicated PRMT(1 µl, 0.1 µg) in a total volume of 20 µl. The methyltransferase dependence of the incorporated ^{3}H was demonstrated by the addition of 1000 µM S-adenosyl-homocysteine (SAH) to the reactions. Incubations were allowed to proceed for 2 h at 30°C. Following the incubation, an equal volume of 2x Laemelli buffer was added to the reaction mixtures and the proteins were resolved in duplicate on SDS-polyacrylamide gels. The gels were fixed, in 30% methanol, 10% acetic acid overnight. After removing the fixing solution the gels were either soaked in En^{3}Hance (Perkin Elmer) for 1 h and then water for 30 min. and then dried and subjected to fluorography as described, or stained with Coomassie Brilliant Blue (Dolzhanskaya et al., 2006). *In vitro* methylation of the biotinylated-SmD1 peptide (100 pmols) by the endogenous methyltransferases (MTs) of the proteins in cultured cell lysates (30 µg), or *in vitro* translation lysates (30-50 µg) or recombinant PRMTs (0.2 µg) was performed in either HMTase-Buffer (50 mM Tris-HCl pH 9, 0.5 mM DTT) or PBS

Table 1. PRMT Primers.

Name	Sequence	Size (bp)
PRMT1-F	5'GAGGCCGCGAACTGCATCAT3'	374, 428, 545
PRMT1-R	5'TGGCTTTGACGATCTTCACC3'	
PRMT3-F	5'GCAGTTGCTGGGTACTTTGATA3'	247
PRMT3-R	5'TCACTGGAGACTGTAAGTCTGG3'	
PRMT4-F	5'GCTGTGGCTGGAATGCCTACT3'	109, 179, 306, 584
PRMT4-R	5'TCCCTGGGCACCTGAGGACCT3'	
PRMT5-F	5'CACGAAGGCCAGAACATCTG3'	220
PRMT5-R	5'ATGTTCTACACCTTCTGTGC3'	
PRMT7-F	5'CAACAGCCTATGCAATCCAAGGGCAC3'	320
PRMT7-R	5'CTCAATAAGAGATCAGCTCAAGGTG3'	
PRMT8-F	5'GACATTTACACTGTGAAGACGG3'	434, 375
PRMT8-R	5'GATACAGATGTTTCACACAGCTG3'	

supplemented with 1 µCi of ^{3}H-SAM in a total volume of 20 µl. Incubations were allowed to proceed for 2 h at 30°C. Subsequently, 150 pmol of Tetralinkavidin resin (Promega) was added and the biotinylated peptide was captured at 4°C for 20 min. on a rotating mixing platform. The resin was centrifuged at 14K for 2 min. and the supernatant was removed. The resulting pellet was washed twice with 100 µl of buffer and the bound radioactivity counted in 10 ml of Filtron X (National Diagnostics). Competition reactions were performed using either 1000 µM SAH or 36 mM biotin (Sigma). Background values were measured in mock reactions containing all of the components except the methyltransferases(s) and were subtracted from the sample values.

Methylarginine amino acid analysis

In vitro methylation reactions (20 µl) containing 1 µCi of ^{3}H-SAM were hydrolyzed with 250 µl of 6 N HCl at 110°C for 21 h in a sealed glass ampule. The hydrolyzed amino acids were dried in an oven after opening the ampule. Ten microliters of water was added to the dried residue, and then 1 µl of the solution was applied to each lane of a Silica 60 TLC plate (Whatman) along with 1.0 µg(0.5 µl) ofeach of the standards ω-N^{G}-monomethylargininеacetatesalt (ω -MMA; SigmaM7033), asymmetric ω-N^{G},N^{G}-dimethylarginine hydrochloride (aDMA; Sigma D4268) and ω-N^{G}, $N^{G'}$-symmetric dimethylargininedi(p-hydroxyazobenzene-p′-sulfonate) salt (sDMA; Sigma D0390) for amino acid analysis by TLC. Sodium citrate buffer (0.35 M Na^{+}, pH5.27) was used to separate the amino acids (Miranda et al., 2004). Under these conditions aDMA and sDMA are not completely resolved and therefore, we consider the composite peak their sum, which we designate dimethylarginine (DMA). Color was developed with a ninhydrin spray (Sigma) following incubation of the plate at 55°C for 15 min.The plates were scanned with an Epson Perfection V300 scanner and the resulting image data converted to a ninhydrin intensity distributions using UN-SCAN-ITGel 6.1 (Silk Scientific Software). MMA and DMA spots were subsequently scraped into liquid scintillation vials containing 5 ml Filtron X (National Diagnostics) and were subsequently counted.

RNA Isolation and RT-PCR

RNA was extracted from cells and tissues using RNAeasy Mrna mini columns (Qiagen). Purified RNA was eluted in 25 µl of DEPC-treated H_2O and quantified spectrophotometrically. One microgram of RNA was used to prepare first strand cDNA (Invitrogen). Aliquots of the cDNAs were amplified in a Perkin Elmer GeneAmp 9700 thermocycler using the primers in Table 1 and the following cycling parameters: 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. A range of cDNA concentrations and cycles was used to insure accurate quantification as previously described (Xie et al., 2009). Reaction products were resolved on 1–2% TAE agarose gels along with appropriate size markers and plasmid controls. Gels were imaged using a Scion CFW -1308M mega pixel camera and captured using FOTO/Analyst PC Image software version 9.04 (FOTODYNE).The resulting image files were digitized and analyzed using UN-SCAN-IT gel version 6.1 (Silk Scientific, Inc).

Cell culture

P19 cells were cultured in ω-MEM supplemented with 10% fetal bovine serum. Differentiation of rapidly growing cultures into neuronal and glial cells was accomplished *via* the method of Jones-Villeneuve (Jones-Villeneuve et al., 1982). All of the results presented here correspond to cells cultured for 6-8 days in the presence or absence of 0.1 µM retinoic acid.

Retinoic differentiated P19 cells were cultured on 40 mm coverslips.Twenty four hours later the cells were loaded with the pH-sensing fluorescent dye SNARF-1AM, 4 µM 30 min, 37°C, according to the manufacturer's instructions. Subsequently, the dye was washed out and the cover slips mounted in a Sykes-Moore chamber (Bellco Glass Inc., Vineland, NJ). The cells were maintained at 37°C with an air curtain incubator (NevTek, Williamsville, Va). Baseline images of the cells were acquired using a Nikon 90i microscope coupled to a Nikon C1 three-laser scanning confocal system (NIKON Instruments Inc., Melville, NY) by exciting at 488 nm and recording at 580 nm and 640 nm. The cells were treated

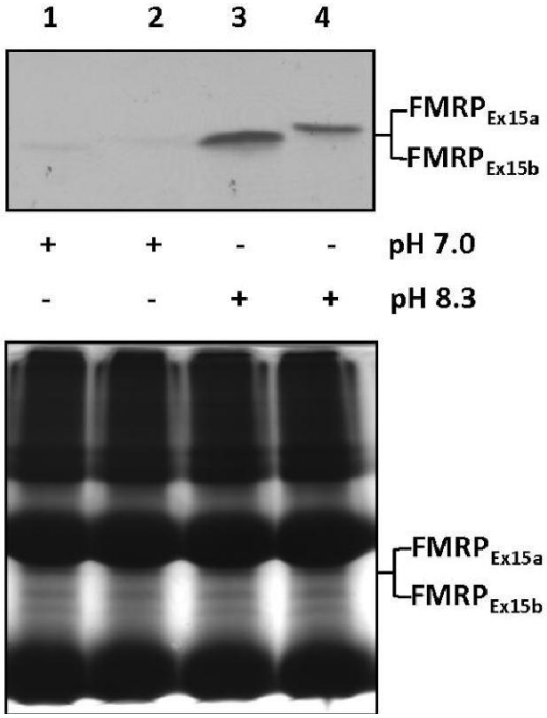

Figure 1A. Increasing pH facilitates FMRP *in vitro* methylation. Equal amounts of $FMRPE_{x15a}$ and $FMRPE_{x15b}$ were methylated with 30 µg of rabbit reticulocyte lysate at pH 7.0 and pH 8.3 as indicated. The reactions were resolved by SDS-PAGE and subject to fluorography.

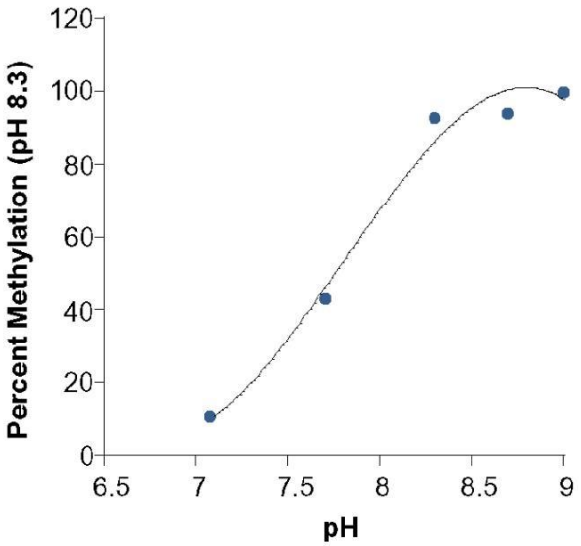

Figure 1B. Increasing pH facilitates FMRP *in vitro* methylation. Relative methylation of $FMRPE_{x15a}$ at various pHs. Fluorgram intensities were compared to the intensity at pH 8.3, which was arbitrarily set to 100%.Values plotted are the average of two experiments whose means differ by less than 10%.

or not treated with NH_4Cl (100 mM) and timelapse images recorded at 580 nm and 640 nm in 1 min intervals over the course of 12-20 min. The images were thresholded using Image-Pro (version 5.1) to define the cells and the ratio of fluorescence intensities, 640 nm/580 nm, within the cells was computed. The results were exported to a Microsoft Excel spread sheet for statistical analysis.

RESULTS

Increasing pH facilitates the *in vitro* methylation of select substrates.

We previously hypothesized that since histone lysine protein methyltransferases such as DIM5 and protein arginine methyltransferases PRMT1 and PRMT3 are especially active at alkaline pH (Cheng et al., 2005; Zhang and Cheng, 2003) increasing the pH of *in vitro* methylation reactions should increase PRMT-dependent incorporation of ^{3}H-SAM into its substrates (Denman, 2006). Figure 1A illustrates the results of the *in vitro* methylation of two truncated FMRP splice variants, $FMRP_{Ex15a}$ and $FMRP_{Ex15b}$, by the protein methyltransferases in rabbit reticulocyte lysate (RRL) assayed in PBS buffer (reaction pH 7.0) and HMTase buffer (reaction pH 8.3). It is quite evident in this case that the higher pH favors incorporation of ^{3}H-SAM into these proteins. In fact, by examining a range pH values it can be shown that maximal ^{3}H-SAM incorporation into $FMRP_{Ex15a}$ occurs at a pH of 8.3, Figure 1B. Similar results were observed when we substituted an HMTase buffer (reaction pH 7.0) for PBS indicating that the effects were pH- rather than buffer-dependent. Thus, all subsequent experiments used this buffer for *in vitro* methylation reactions at the lower pH.

These data agree with that reported for the pH dependence of myelin basic protein (MBP) *in vitro* methylation by CARM1 (Denman, 2008). To examine whether other substrates were similarly affected by altering the reaction pH we compared the methylation of the FXRP family of proteins (FMRP, FXR1P, FXR2P), along with PABP by RRL at pH 7.0 and pH 8.3. Interestingly, in each of these cases, ^{3}H-SAM incorporation into the full-length proteins preferentially occurred at pH 7.0 rather than at pH 8.3, Figure 1C; nevertheless, the range of differences were profound. Full-length FMRP was much more significantly affected by changing pH than were the other FXRPs, whereas PABP methylation was not greatly affected. Similar results were obtained when we assessed the methylation of histone H3, histone H4, MBP and NOLA by RRL at pH 7.0 and pH 8.3, Figure 1D. Finally, we also examined the effect of pH on the methylation of the cold-inducible RNA binding protein, CIRP, expressed in

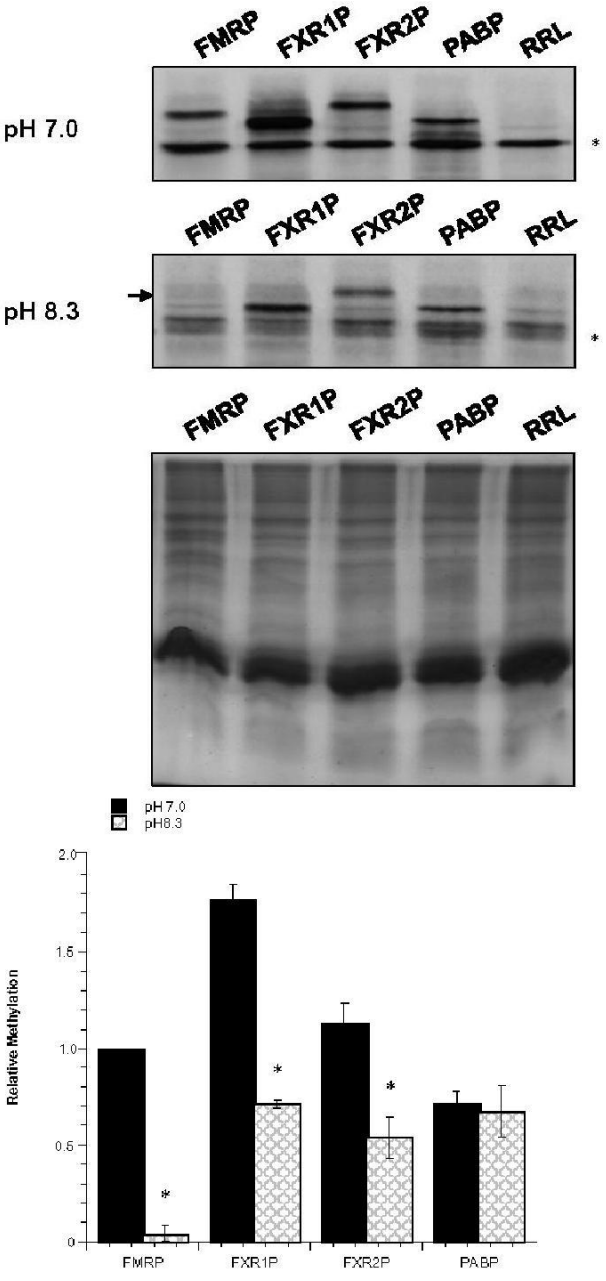

Figure 1C. Increasing pH facilitates FMRP *in vitro* methylation. Rabbit reticulocyte lysates (30 µg) programmed to express full-length FMRP, FXR1P, FXR2P, PABP or no protein were incubated in the presence of ^{3}H-SAM at pH 7.0 and pH 8.3. The methylated proteins were resolved by SDS-PAGE and subject to fluorography. The asterisk marks a non-specific methylated RRL protein. The arrow in the second panel marks the location of FMRP. The relative protein loads are shown by the Coomassie gel in the third panel. The graph shows the relative methylation of each protein normalized to FMRP at pH 7.0.Values represents the means ± sem for three determinations/protein. For each member of the FXRP family methylation preferentially occurred at pH 7.0 (P=0.007 for FMRP, P=0.003 for FXR1P, P=0.05 for FXR2P by ANOVA).

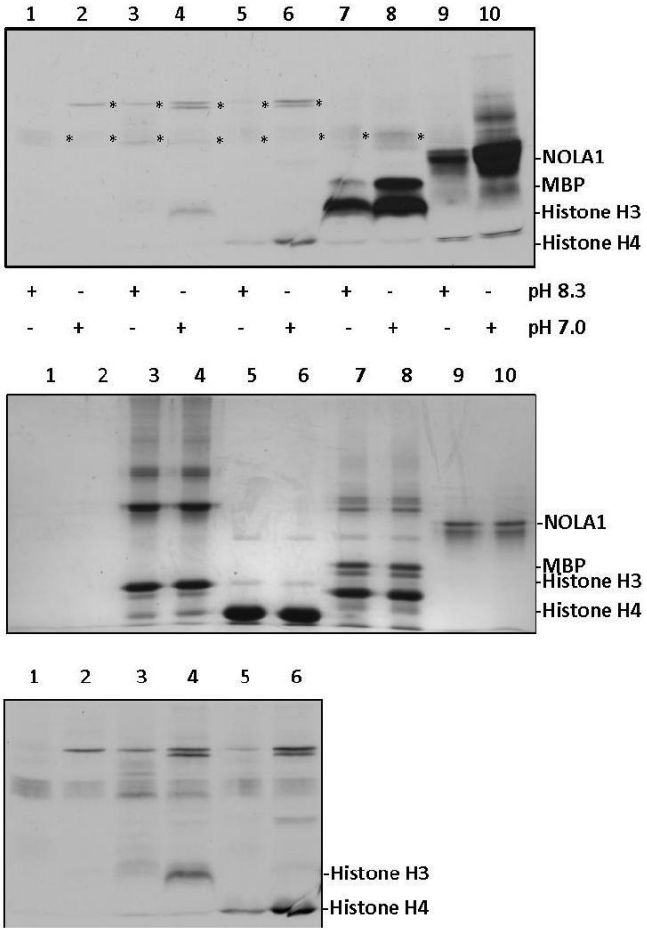

Figure 1D. Increasing pH facilitates FMRP *in vitro* methylation. Rabbit reticulocyte lysates (30 µg) were incubated in the presence of ^{3}H-SAM and in the absence or presence of 1 µg of histone H3, histone H4, myelin basic protein (MBP) or NOLA1 at pH 7.0 and pH 8.3. The methylated proteins were resolved by SDS-PAGE and subject to fluorography.The asterisks mark non-specific methylated RRL proteins.The relative protein loads are shown by the Coomassie gel beneath the fluorgram. An over exposure of lanes 1-6 is shown below the protein loads.

HEK293 cells. In this case, the protein was more highly methylated at pH 8.3 than at pH 7.0, Figure 1E.

pH uniquely modulates the methylation of cellular proteins.

The aforementioned data suggested that *in vitro* methylationof different protein substrates is uniquely modulated by pH. To explore this question more generally we compared the ability of the endogenous protein methyltransferases in *Spodoptera fugiperda* cell lysates to methylate insect cell proteins at pH 7.0 and pH 8.3. We chose insect cell lysates because unlike most cell lysates there are a host of endogenous proteins that

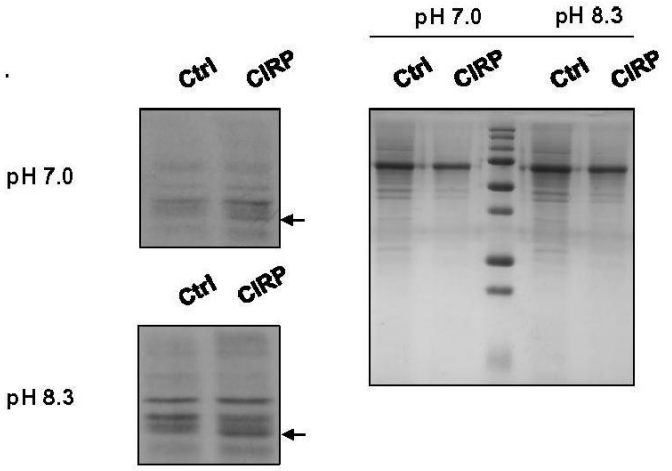

Figure 1E. Increasing pH facilitates FMRP *in vitro* methylation. HEK293 control and CIRP overexpressing cell lysates (30 µg) were incubated in the presence of ^{3}H-SAM at pH 7.0 and pH 8.3. The methylated proteins were resolved by SDS-PAGE and subject to fluorography. The arrow marks CIRP. The relative protein loads are shown by the Coomassie gel to the right of the fluorgram.

are hypomethylated, yet the lysates have abundant protein methyltransferase activity (Denman, 2008). In addition, to insure that the incorporation of ^{3}H-SAM into protein represented bona fide methylation, the reactions were carried out in the absence or presence of the methylation inhibitor S-adenosyl-homocysteine (SAH). Figure 2 shows the results of this experiment. In this case, the*in vitro* methylation of most *Spodoptera fugiperda* proteins was significantly better at pH 7.0; however, there were several notable exceptions to this rule and again, in some cases, the methylation of particular substrates was not substantially affected by the reaction pH.

These data indicated that the various protein MTs and/or substrates in the insect cell lysate were differentially affected by pH. To continue to evaluate the effect of pH on protein methylation we compared the ability of the MTs in RRL to methylate insect cell proteins. To insure that the methylation was a direct result of the input MTs from RRL we first denatured the insect cell proteins. Figure 3A shows that following denaturation at 75°C insect cell proteins are no longer able to be methylated by insect cell MTs.Two interesting results occurred when the MTs in RRL were used to methylate the insect cell proteins, Figure 3B. First, it was obvious that many of the insect cell substrates that were methylated by the endogenous insect cell MTs were not methylated by the MTs in RRL (compare Figure 2 with Figure 3B). Secondly, of the substrates that were methylated by RRL MTs, most were preferentially methylated at pH 8.3.

The difference between insect cell methylation patterns

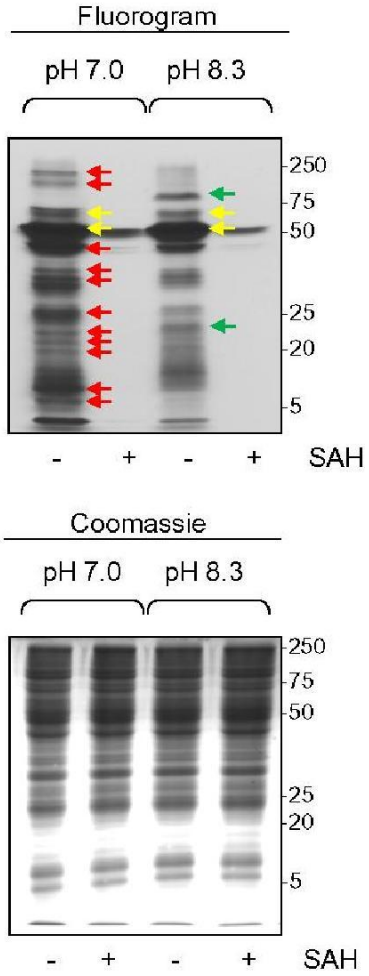

Figure 2. Alterations in pH affect the *in vitro* methylation of native insect cell proteins. Native insect cell lysates (30 µg) were incubated in the presence of ^{3}H-SAM and in the absence and presence of 1000 µM SAH at pH 7.0 and pH 8.3. The methylated proteins were resolved by SDS-PAGE and subject to fluorography. Red arrows indicate substrates that are preferentially methylated at pH 7.0; green arrows indicate substrates that are preferentially methylated at pH 8.3; yellow arrows indicated substrates that are equally methylated at both pHs. The relative protein loads are shown by the Coomassie gel beneath the fluorgram.

observed when the extracts were methylated by endogenous insect cell MTs as opposed to the RRL MTs might be due to changes in the type, amount or specificity

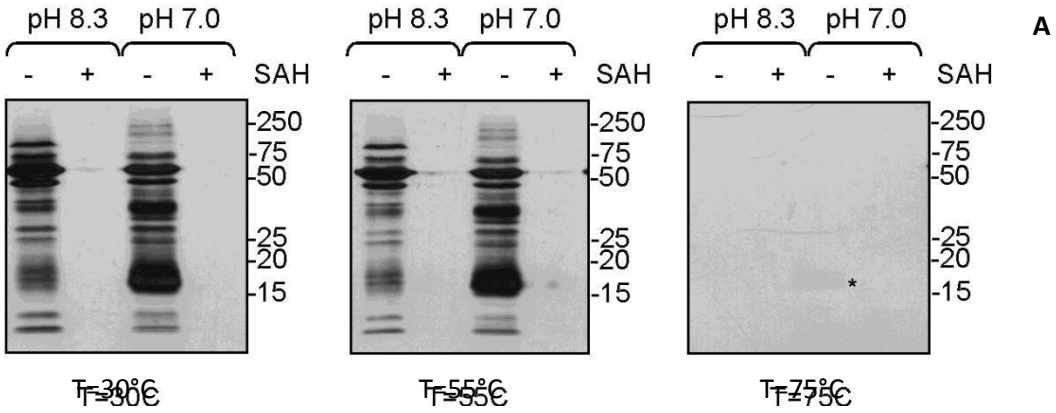

Figure 3A. Rabbit reticulocyte lysate differentially methylates denatured insect cell proteins depending on the pH of the reaction. Insect cell lysates (30 µg) were incubated in the presence of ^{3}H-SAM, and in the absence and presence of 1000 µM SAH at pH 7.0 and pH 8.3 at the indicated temperatures. The methylated proteins were resolved by SDS-PAGE and subject to fluorography. The asterisk marks residual methylation at 75°C.

of the two populations of MTs, or it might be due to the fact that for the RRL methylation experiment the insect cell proteins were denatured by the heat inactivation of the insect cell MTs. To examine this question more closely we next assessed the effect recombinant human PRMTs had on denatured insect cell proteins at pH 7.0 and pH 8.3 to determine whether the methylation patterns produced would mimic those with the endogenous insect cell MTs or RRL MTs. Specifically, we examined two type I (hPRMT1 and hPRMT4) and two type II (hPRMT5 and hPRMT7) protein arginine methyltransferases.In every case, many more proteins were methylated by the recombinant PRMTs than were methylated by the MTs in RRL, Figure 4. Also, with the exception of hPRMT5, most of the methylation that occurred preferentially occurred at pH 7.0; nevertheless, for each hPRMT there were multiple substrates that were more highly methylated at pH 8.3.Again, these data demonstrate that pH uniquely modulates *in vitro* substrate methylation.

Since none of the methylated insect cell lysate proteins are known we decided to examine the effect pH had on the methylation of a well-characterized substrate by the human recombinant PRMTs. For this we chose a peptide corresponding to the RG-rich region of the SmD1 protein (Friesen et al., 2001). This protein is methylated by both type I and type II PRMTs *in vitro* and is differentially methylated by type II PRMTs in the cytoplasm and type I PRMTs in the nucleus (Miranda et al., 2004). Using an affinity-capture assay we demonstrated that the incorporation of ^{3}H-SAM into the biotinylated-peptide was due to methylation as it could be competed out with the methylation inhibitor SAH or with free biotin, Figure 5A. Next, we showed that the methylation of this particular substrate by the endogenous MTs in insect cell lysates was preferentially active at pH 8.3, Figure 5B. Finally, we assessed the effect of pH on SmD1 methylation by human recombinant PRMTs, Figure 5C. Here we found that the pH that the peptide was preferentially methylated depended entirely on the human PRMT that was being assayed.

pH alters the distribution of monomethylarginine and asymmetric dimethylarginine incorporated into cellular proteins

Although we have demonstrated that pH modulates the activity of *in vitro* methylation in both substrate choice and the extent of methylation we had no information regarding whether and if pH modulates the distribution of monomethylarginine (MMA), asymmetric dimethylarginine (aDMA) or symmetric dimethylarginine (sDMA) incur-porated into cellular proteins. To begin to address this question we methylated NOLA1 using the endogenous protein methyltransferases in insect cell lysates at pH 7.0 and pH 8.3. Figure 6A shows that NOLA1, which migrates as a two bands ca. 22 and 18 kDa, is preferentially methylated at pH 7.0.A Western blot of these extracts, which was probed with an antibody that detects a subset of asymmetrically dimethylated arginine residues (ASYM24), demonstrated that (1) NOLA1 is specifically detected by anti-ASYM24 and (2) there is no corresponding increase in the level of aDMA in the pH 7.0 NOLA1-treated sample over the pH 8.3 NOLA1-treated sample, Figure 6B. These data imply that at pH 7.0a DMA is likely not incorporated into NOLA1.

To further examine this question we next hydrolyzed insect cell lysates that had been methylated at either pH

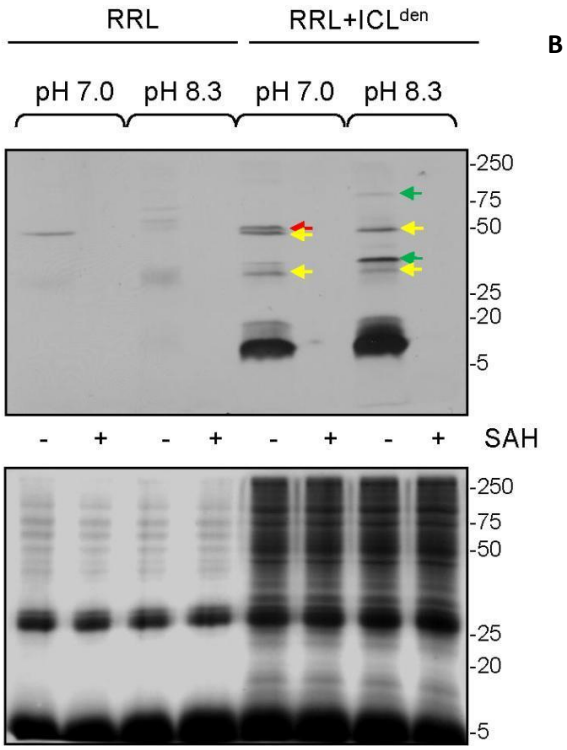

Figure 3B. Rabbit reticulocyte lysate differentially methylates denatured insect cell proteins depending on the pH of the reaction. Denatured insect cell lysates (30 µg) were incubated in the presence of ^{3}H-SAM, RRL and in the absence and presence of 1000 µM SAH at pH 7.0 and pH 8.3 as indicated. The methylated proteins were resolved by SDS-PAGE and subject to fluorography. Red arrows indicate substrates that are preferentially methylated at pH 7.0; green arrows indicate substrates that are preferentially methylated at pH 8.3; yellow arrows indicated substrates that are equally methylated at both pHs. Control lanes 1-4 show endogenous methylation of RRL proteins at both pHs.The relative protein loads are shown by the Coomassie gel beneath the fluorgram.

7.0 or pH 8.3 in the presence of ^{3}H-SAM into their amino acids and separated the methylarginine residues (MMA and aDMA) by TLC. The results, shown in Figure 6C, demonstrate that there is an approximate two-fold increase in the amount of MMA incorporated into protein at pH 7.0 relative to that at pH 8.3, confirming the results obtained with NOLA1 methylation.

Transient increases in intracellular pH produce sustained changes in cellular protein methylation

Can changes in intracellular pH drive alterations in protein arginine methylation? To begin to address this question we used NH_4Cl to produce a transient intracellular alkalinization (Raley-Susman et al., 1991) in retinoic acid-differentiated mouse P19 cells.The alkalinization was monitored using SNARF-1AM, one of a family of fluorescent indicator dyes the ratio of whose emission wavelengths change as a function of pH (Roberts, 1999). Quite simply cells were loaded with SNARF-1AM and after a specified time the unabsorbed dye was washed out. The dye-loaded cells were then stimulated at a wavelength of 480 nm and the fluorescence intensities at 640 nm and 580 nm simultaneously recorded over time. Figure 7A shows that for untreated P19 cells the ratio the of fluorescence intensities at 640 nm and 580 nm (F_{640}/F_{580}) does not markedly change over the time we observed indicating that the intracellular pH was relatively stable. Nevertheless, when P19 cells were exposed to 100 mM NH_4Cl they exhibited a robust increase in F_{640}/F_{580} which lasted for 10 min, consistent with the hypothesis that NH_4Cl induces a sustained intracellular alkalinization, Figures 7A and 7B.

If NH_4Cl treatment alters either the extent or the specificity of substrate methylation one would expect to see a change in either the pattern or extent of methylation of the endogenous substrates in these extracts. To examine this question we first performed *in vitro* methylation reactions on P19 cell control and NH_4Cl-treated protein extracts in the presence of ^{3}H-SAM. The methylated proteins were then resolved by SDS-PAGE and subjected to fluorography. We discovered a general diminution of most of the methylated proteins, indicating that more of the P19 MT substrates had been methylated upon NH_4Cl treatment and were therefore unavailable for *in vitro* methylations than the control extracts, Figure 7C. To confirm that the implied changed occurred, we next assessed the PRMT activity in these extracts by supplementing them with ^{3}H-SAM and various exogenous protein substrates and comparing the extent of methy-lation. Strikingly, we again observed substrate-specific differences in the ability of these extracts to methylate particular proteins, Figure 7D. Specifically, NH_4Cl treat-ment greatly increased the ability of histone H4 and the SmD1 peptide to be methylated; whereas methylation of MBP was only marginally increased and NOLA1 methylation actually decreased slightly.

The differences in the methylation potential of the alkalinized and control P19 cell extracts could be explained by differences in either the activity or the expression of P19 PRMTs. To determine which PRMTs were expressed in P19 cells we used RT-PCR to assess PRMT mRNA expression. We found that both undifferentiated and retinoic acid-differentiated P19 cells express nearly identical amounts of PRMT1, PRMT3, PRMT4, PRMT5, PRMT7 and PRMT8 and that the splicing pattern between the two cell types also did not

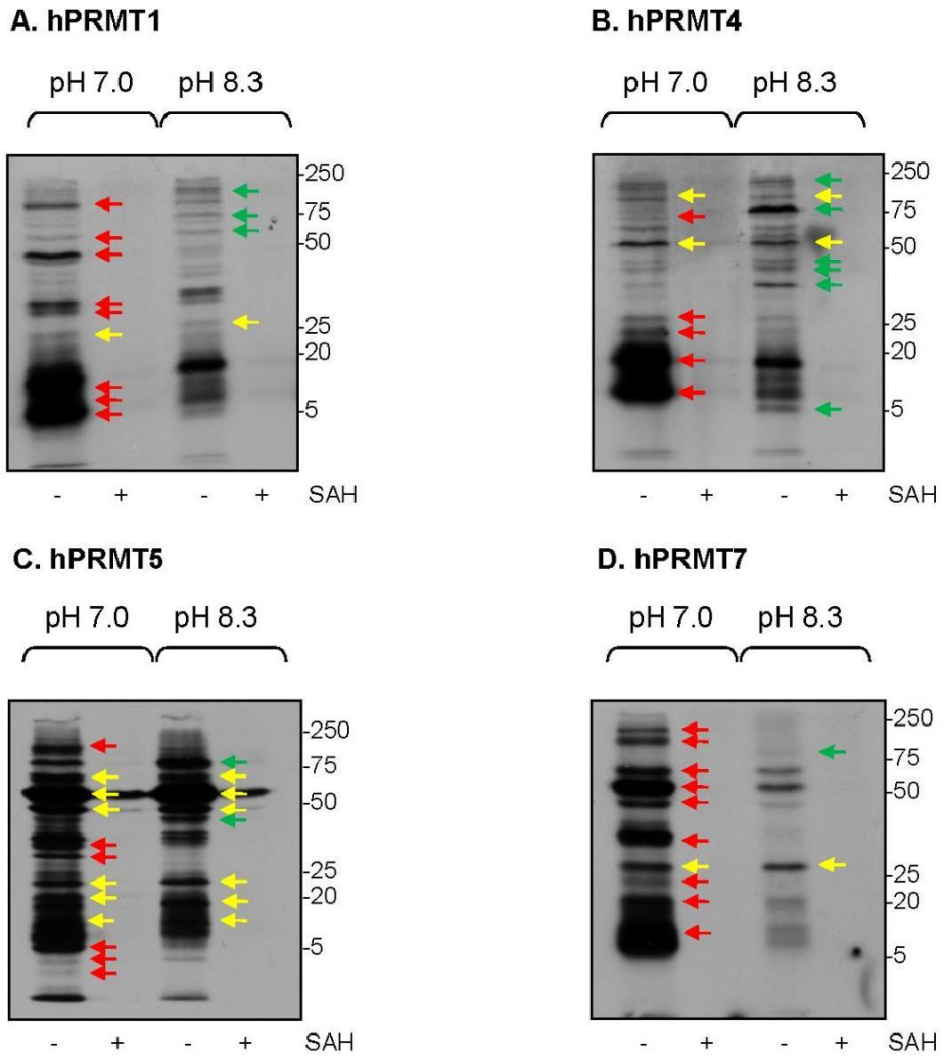

Figure 4. Recombinant human PRMT methylation of denatured insect cell proteins is affected by changes in pH. Denatured insect cell lysates (30 µg) were incubated in the presence of ^{3}H-SAM, a particular hPRMT and in the absence and presence of 1000 µM SAH at pH 7.0 and pH 8.3 as indicated. The methylated proteins were resolved by SDS-PAGE and subject to fluorography. Red arrows indicate substrates that are preferentially methylated at pH 7.0; green arrows indicate substrates that are preferentially methylated at pH 8.3; yellow arrows indicated substrates that are equally methylated at both pHs.

change significantly, Figure 8A. We then assessed the protein expression of PRMT1, PRMT3, PRMT4, PRMT5 and PRMT7 in the absence or presence of NH_4Cl, Figure 8B. PRMT1 and PRMT5 expression was robust and did not change as a function of treatment. On the other hand, we were unable to detect PRMT3 and PRMT7, indicating that these proteins are expressed at relatively low levels in P19 cells. Finally, PRMT4 protein expression, which was also weak, appeared to decrease slightly following NH_4Cl treatment. While not a comprehensive assessment of all 11 PRMTs these data indicate that the alterations in substrate methylation seen following NH_4Cl treatment are most likely due to changes in the activities of the PRMTs rather than in their expression.

DISCUSSION

The family of SAM-dependent protein arginine methyltransferases has several well-conserved domains including a catalytic core, which has two conserved motifs a Rossman fold and a β-barrel, three signature

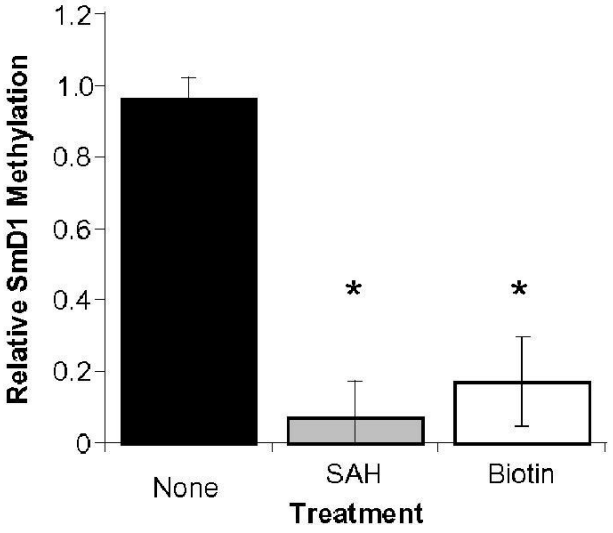

Figure 5A. Recombinant human PRMTs differentially methylate SmD1 at different pHs *in vitro*. SmD1 peptide was methylated by insect cell lysate(30 µg) in the presence of of 3H-SAM and in the absence or presence of 1000 µM SAH or 36 mM biotin as indicated. The incorporation of tritium into the peptide was measured by liquid scintillation counting.Values represent the means±sem for six determinations/group. ($P=1.12x10^{-9}$ for biotin compared to control and $P=3.1x10^{-6}$ for SAH compared to control by ANOVA.

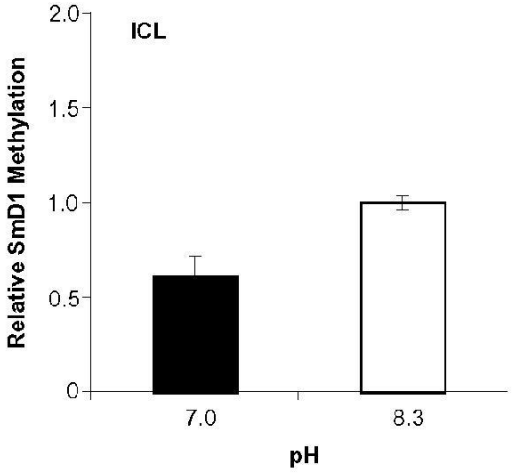

Figure 5B. Recombinant human PRMTs differentially methylate SmD1 at different pHs *in vitro*. SmD1 peptide was methylated by insect cell lysate (30 µg) in the presence of of ^{3}H-SAM at pH 7.0 and pH 8.3. Values represent the means±sem for six determinations/group. The extent of methylation at pH 7.0 was significantly different from that at 8.3 ($P=1x10^{-3}$ by ANOVA). The incorporation of tritium into the peptide was measured by liquid scintillation counting.

SAM-dependent methylation motifs (I, II and III) and two PRMT-specific motifs (VRT and THWY) (Troffer-Charlier et al., 2007). The catalytic forms of these enzymes are dimers, although culture cell studies have shown that the PRMTs exist in a wide range of higher-order complexes and in multimers that are also active (Lim et al., 2005). Several PRMTs have been crystallized and the resulting structures have led to the hypothesis that deprotonation of the target ω-amino group, which is require for catalysis, occurs as a result of ionic interactions with the Ado-Met sulfonium ion, hydrophobic interactions and ionic interactions with a conserved Glu residue; all these serve to lower the pKa of the target ω-amino group (Zhang and Cheng, 2003).

The requirement that the target ω-amino group be deprotonated implies that these enzymes would be more active at alkaline pH and indeed, several studies have shown that particular PRMT substrates were more actively methylated when the pH of the reaction buffer was raised (Denman, 2008; Dolzhanskaya et al., 2006; Dolzhanskaya et al., 2008). Here, however, we have demonstrated that simply raising the reaction pH is not a general mechanism for accelerating protein arginine methylation. Rather, our data show that the extent of methylation depends on the substrate assayed. This was particularly evident when we examined the FXRP family of proteins. While full-length FMRP was much more highly methylated at neutral pH, truncated forms of the protein were preferentially methylated at pH 8.3. This difference may be related to structural differences between the full-length and truncated proteins. X-ray crystallographic and NMR studies have shown that the C-terminal end of FMRP, which includes the methylation region, is unstructured (Adinolfi et al., 1999; Ramos et al., 2006). In contrast, molecular modeling studies of the truncated proteins have indicated that the sites of methylation lie within an extended β–sheet (Dolzhanskaya et al., 2008).

In addition to pH-induced substrate influences on methylation, we also demonstrated using a well-characterized single substrate, SmD1, that individual human recombinant PRMTs were differentially affected by changing pH, Figure 5. Thus, hPRMT1 was more active at pH 7.0 than pH 8.3, while hPRMT4, hPRMT5 and hPRMT7 were more active at pH 8.3 than at pH 7.0.

Cellular protein methylation occurs in a milieu containing multiple substrates and multiple methyltransferases. Using various cell lysates and cell extracts we next demonstrated that the pH at which *in vitro* methylation reactions were carried out greatly influence whether and to what extent endogenous proteins are methylated, Figures 2 to 4. Here, both the substrate variety and the extent of methylation varied significantly. At both high and low pH there were multiple substrates that were either preferentially or uniquely methylated. On the other hand, some substrates were

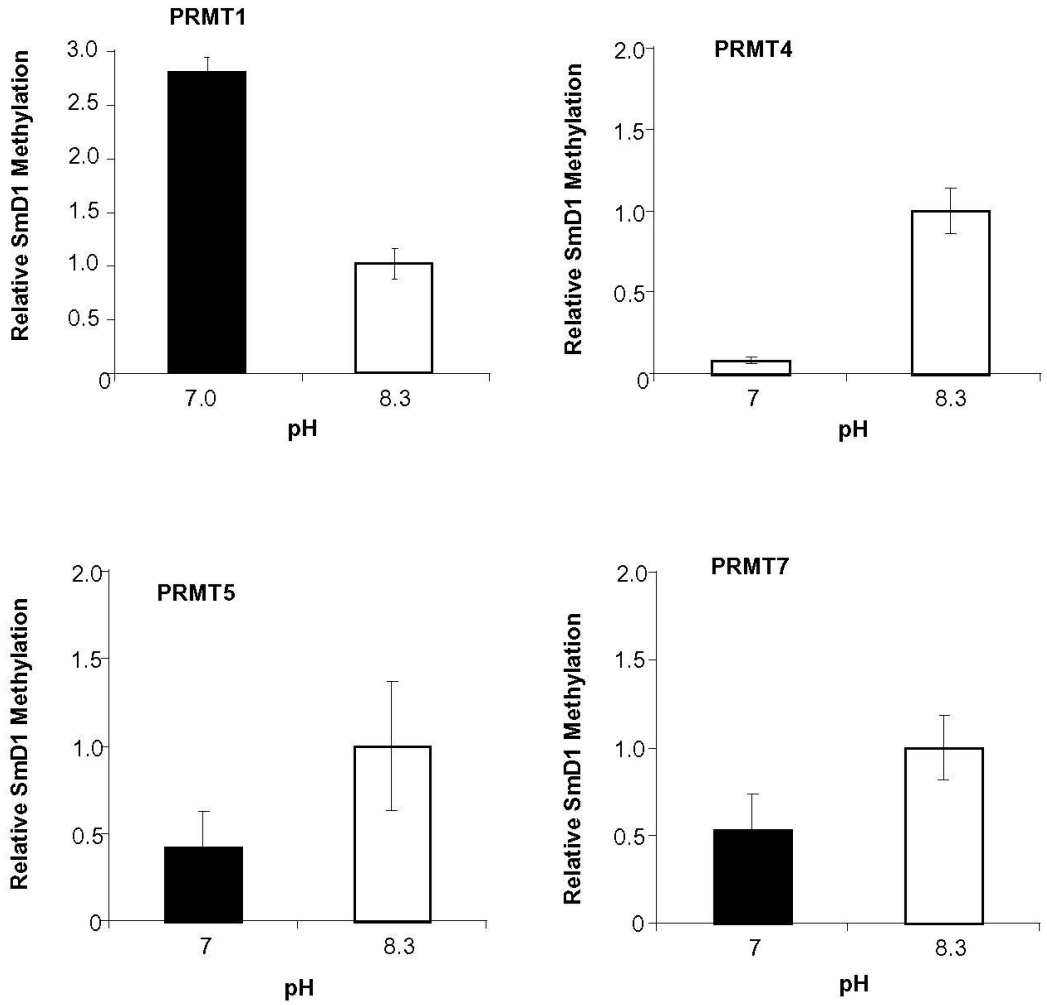

Figure 5C. Recombinant human PRMTs differentially methylate SmD1 at different pHs*in vitro*. SmD1 peptide was methylated by PRMT1, PRMT4, PRMT5 and PRMT7 in the presence of of ^{3}H-SAM at pH 7.0 and pH 8.3. The incorporation of tritium into the peptide was measured by liquid scintillation counting. Values represent the means±sem for four determinations/PRMT/group. The extent of methylation at pH 7.0 was significantly different from that at 8.3 ($P=2x10^{-4}$ for PRMT1, $P=7x10^{-5}$ for PRMT4, $P=9x10^{-5}$ for PRMT5 and $P=1x10^{-2}$ for PRMT7 by ANOVA).

not markedly affected by changing the pH. All of these data suggest that modulating a cell's intracellular pH should change both the extent and type of substrate proteins that are methylated.

What physiologically-relevant mechanisms do cells possess that would lead to pH-dependent methylation? As mentioned in the Introduction, intracellular and intra-organellar pH is tightly controlled and it is so because as little as a 0.5 unit change in pH can result in cell death (Garcia-Moreno, 2009). We investigated this question by artificially changing the extracellular pH that mouse embryonic P19 neurons were grown in using NH_4Cl. Previous studies had shown that bath application of NH_4Cl to the medium that hippocampal neurons were grown in resulted in sustained alkalinization of the cytosol (Raley-Susman et al., 1991). Using various inhibitors these investigators determined the principal regulator of the alkalinization was the Na^+/H^+ antiporter, which is known to be, expressed in both retinoic acid differentiated and undifferentiated P19 cells (Bierman et al., 1987; Wang et al., 1997).

We first verified that NH_4Cl treatment of retinoic acid-induced P19 cells led to a sustained intracellular alkalinization, Figures 7A and 7B. More importantly however, we found that the alkalinization of the cytosol led to alterations in endogenous protein methylation and

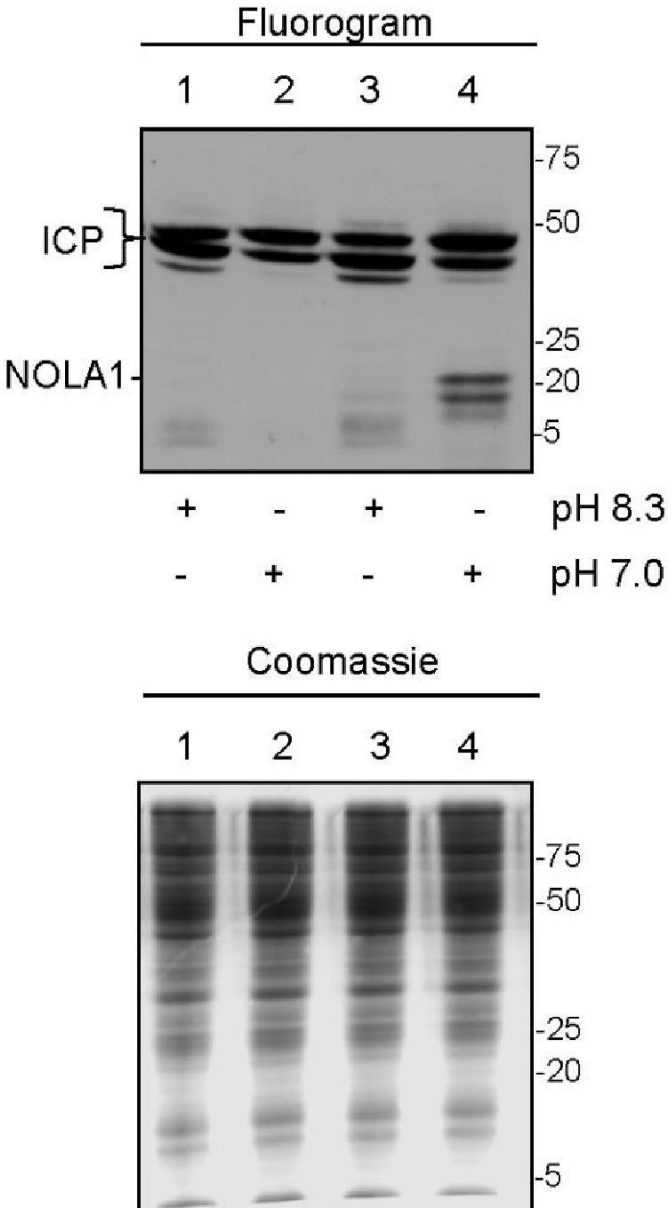

Figure 6A. pH alters the ratio of monmethyl to dimethylarginine. Insect cell lysates (30 µg) were incubated in the presence of ^{3}H-SAM and in the absence and presence of NOLA1 (1 µg) at pH 7.0 and pH 8.3 as indicated. The methylated proteins were resolved by SDS-PAGE and subject to fluorography. ICP marks insect cell proteins that are prominently methylated during the reaction; recombinant NOLA1 migrates as a doublet centered at 20 KDa. The relative protein loads are shown by the Coomassie gel beneath the fluorgram. Coomassie gel to the right shows the NOLA1 protein before methylation.

A

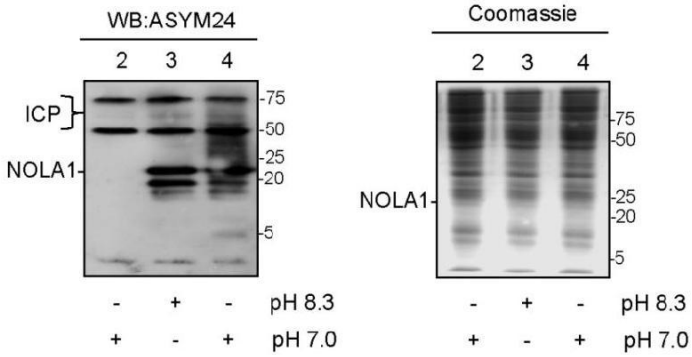

Figure 6B. pH alters the ratio of monmethyl to dimethylarginine. lanes 2-4 from Figure 6A were subject to Western blotting using anti-ASYM24.

in the methylation of exogenously added substrates. Furthermore, the changes were not accompanied by changes in the expression of PRMT1, PRMT3, PRMT4, PRMT5 or PRMT7, implying the changes arise from alterations in PRMT activity. The data also suggest that other systems that induce transient or sustained changes in intracellular or intra-organellar pH will secondarily alter protein methylation and we are in the process of verifying this prediction.

How might the physiological pH changes accompanying the activation of the Na^{+}/H^{+}antiporteror other pH sensors change protein arginine methylation without affecting PRMT expression? We speculate that the change in pH affects protein-protein interactions between various PRMTs and their regulators i.e. both activators and inhibitors. Precedence for this hypothesis can be found in papers by Jiang, Berthet and Guderian. Specifically, Jiang et al showed that adding the PRMT3/PRMT5 modulator Dal4.1B to *in vitro* methylation reactions of hypomethylated RAT-1 cells led to increases and decreases in the extent of methylation of particular substrates (Jiang et al., 2005). Likewise, BTG1, which binds to PRMT1, altered the extent of methylation of histone H2A (Berthet et al., 2002). Finally, RioK1, which binds to PRMT5, modulates the activity of the PRMT5 complex by enabling it to recruit and subsequently methylate the RNA binding protein nucleolin (Guderian et al., 2011). These changes in substrate methylation are reminiscent of the variations in substrate methylation we observe when the reaction pH is altered. Thus, it would be interesting to examine the influence pH has on the ability of each of these interactors to bind their respective PRMTs. Accordingly, one would expect that particular interactors would be either more or less associated with the particular PRMT at a given pH or that the expression levels of the interactors would fluctuate as a function of pH.

Besides changing substrate specificity and/or the extent of substrate methylation we also determined that pH influences the type of methylarginine product that is produced. Thus, at pH 7.0 there was an approximate

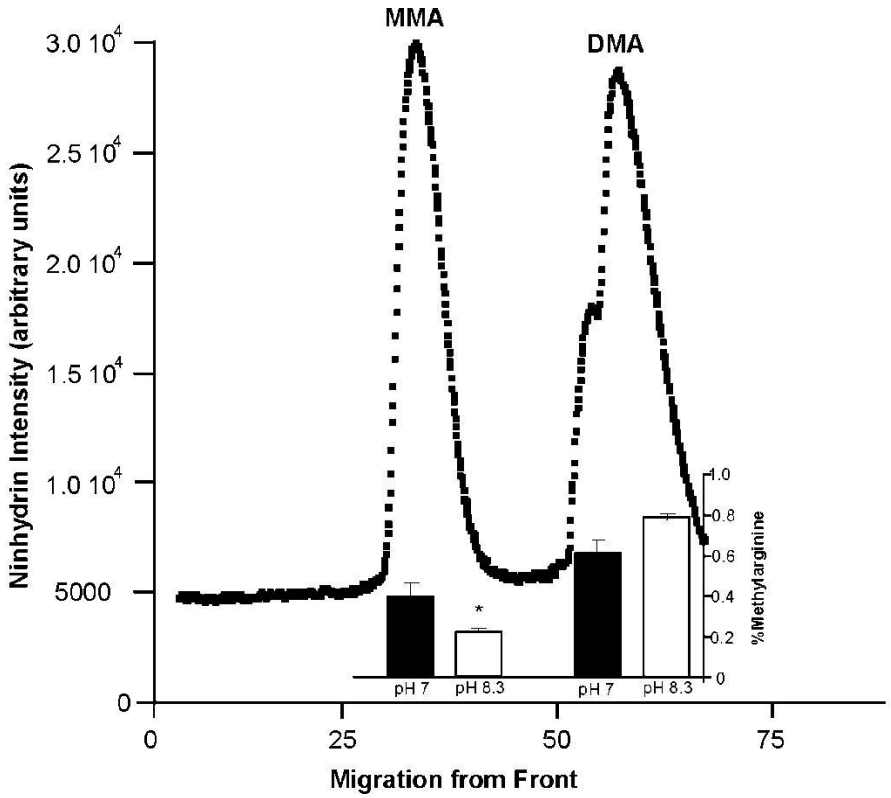

Figure 6C. pH alters the ratio of monmethyl to dimethylarginine. The distribution of MMA and DMA in *in vitro* methylated insect cell lysates at pH 7.0 and pH 8.3 was monitored byTLC. The graph shows the ninhydrin staining of the MMA and aDMA standards. The inset graph depicts the % distribution at the two pHs. Values represent the means±sem for four determinations/group.The distribution of MMA at pH 7.0 is significantly different from that at pH 8.3 ($P=3x10^{-4}$ by ANOVA).

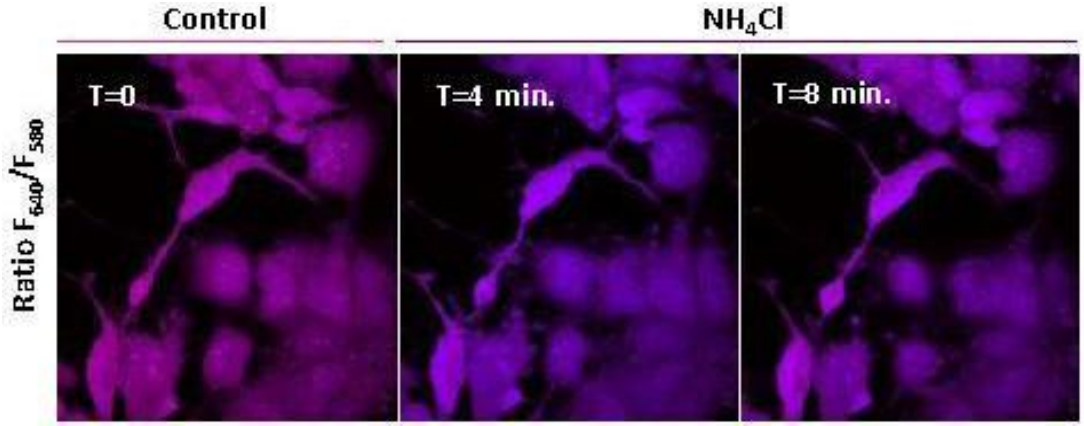

Figure 7A. Ammonium chloride induces a transient increase in intracellular pH and sustained increases in PRMT activity. Retinoic differentiated P19 cells (day 6) were loaded with the pH-sensing fluorescent dye SNARF-1AM. Subsequently, the cells were treated or not treated with NH_4Cl (100 mM) and subject to confocal fluorescence microscopy. The cells were excited at 488 nm and images recorded at 580 nm and 640 nm in 1 min intervals over the course of 12 min. (upper panel) Selected images of treated cells at 0, 4 and 8 min. are presented.

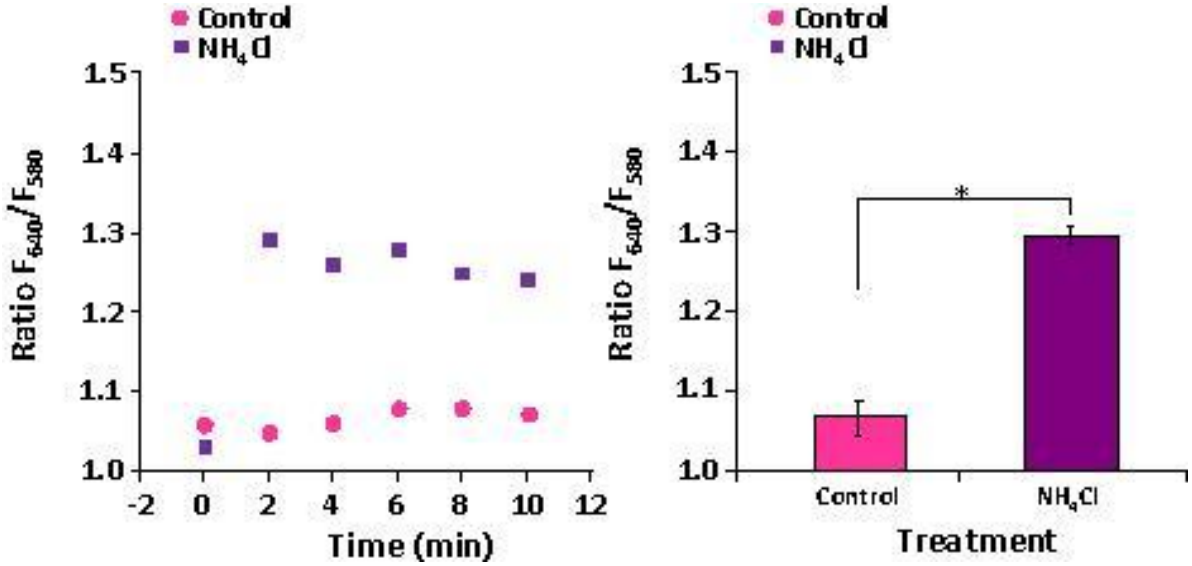

Figure 7B. Ammonium chloride induces a transient increase in intracellular pH and sustained increases in PRMT activity. The graph on the left shows representative values of the ratio of fluorescence intensities at 640 nm and 580 nm i.e. F_{640}/F_{580}, within the cells as a function of time for one experiment. The time-averaged data for three experimentsis shown in the graph on the right. The F_{640}/F_{580} ratio of NH_4Cl-treated cells were significantly different from control cells($P=3x10^{-3}$ by ANOVA).

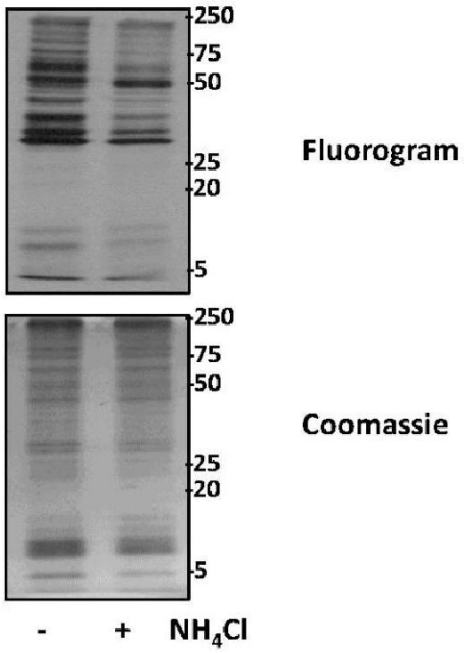

Figure 7C. P19 cell extracts from untreated control cells or NH4Cl-treated cells were supplemented with ^{3}H-SAM and the endogenous substrates in the extracts subject to *in vitro* methylation. The methylated proteins were resolved by SDS-PAGE and subject to fluorography.

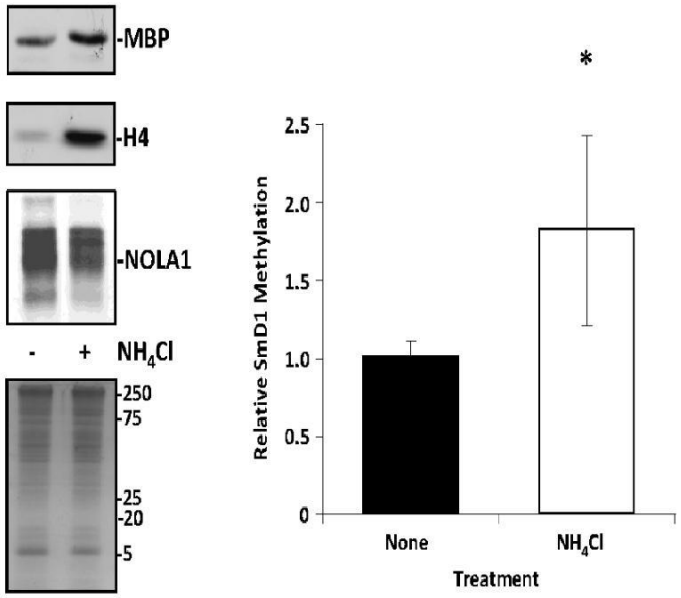

Figure 7D. P19 cell extracts from untreated control cells or NH4Cl-treated cells were supplemented with ^{3}H-SAM and various substrate proteins (1 µg) or the SmD1 peptide and subject to *in vitro* substrate methylation as described above. The methylated proteins were resolved by SDS-PAGE and subject to fluorography or liquid scintillation counting. The relative protein loads are shown by the Coomassie gel beneath the fluorogram. The methylation of the SmD1 peptide in the NH4Cl-treated samples was significantly different from the control ($P=3x10^{-3}$ by ANOVA).

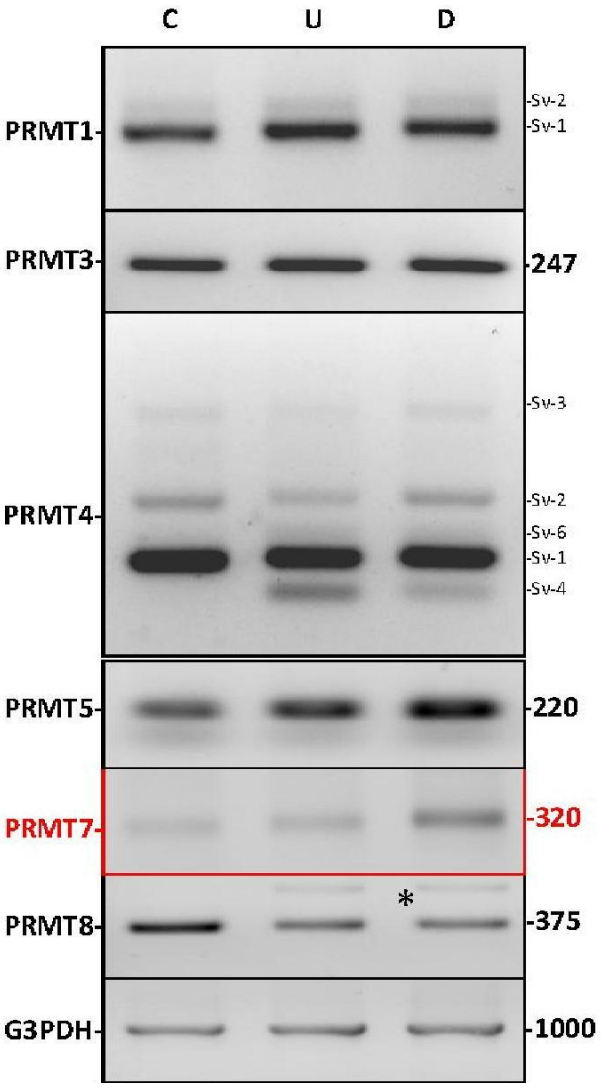

Figure 8A. PRMT mRNA expression in P19 cells. RNA isolated from mouse brain cortex (C), undifferentiated P19 cells (U) and retinoic acid differentiated P19 cells day 6 (D) was subject to RT-PCR using primers to PRMT1, PRMT3, PRMT4, PRMT5, PRMT7, PRMT8 and G3PDH. Primers amplifying PRMT1, PRMT4 and PRMT8 were designed to detect the various known alternatively spliced transcripts (sv) of each message. All amplifications were carried out with the same amount of cDNA for 35 cycles with the exception of PRMT7 (red box), which was carried out for 40 cycles in order to visualize the product.

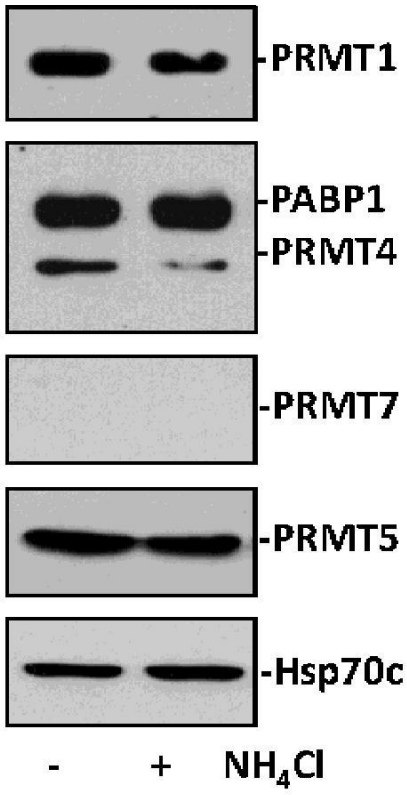

Figure 8B. PRMT mRNA expression in P19 cells. P19 cell extracts from untreated control cells or NH_4Cl-treated cells were subject to Western blotting and probed for PRMT1, 3, 4, 5, and 7 expression; Hsp70cp and PABP1 are shown as a protein load controls.

two-fold increase in the amount of monomethylarginine (MMA) that is incorporated into insect cell proteins relative to the amount of dimethylarginine (DMA), Figure 6.

All together our data are consistent with a hypothesis in which transient changes in intracellular or intraorganellar pH produce sustained changes in a cell's methylproteome. As protein methylation can affect protein-protein, protein-RNA interactions and subcellular localization (for reviews see (Xie and Denman, 2011; Yu, 2011) altered substrate methylation resulting from pH-induced changes of PRMT activities coupled with low levels of demethylation may provide the long-term tag(s) necessary for the formation and maintenance of "molecular memory".

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