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De novo DNA methyltransferase DNMT3A: Regulation of oligomeric state and mechanism of action in response to pH changes

Celeste Holz-Schietinger [a], Norbert O. Reich [a,b,*]

[a] Interdepartmental Program in Biomolecular Science & Engineering, University of California, Santa Barbara, CA 93106-9510, United States
[b] Department of Chemistry & Biochemistry, University of California, Santa Barbara, CA 93106-9510, United States

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ABSTRACT

Background: The oligomeric state of the human DNMT3A is functionally important and cancer cells are known to undergo changes in pH (intracellular).
Methods: Light scattering, gel filtration, and fluorescence anisotropy. Also, methylation and processivity assays.
Conclusions: Physiologically relevant changes in pH result in changes in DNMT3A oligomer composition which have dramatic consequences on DNMT3A function.
General significance: The pH changes which occur within cancer cells alter the oligomeric state and function of DNMT3A which could contribute to changes in genomic DNA methylation observed in vivo.
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1. Introduction

Covalent modification of DNA by methylation of cytosine residues is heritable and reversible and regulates a diverse range of biological processes. In mammals, DNA methylation is essential to cellular programing, memory formation, imprinting, silencing transposable elements, and gene regulation [1,2]. Malignant tumors undergo dramatic changes in their methylation patterns that correlate with altered transcriptional profiles [3]. DNA methylation is initially (de novo) established by DNMT3A and the closely related DNMT3B in a spatial and temporally regulated manner [4–8]. The DNMTs transfer a methyl group from the cofactor *S*-adenosyl-methionine (AdoMet) to cytosines primarily located in a CpG dinucleotide pair.

For many proteins, physiological conditions regulate function by altering substrate affinity, interactions with binding partners, and oligomeric state [9–11]. Hypoxia, extracellular acidification, and transient changes in intracellular pH occur frequently in tumors, and impact tumor growth and response to therapeutics [12,13]. Cancer cells usually have a decrease in extracellular pH from 7.8 to 6.8 [12]. Gene expression is greatly altered by intracellular changes in pH, due in part from changes in mRNA stability [14,15] and DNA binding proteins changing their protein–protein and protein-DNA complex affinities, including NF-κB, AP-1 [15] and Sp1 [16]. Sp1 binds CpG sites, protecting individual regions from methylation and is activated by increasing acidity [16]. pH changes are also known to alter the oligomeric state of many proteins, including a p53 mutant [17], where tetramerization is disrupted and thus DNA binding. It is proposed that the combination of the p53 mutation and altered pH in cells leads to adrenocortical carcinoma. DNA methylation patterns change during cancer progression but the mechanism that drives altered patterning is mostly unknown. Thus, small changes in pH that alter protein–protein and protein–nucleic acid interactions may contribute to the changes in methylation patterning observed in malignant cells. In addition, during development intercellular pH changes have been observed ranging from about 0.1 to 1.6 pH units in many diverse organisms [18].

Recently, the oligomeric state of DNMT3A was shown to be important in regulating the catalytic properties of the enzyme [19].[1] DNMT3A interacts with DNMT3L, a non-catalytically active homologue [20–22] to form a DNMT3L:DNMT3A:DNMT3A:DNMT3L heterotetramer complex and the interface along which DNMT3L binds (the tetramer interface) also supports DNMT3A homotetramerization in the absence of DNMT3L [19]. At the opposite side of DNMT3A's catalytic domain in the recognition domain, DNMT3A also oligomerizes (dimer interface) [23].

* Corresponding author at: Department of Chemistry & Biochemistry University of California, Santa Barbara, California 93106-9510, United States. Tel.: +1 805 893 8368; fax: +1 805 893 4120.
E-mail address: reich@chem.ucsb.edu (N.O. Reich).

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DNMT3A can even further oligomerize. Disruption of either interface by mutations has modest effects on the catalytic activity of the enzyme but significant consequences to the ability of the enzyme to carry out multiple turnovers on the same strand of DNA. Disruption of homo-oligomerization in DNMT3A also has been shown to disrupt binding to nucleosomes and is necessary for heterochromatic localization [24]. Recurrent DNMT3A mutations are also found in AML and myelodysplastic syndrome MDS patients with poor survival outlook [25,26]. The majority of the mutations, mainly at the dimer interface and accounting for ~15% of adult AML cases, were found to disrupt oligomerization in vitro and suggest the disruption of oligomerization has a significant role in the function of DNMT3A in vivo.[1] Therefore, disruption of the oligomeric state of DNMT3A may provide a mechanism for the elimination of clustered methylation observed in cancer patients [19].

In this study, we evaluated the functional significance of pH on DNMT3A. Acidification by one pH unit, from 7.8 to 6.8, completely disrupts the dimer interface of DNMT3A as observed with light scattering, gel filtration, and fluorescence anisotropy. Disruption of the dimer interface by pH decreases catalytic activity as a result of changes in oligomerization and DNA binding. Homotetramers carry out multiple cycles of methylation on the same piece of DNA (processive catalysis), while dimers have faster product release and a non-processive mechanism [19]. Our results show environmental conditions regulate the oligomeric state, and thus the catalytic properties, of DNMT3A. pH titration and mutagenesis studies suggest the observed effect may be due to the protonation of His-873 and/or His-821 at the dimer interface. The activities of mutants previously shown to disrupt the dimer interface, including AML mutation R882H, are recovered with acidification, resulting in similar activity between R882H and the wild type enzyme at pH 6.8. Our data suggests the DNMT3A dimer interface is dynamic and small pH changes that occur through development, localized physiological processes, or cancer progression could change methylation patterns created by of DNMT3A.

2. Material and methods

2.1. Cloning and protein purification

The catalytic domain of DNMT3A and full length DNMT3L were purified as stated in Holz-Schietinger and Reich [27]. Plasmids used for protein expression and site-directed mutagenesis, include codon-optimized pET28a-hDNMT3A_CD (Δ1-611) [28], and pTYB1-3L for hDNMT3L [22]. DNMT3A was purified from BioRex and nickel affinity columns, DNMT3L was purified from nickel affinity and chitin columns, all purified to >95%. The catalytic domain of DNMT3A has similar kinetic parameters as the full-length enzyme, including k_{cat}, K_m^{DNA}, K_m^{AdoMet}, processivity, and DNMT3L activation [22,27,28], and was used for the DNMT3A/DNMT3L co-crystal structure [23]. Both the homo- and DNMT3L hetero-oligomerization interfaces are located on the catalytic domain.

2.2. DNA sequences

Substrates include duplex poly-dIdC (~1000 bp) (Sigma-Aldrich), plasmid $pCpG^L$ (non-CpG substrate [29]), linear human promoters (*RASSF1A*, *p15*, *Hoxd4* and *Oct-4*) in plasmid $pCpG^L$, single site substrates GCbox2 unmethylated (5′-GGGAATTCAAGGGGCGGGGCAATGTTAGGG-3′) and fluorescent DNA (GCbox30) with fluorescein (6-FAM) on the 5′ end of the top strand, and (5′/6-FAM/TGGATATCTAGGGGCGCTATGATA TCT-3′) GCbox2 purchased from Integrated DNA Technologies (HPLC purified). See Supplemental methods for human promoter sequences and creation.

2.3. Size-exclusion chromatography coupled to multi-angle light scattering

Experiments were done as described in Holz-Schietinger et al. [19]: here we used a light scattering buffer at either pH 7.8 or 6.8 (50 mM KH_2PO_4/K_2HPO_4, with 200 mM NaCl, 1 mM DTT, 1 mM EDTA and 0.2% azide).

2.4. Methylation assays

DNMT3A methylation assays measured the amount of tritiated methyl groups transferred from cofactor AdoMet to the DNA by the enzyme. Reactions were carried out at 37 °C in reaction buffer (50 mM KH_2PO_4/K_2HPO_4, 1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 20 mM NaCl) at pH indicated. Enzyme was at 150 nM total, which is 27 nM active enzyme (previously determined in Purdy et al. 2010) [28]. DNA was the multiple site substrate poly-dIdC at saturation (40 μM base pairs (bp)) unless stated otherwise, all other substrates were also used at saturation (40 μM bp). AdoMet was used at saturation (5 μM). Processivity assays and DNMT3L activation assays were carried out as described in Holz-Schietinger and Reich [27]. Briefly, processivity assays involve three separate reactions; positive control: 20 μM bp substrate, experiment: substrate followed at 20 min by chase DNA ($pCpG^L$) at 20-fold excess over substrate concentration, and negative control: chase and substrate at the start of reaction. Mathematical modeling was done as described in Holz-Schietinger and Reich [27], and detailed in Supplemental methods. A 1:1 ratio with a one hour pre-incubation (with AdoMet) was used for all DNMT3L assays. All reactions were quenched by addition of 500 μM AdoMet and 50 μg/ml proteinase K. Samples were spotted onto Whatman DE81 filters then washed, dried, and counted as described previously.

For K_m determination, data was fit to the Michaelis–Menten equation, k_{cat}, and processivity data was fit to either linear regression or a fit to a nonlinear regression using Prism v5 (GraphPad). Error bars are standard error from three reactions. Bar charts of kinetic values compared mutants to wild type using one-way ANOVA to determine p-value using Prism.

2.5. Fluorescence anisotropy

Enzyme was titrated with 5′ 6-FAM labeled GCbox30 duplex DNA at 20 nM in reaction buffer with saturating AdoMet (1 μM). Data fit to single exponential, pH 6.8 fit to two curves, before 230 nM and after due to the two phasic behavior.

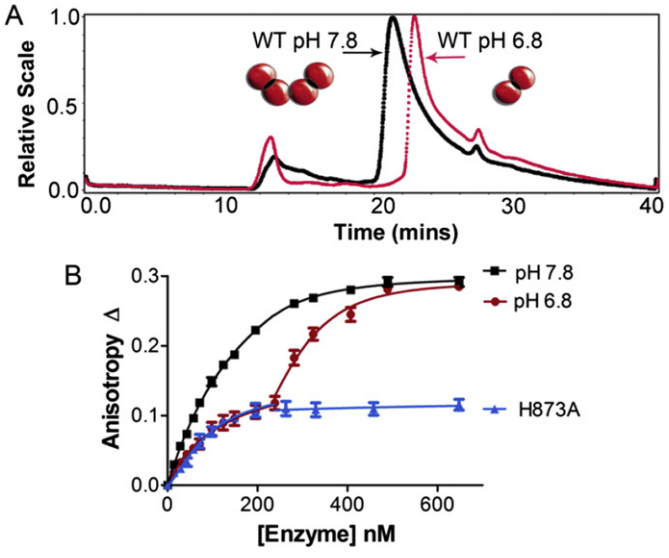

Fig. 1. Acidification disrupts tetramerization. A. Light scattering data of DNMT3A at pH 7.8 and pH 6.8. Size-exclusion chromatography of light scattering traces of the wild type catalytic domain at pH 7.8 (black trace) and at pH 6.8 (red trace). Molecular weights were determined from the amount of scattered light, in relation to the protein concentration determined by A_{280}. B. Anisotropy change observed with wild type DNMT3A at pH 7.8 (black) and pH 6.8 (red), and a mutant that disrupts the dimer interface, H873A (blue). DNA is a single site duplex labeled with 6-FAM at 20 nM and enzyme-varied.

k_{off} values were determined as described in Holz-Schietinger et al. [19], with the enzyme at 250 nM and 5′ 6-FAM labeled GCbox30 duplex DNA at 20 nM, 5 μM AdoHcy, reaching maximum anisotropy, followed by adding in chase DNA (unlabeled GCbox30) at 100× concentration of labeled DNA. The decrease in anisotropy was measured with time on a PerkinElmer LS 55 fluorescence spectrometer using Fl Winlab software. Data was fit to a one-phase exponential decay ($Y = \text{span}\, e^{-kt} + \text{plateau}$) plateau) using GraphPad, from two independent experiments.

3. Results

3.1. DNMT3A oligomerization is disrupted with acidification

We combined size exclusion chromatography with multi-angle light scattering to evaluate how pH changes the oligomeric state of DNMT3A. As previously shown, our preparations of the wild type DNMT3A catalytic domain have a weight-average molecular weight of 127 ± 2 kD in solution at pH 7.8, in agreement with four 36 kD monomers forming a tetramer [19]. However, the broad, tailing peak suggests the tetrameric form is in equilibrium with both the dimeric and monomeric forms of the enzyme on the column in the absence of DNA. Surprisingly, acidification by 1 pH unit (6.8) resulted in DNMT3A having a weight-average molecular weight of 72 ± 5 kDa, close to the theoretical dimer mass. Also at pH 6.8 DNMT3A eluted from the column slower than at pH 7.8 (Fig. 1A). Previously, mutations made along DNMT3A's dimer interface (H873A, H873R, W860A, R882H, N879A and D876G) were shown to have a weight-average molecular weights close to the theoretical dimer mass 82–66 kDa. These mutants that resulted in dimers show a similar elution profile as the wild type enzyme at pH 6.8.

Florescence anisotropy was used to show the differences in oligomeric state with pH when binding to DNA. Changes in anisotropy were measured as DNMT3A bound to a single site duplex DNA (GCbox30) with a 6-FAM label. The florescence anisotropy assays measure the rotational diffusion of a molecule or complex, as the complex becomes larger the rotation decreases causing an increase in anisotropy. The binding of a larger protein complex, such as a tetramer compared to a dimer, can further increase the observed of anisotropy.

As shown in Fig. 1B, titration of wild type enzyme at pH 7.8 results in a maximal change in anisotropy of 0.3, where the observed anisotropy increases monotonically with enzyme concentration. Wild type enzyme

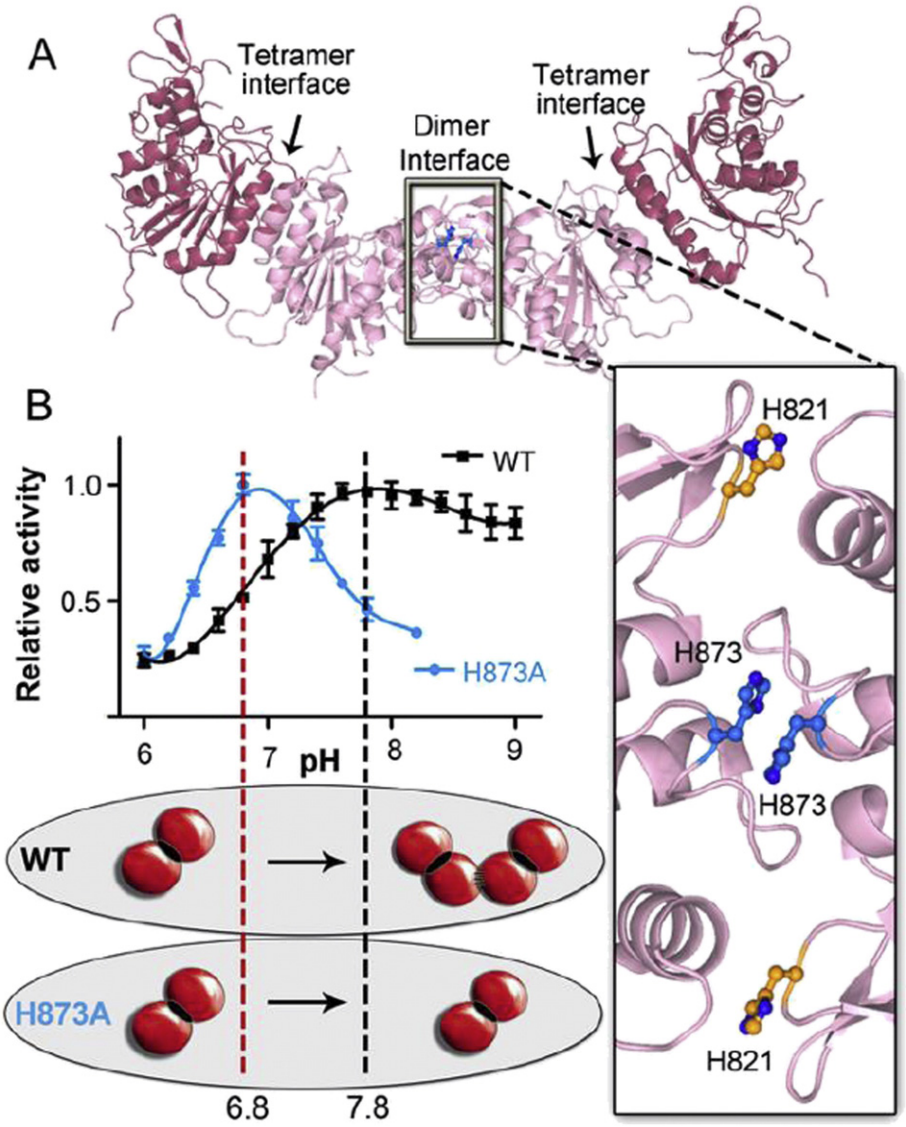

Fig. 2. DNMT3A activity is pH sensitive, which correlates with changes in oligomeric state. A. Picture of the DNMT3A homotetramer model derived from the crystal structure (PDB: 2QRV) and close up of the dimer interface showing the His residues along the interface. B. Wild type DNMT3A activity decreases on multiple site substrate with acidification, unlike dimer interface disrupting mutant H873A. H873A shows the highest activity at pH 6.8. Below graph shows the oligomeric changes that are occurring at the different pH values as determined from the data in Fig. 1.

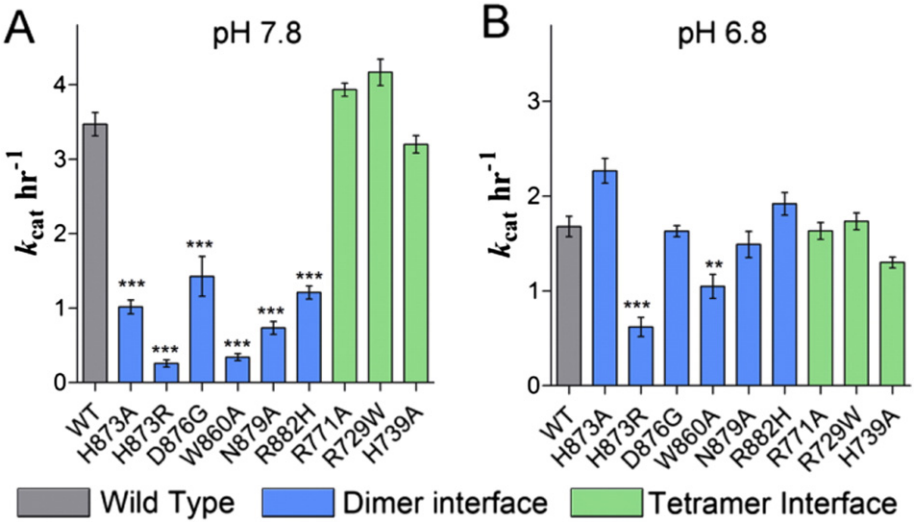

Fig. 3. Dimer disrupting mutations cause differential pH sensitivities. Wild type (gray) DNMT3A has 2.5-fold higher activity at pH 7.8 compared to 6.8 on multiple site substrate. Mutants that disrupt the dimer interface (blue) have a higher activity at pH 6.8 compared to at pH 7.8. Mutants that disrupt the tetramer interface (green) have similar pH activity as the wild type enzyme. All enzymes show similar activity at pH 6.8. A. k_{cat} values at pH 7.8, B. k_{cat} values at pH 6.8.

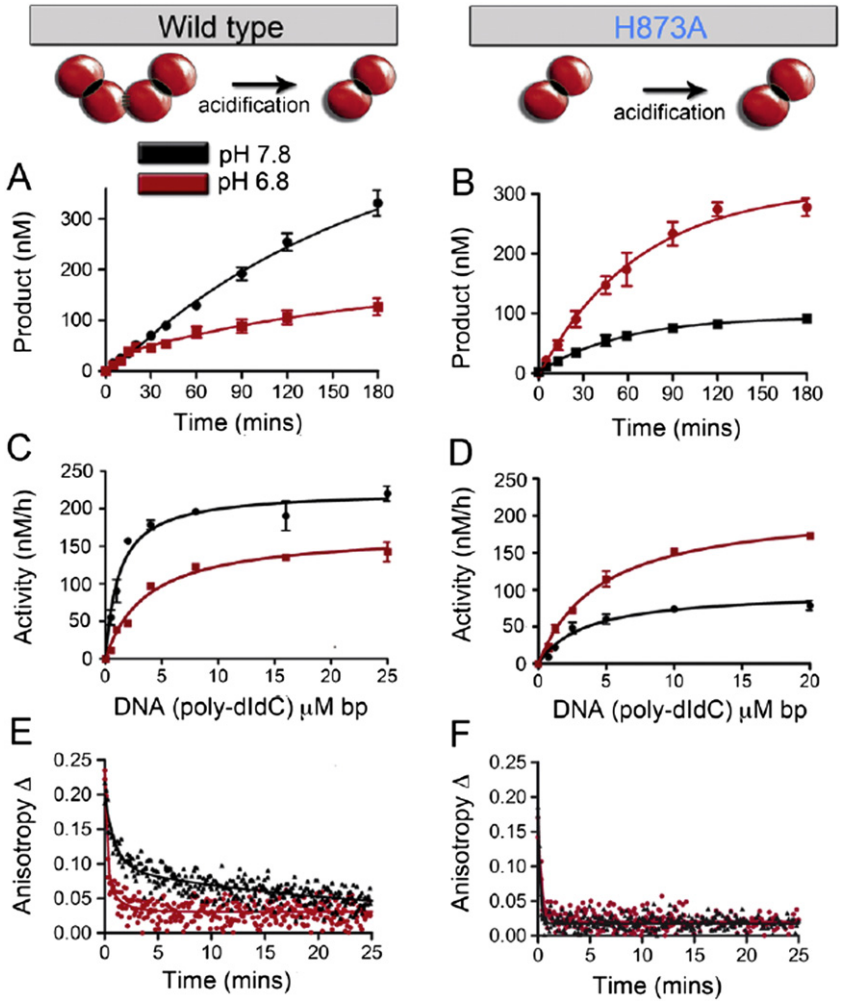

Fig. 4. Acidification dramatically increases the dissociation rates. All black data points are from experiments run at pH 7.8, red data points were run at pH 6.8. A. Product formation of the wild type enzyme on multiple site DNA, showing before 20 min (pre-steady state) the activity is the same at both pH values and after 20 minutes acidification decrease the overall rate of catalysis. B. H873A (dimer disrupting mutant) is activated at all time points with acidification. C. Wild type has a 3-fold increase in K_m at pH 6.8. D. H873A at pH 6.8 has a 1.2-fold increase in K_m^{DNA}. E. Wild type dissociation plots, showing that DNMT3A has an increase in k_{off} at pH 6.8. F. H873A has similar k_{off} at both pH values.

titrated at pH 6.8 showed a very different profile, with distinct two phases. The addition of enzyme up to 230 nM resulted in a mono-exponential increase in anisotropy to a plateau of 0.13 and further addition resulted in another titration curve plateauing at 0.3. This first plateau is the same magnitude of anisotropy change observed for the dimer-interface-disrupted mutant H873A (Fig. 1B). The 0.13 anisotropy plateau observed for DNMT3A at pH 6.8 is consistent with a lower molecular weight complex forming compared to the complex at pH 7.8. The observed data could result from either additional dimers binding to the same substrate or higher order complexes forming. However, comparison with the gel-filtration/light scattering data suggests the former, multiple dimers binding, as the most likely scenario.

3.2. DNMT3A is most active at pH 7.8 on multiple site substrates

We tested how pH changes the activity of DNMT3A. The number of methyl groups transferred by DNMT3A to multiple site substrates was monitored as the pH of the reaction was varied. As shown in Fig. 2B, DNMT3A is most active in a basic environment (pH 7.8) and methylation activity decreases with increasing acidity. At pH 6.8, where DNMT3A is a dimer, DNMT3A activity is decreased 2-fold. The decrease in activity is due to a 2.5-fold decrease in the steady state (after 20 min) turnover rate (k_{cat}) at pH 6.8 compared to pH 7.8. The pre-steady rate (before 20 min) is nearly identical at both pH values (Fig. 3A) indicating that the rate of methyl-transfer, or other steps that limit methylation, are unaffected. Instead, we found that the change in steady state rate results from the enzyme dissociating from its substrate, which results from greatly reduced ability to translocate on DNA and carry out multiple methylations per binding event as demonstrated below.

To understand if the decrease in DNMT3A's activity with acidification is a consequence of disrupting tetramerization, the affect of pH on a mutant that already has a disrupted dimer interface was tested. The H873A mutant has a disrupted dimer interface by light scattering and electrophoretic gel mobility assays.[1] This residue is located at the center of the dimer interface (Fig. 2A). The pH profile of H873A was dramatically different then the wild type enzyme; the mutant was most active at pH 6.8 and less active in a basic environment (Fig. 2B). The H873A mutant has a 3.4-fold decrease in activity compared to the wild type enzyme at pH 7.8. As shown in Fig. 3B at pH 6.8, H873A is activated at all time points, with an overall 2.2-fold increase in k_{cat} at pH 6.8 compared to pH 7.8. These results suggest that changes in activity observed for the wild type enzyme with pH is the result of oligomeric changes.

3.3. Acidification disrupts the dimer interface of DNMT3A

DNMT3A mutants with a disrupted dimer or tetramer interface were tested for changes in activity upon acidification to determine which interface is controlled by pH. We found all dimer interface mutants (H873A, H873R, N879A, W860A, D876G, and R882H) had a 2-4-fold increase in activity at pH 6.8 compared to pH 7.8 (Fig. 3). Mutations

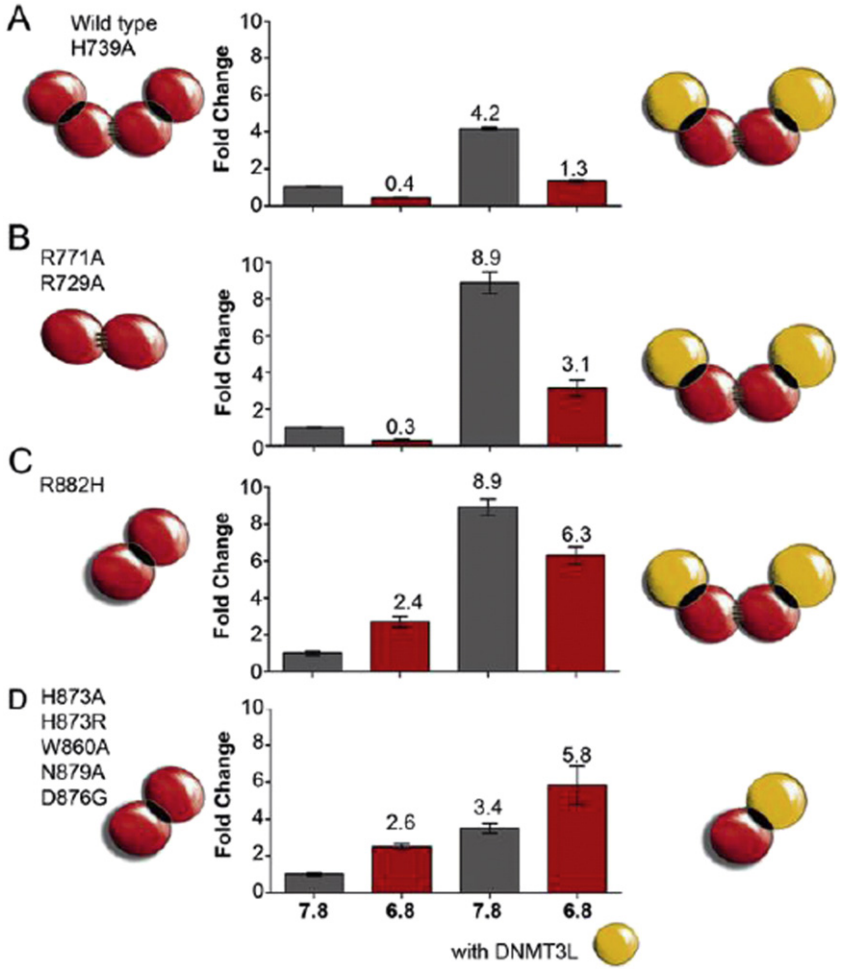

Fig. 5. The dimer interface is disrupted with acidification. Bar chart shows the relative change in activity on multiple site substrates. The gray bars are for the enzyme at pH 7.8 and the red for the enzyme at pH 6.8. The two left bars are without DNMT3L, the two right bars are in the presence of DNMT3L. Drawings show the oligomeric state of each of the mutants as previously shown. A. Wild type enzyme or H873A (only His at the tetramer interface) are *both* most active at pH 7.8 with and without DNMT3L. B. Mutants that disrupt the tetramer interface, also show the highest activity at pH 7.8, even with DNMT3L. C. R882H is located at the dimer interface and shows the highest activity at pH 6.8. R882H with DNMT3L forms a heterotetramer and there is a higher activity at pH 7.8. D. Dimer interface mutants have the highest activity at pH 6.8 with and without DNMT3L.

Table 1
Acidification decreases activity for wild type enzyme and increases the activity for dimer disrupting mutants. k_{cat} and K_m values were determined by monitoring the ability of the enzyme to incorporate tritiated methyl groups transferred from cofactor AdoMet onto DNA (poly-dIdC).

	k_{cat} h^{-1} (pH 7.8)	Fold Δ	k_{cat} h^{-1} (pH 6.8)	Fold Δ
WT	3.47 ± 0.27	1.0	1.68 ± 0.19	1.0
H873A	1.02 ± 0.16	3.4	2.27 ± 0.23	0.7
H873R	0.26 ± 0.08	13.5	0.62 ± 0.18	2.7
W860A	0.44 ± 0.08	7.8	1.05 ± 0.22	1.6
N879A	1.53 ± 0.15	2.3	1.49 ± 0.24	1.1
R882H	1.41 ± 0.15	2.5	1.92 ± 0.21	0.9
D876G	1.63 ± 0.46	2.1	1.63 ± 0.10	1.0
R885A	No activity		No activity	

that disrupt the tetramer interface, R729A and R771A, as well as a mutation that does not disrupt the interface, H739A, all showed 2-fold higher activity at pH 7.8, similar to what is observed for the wild type tetramers (Fig. 3). H739 is the only histidine at the tetramer interface and mutating it to alanine did not disrupt the interface. The results are consistent with the dimer interface being pH sensitive, as the disruption of the tetramer interface did not change how pH affects the enzyme. Our results suggest the most likely residue/s responsible for the pH sensitivity that controls oligomerization are His-873 and/or His-721 at the dimer interface; His-739 is an unlikely candidate since it is located at the tetramer interface.

For additional evidence that the dimer interface is pH controlled, DNMT3L activation of DNMT3A was also tested at pH 7.8 and 6.8. DNMT3L is known to bind at the tetramer interface and activate both sets of oligomeric mutants and wild type enzyme at pH 7.8 [19].[1] If pH controls the dimer interface, then DNMT3L would be expected not to change the effect of acidification on DNMT3A activity, since the dimer interface would still be disrupted.

DNMT3L activates all the enzymes at pH 6.8 but the pH sensitivity does not change (Fig. 5), consistent with acidification disrupting the dimer interface. R882H is the odd mutant, where DNMT3L facilitates the formation of the dimer interface,[1] and to be expected, this mutant in complex with DNMT3L had the greatest activation at pH 7.8 (Fig. 5C). Our data strongly suggest lowering pH disrupts the dimer interface, causing a decrease in activity on multiple site substrates for the wild type enzyme and an increase in activity for the dimer interface mutants. The wild type enzyme at pH 6.8 also has similar catalytic properties, as the dimer interface mutants provide further evidence that the dimer interface is disrupted with pH, see below.

3.4. The dimer interface facilitates processivity

We also found pH had a significant effect on the affinity of DNMT3A for DNA, as the K_m for DNA increased 3-fold, to 3.5 μM for the wild type enzyme at pH 6.8 (Fig. 4C) and similar to the 3.4–6.6 μM K_m observed for dimer interface mutants at pH 7.8 (Fig. 4D). pH has little effect on the K_m^{DNA} for H873A from pH 7.8 to 6.8 (Fig. 4D). The observed increase in K_m^{DNA} for the wild type enzyme indicates a perturbation to substrate binding and/or product dissociation.

As shown elsewhere, DNMT3A is processive on multiple sites substrates [19,27]. An increase in k_{off} would be expected to decrease or eliminate processivity, as seen with the dimer interface as well as tetramer interface mutants. To further define the effect of pH on DNA affinity, dissociation rates (k_{off}) were determined by binding excess enzyme to 6-FAM labeled single sites duplex (GCbox30) and measuring the rate of change in anisotropy upon the addition of 100-fold excess unlabeled GCbox30. At pH 7.8 DNMT3A has a k_{off} of 0.20 ± 0.1 min^{-1}, we show at pH 6.8 that the rate increases 10-fold to 2.2 ± 0.2 (Fig. 4E, Table 2). The increase in k_{off} with acidification is similar to the change (6–20-fold) observed for the dimer interface disrupting mutants at pH 7.8 (Table 2). At pH 6.8 the dimer interface mutants tested, H873A, W860A, and R882H, show a modest 1.2 to a 1.6-fold increase in the k_{off} rate (Fig. 4F, Table 2). The data indicates that the disruption of the dimer interface greatly accelerates the off rate from DNA and that acidification has a small affect on dissociation for the mutants that already have a disrupted dimer interface (Table 1).

The processivity of the enzyme was assessed using both mathematical modeling and chase experiments [27,30]. Using mathematical modeling, both full length and the catalytic domain of wild type DNMT3A were shown to act processively [27] on several DNA substrates. The enzyme methylates an average of 30 ± 6 cytosines before dissociating from poly-dIdC DNA and 15 ± 5 sites on the human *RASSF1A* promoter (Fig. 6A, Table 3) at pH 7.8. The *RASSF1A* promoter is 1000 base pairs long with 104 CpG sites spread throughout the sequence (diagramed in Fig. 6B). We found that DNMT3A is very poorly processive at pH 6.8. At this pH, an average of 2.9 ± 1.5 turnovers occur on poly-dIdC and 1.5 ± 0.5 turnovers occur on the *RASSF1A* promoter before 50% of the enzyme dissociates (Fig. 6A, Table 3). Modeling shows the loss of processivity is due to a 6-fold increase in the off rate of the dimers along with 1.6-fold decrease in the combined rate of methylation and translocation (Table 3), in general agreement with our findings via the anisotropy-based methods.

3.5. pH sensitivity on human promoters

Processivity was also tested using the chase experiment, which shows wild type DNMT3A at pH 7.8 has an essentially unaltered methylation rate for 90 min after 20-fold excess of chase (non-CpG) DNA is added, indicating that the enzyme is continuing to methylate the same substrate for multiple rounds of methylation. The wild type enzyme is shown here to be processive on both poly-dIdC and the human promoter *RASSF1A* (Fig. 6C). When the chase assay was carried out at pH 6.8 on either poly-dIdC or the *RASSF1A* promoter, product formation was eliminated when the chase DNA was added (Fig. 6D). These data are consistent with the dimer interface mutants, which also showed a loss of processivity. Our observations with a human promoter indicate that disruption of the dimer interface with either pH or mutations could eliminate clustered methylation in a genome.

Table 2
Acidification increases the dissociation rate. k_{off} values were determined by binding excess enzyme to 5′ FAM-6 labeled GCbox30 duplex and measuring the rate of change in anisotropy upon the addition of excess unlabeled GCbox30 (100-fold labeled DNA).

					With DNMT3L				
	k_{off} min^{-1} (pH 7.8)	Fold Δ	k_{off} min^{-1} (pH 6.8)	Fold Δ	k_{off} min^{-1} (pH 7.8)	Fold Δ	Fold Δ	k_{off} min^{-1} (pH 6.8)	Fold Δ
WT	0.21 ± 0.01	1.0	2.69 ± 0.29	1.0	0.17 ± 0.01	1.0	1.3	2.23 ± 0.22	1.0
H873A	3.48 ± 0.29	16.4	4.21 ± 0.96	1.6	2.25 ± 0.25	13.4	1.6	4.53 ± 0.68	2.0
H873R	4.17 ± 0.78	19.7	4.00 ± 0.45	1.5					
W860A	3.07 ± 0.19	14.5	3.11 ± 0.46	1.2	2.16 ± 0.34	12.9	1.4	3.97 ± 0.56	1.8
N879A	1.52 ± 0.13	7.2	4.35 ± 0.68	1.6					
R882H	2.36 ± 0.04	11.1	3.1 ± 0.37	1.2	0.43 ± 0.07	2.6	5.5	2.99 ± 0.32	1.3
D876G	1.35 ± 0.10	6.4	3.42	0.26	1.3				
R885A	11.17 ± 2.32	52.7							

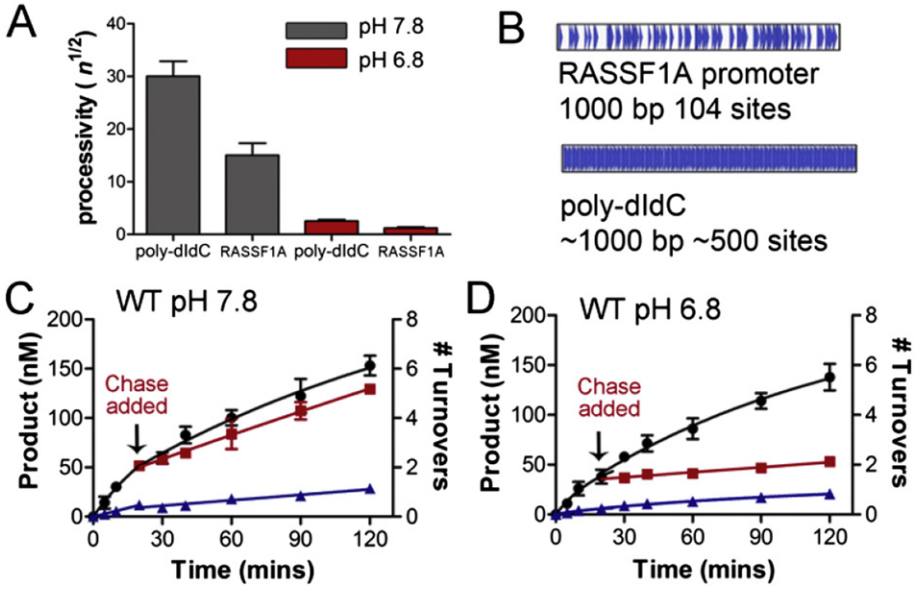

Fig. 6. Processivity is lost with acidification. A. Processivity values determined from mathematical models based upon the activity curves on the continual recognition site substrate poly-dIdC and multiple site human promoter *RASSF1A*. DNMT3A is processive at pH 7.8 ($n^{1/2} = 30$ or 15) and DNMT3A is non-processive at pH 6.8 ($n^{1/2} = 2.5$ and 1.5). B. Substrates diagramed. C and D, chase assays on *RASSF1A* promoter B. DNMT3A at pH 7.8 shows the chase DNA has little affect on activity, indicating a processive enzyme. C. DNMT3A at pH 6.8 shows the addition of chase DNA eliminates activity, indicating a non-processive enzyme. Chase assay, ● = only substrate, 20 μM bp poly-dIdC; ■ = substrate and then 400 μM bp chase ($pCpG^L$) at 20 min; ▲ = substrate and $pCpG^L$ at the start of the reaction.

We examined additional human promoters by measuring the activity for DNMT3A as a tetramer and as a dimer created by mutations or by pH. The use of human promoters of different sizes and densities of CpG sites gives some indication of how pH changes could manifest themselves in vivo to alter DNA methylation patterns. Four human promoters amplified from human cells (*RASSF1A*, *p15*, *Hoxd4*, and *Oct-4*), a single site (GCbox2), and a non-CpG substrate ($pCpG^L$) (diagramed in Fig. 7A, sequences in Supplemental methods) were used as substrates with wild type DNMT3A and the dimer mutant H873A at both pH 6.8 and 7.8.

We observed that the wild type DNMT3A was most active on multiple site substrates (poly-dIdC, *RASSF1A*, and *p15*) at pH 7.8 (i.e., as a tetramer). However, at pH 6.8 the wild type enzyme (dimer) has a 2-3-fold reduction in activity on these substrates. In contrast, the wild type DNMT3A is less active and has little pH sensitivity on the single site and few sites substrates (*Hoxd4* and *Oct-4*) (Fig. 7B). In fact, the enzyme has slightly higher activity on the single site substrate at pH 6.8. The decrease in DNMT3A activity with acidification is directly correlated with the number of CpG in the substrates, as shown with an R^2 of 0.87 for a linear regression (Fig. 7D). In comparison, we observed the dimer interface mutant H873A had the highest activity at pH 6.8 for all CpG containing substrates tested (Fig. 7C). For both enzymes we observed minimum activity and no pH sensitivity on the non-CpG substrate ($pCpG^L$) (Fig. 7). The methylation of these human promoters is altered based on the clustering of methyl-sites, indicating the oligomeric state of the enzyme controls substrate specificity through changes in processive methylation.

4. Discussion

DNA methylation patterning plays an essential role in transcriptional regulation and cellular differentiation [31,32]. However, the organization and function of higher-order DNMT3A complexes in controlling the enzymatic activity are not understood. Physiological conditions including pH can regulate protein function by many mechanisms, including altering the affinity of binding partners [9]. We show multiple lines of evidence that acidification by only 1 unit from 7.8 to 6.8, completely disrupts the dimer interface of DNMT3A and smaller changes in pH also alters the activity of the enzyme. Cancer cells usually have a decrease in extracellular pH from 7.8 to 6.8 [12], and transient changes in intracellular pH also occurs [12,13]. Disruption of the dimer interface increases DNA dissociation 10-fold and decreases activity 2–3-fold on multiple site substrates. This is the same interface disrupted in the majority of mutations correlated with Acute Myeloid Leukemia [26]. Acidification alters methylation patterning by eliminating the ability of DNMT3A to methylate clustered sites on human promoters. This is consistent with both the dimer and tetramer interfaces being essential for processivity in DNMT3A [19].

Whole genome methylation analysis reveals a bimodal distribution in which genomic regions are either fully methylated or unmethylated, consistent with the processive methylation described for DNMT1, DNMT3A, and DNMT3B [33–36]. Therefore, disruptions to the processivity of DNMT3A may have important consequences in the establishment of methylation patterning. Oligomerization at the dimer interface occurs at the DNA target-recognition domain, doubling the DNA-binding surface [37]. It is well established that increasing the protein–substrate interface can enhance processivity in DNA modifying enzymes by decreasing product release and facilitating translocation [38–40]. Two reports that showed DNMT3A is non-processive where carried out at pH 7.0 and did not proceed long enough for the enzyme to show processive activity [41,42].

During development and tumorigenesis, cellular conditions can dramatically change, altering the function of proteins [12]. This is the case for many DNA binding proteins including transcription factors [14] AP-1 [43], NF(kappa) B [14,15], SpI [16], and p53 mutant R337H, where their activity changes with alterations in cellular pH during

Table 3
Acidification controls processivity. Modeling was done as shown in Holz-Schietinger et al. [19]. The modeling is based on the shape of the curve for the non-chase experiment, which determines the methylation with the translocation rate (k) and dissociation rate (k_{off}). A processivity value (p) was determined from the k and k_{off} rates. The processivity values (p) were used to calculate $n^{1/2}$, which indicates the average numbers of catalytic turnovers before half of the enzymes are dissociated from the original substrate.

		$k\ h^{-1}$	$k_{off}\ h^{-1}$	p	$n^{1/2}$
Poly-dIdC	Wild type pH 7.8	11 ± 0.8	0.3 ± 0.01	1.0	30 ± 4.2
	Wild type pH 6.8	6.4 ± 0.3	1.6 ± 0.12	0.79	3 ± 1.5
RASSF1A	Wild type pH 7.8	5.1 ± 0.5	0.2 ± 0.09	0.96	15 ± 5.0
Promoter	Wild type pH 6.8	3.4 ± 0.4	1.5 ± 0.15	0.75	2 ± 0.5

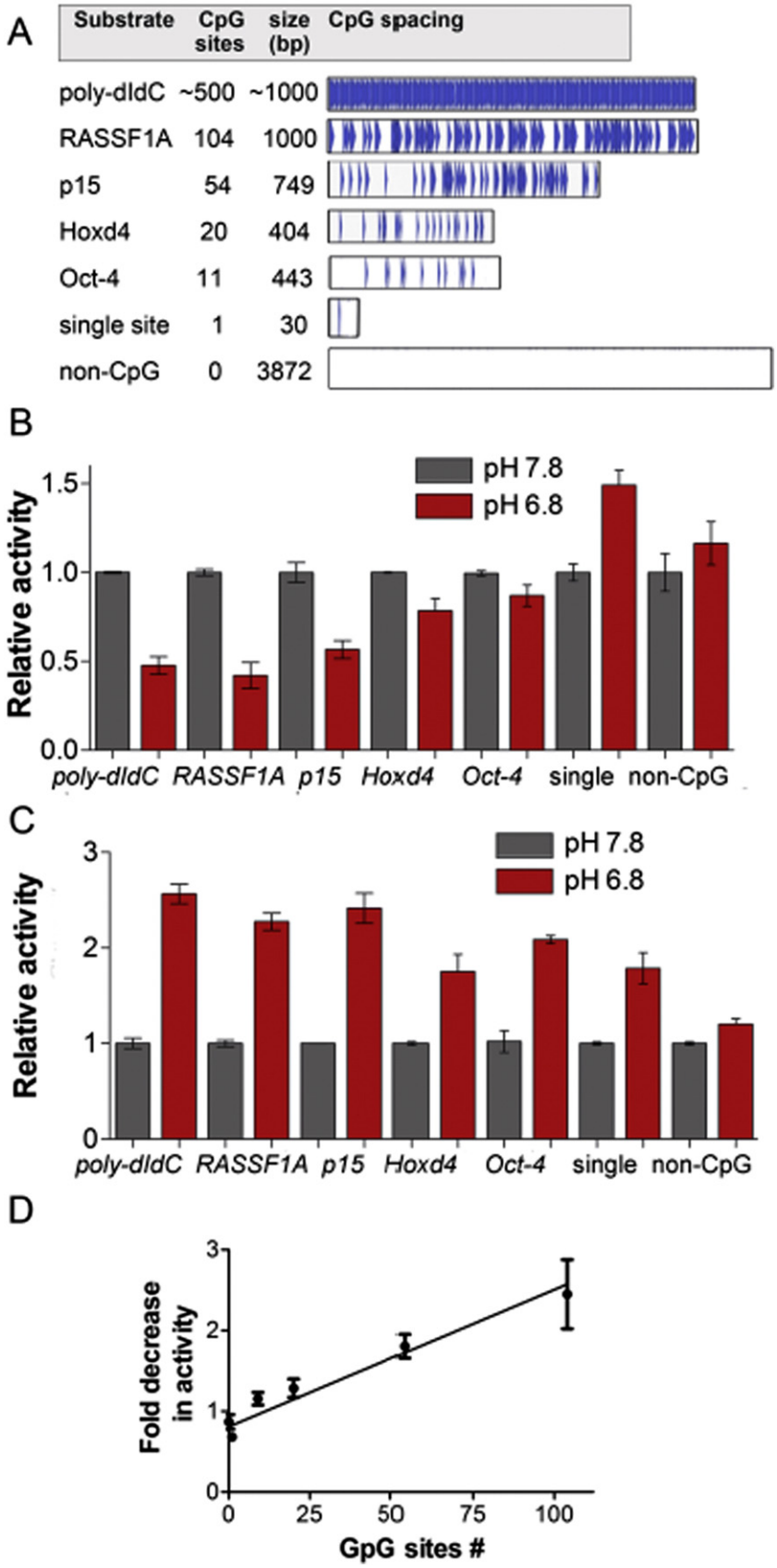

Fig. 7. DNTM3A has human promoter-dependent pH-sensitivity. A. Diagram of substrates. B. H873A has the greatest activity on all substrates tested at pH 6.8. C. pH has the greatest affect on wild type DNMT3A on multiple site substrates, relative activity is based off the activity at pH 7.8 (gray) for each substrate. D. Activity of the WT at pH 7.8 correlates with the number of CpG sites in each substrate (R^2 of 0.87 for a linear regression).

cancer [17]. In the p53 mutant tetramerization is disrupted in a pH-dependent fashion that results in no activity, and is proposed as a molecular basis for adrenocortical carcinoma [17]. In addition, acidification can lead to cell death due to activation of the p53-dependent apoptosis pathways, as well as loss of function of critical pH-sensitive genes [12, 17]. Increasing acidification disrupts tetramerization in DNMT3A due to weakening of the interactions at the enzyme's dimer interface. The only residues at the dimer interface expected to be sensitive to pH changes from 7.8 to 6.8 are His-873 and His-721 via protonation, which would introduce a pair of charges (one from each monomer) into this highly sensitive interface.

The link between mis-regulation of DNA methylation and cancer has been established [31] and mutations in DNMT3A are correlated with AML development and a poor clinical outlook [26]. Residue Arg-882H is altered in ~60% of the cases [26], with most prevalent R882H substitution disrupting the dimerization interface.[1] We show that R882H and all other dimer interface mutants are activated in acidic environments, counter to the wild type enzyme. Because the R882H mutant introduces differential pH sensitivity into DNMT3A, the origins of poor survival in AML cases with the mutants may be associated with an altered pH within the cancer cells. As it has previously been shown DNTM3A oligomerization is necessary for heterochromatin localization in cells [24], small localized pH changes are predicted to alter the localization and thus the methylation patterning in the cell. Changes in the oligomeric state of DNMT3A in cells need to be addressed, as DNMT3A oligomerization is dynamic and alters the function of the enzyme.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary methods including promoter sequence and processivity analysis. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbagen.2015.02.003.

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