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RNA modulation of the human DNA methyltransferase 3A

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ABSTRACT

DNA methyltransferase 3A (DNMT3A) is one of two human *de novo* DNA methyltransferases essential for transcription regulation during cellular development and differentiation. There is increasing evidence that RNA plays a role in directing DNA methylation to specific genomic locations within mammalian cells. Here, we describe two modes of RNA regulation of DNMT3A *in vitro*. We show a single-stranded RNA molecule that is antisense to the *E-cadherin* promoter binds tightly to the catalytic domain in a structurally dependent fashion causing potent inhibition of DNMT3A activity. Two other RNA molecules bind DNMT3A at an allosteric site outside the catalytic domain, causing no change in catalysis. Our observation of the potent and specific *in vitro* modulation of DNMT3A activity by RNA supports *in vivo* data that RNA interacts with DNMT3A to regulate transcription.

INTRODUCTION

DNA methylation, in conjunction with other epigenetic controllers including histone modifications and non-coding-RNA (ncRNA), is critical to the hierarchical regulation of transcription (1,2). In mammals, *de novo* 5-methylcytosine (5mC) patterning is established by DNA methyltransferase 3A (DNMT3A) and the closely related DNMT3B (1,3,4) and the patterning is maintained by DNMT1 (5). The mechanisms that control genomic site selection by the *de novo* methyltransferases are not understood, but there is increasing evidence that binding partners, both RNA and protein, play a major role in controlling DNA methylation (6–9).

Non-coding RNAs play an essential role in transcriptional regulation, including the formation of ternary complexes (protein/RNA/DNA) that modulate genomic targeting by directing hybridization and providing scaffolding for histone modifying enzymes and transcription factors (10–13). In plants, fission yeast and flies, interaction with ncRNA directs the DNMTs to specific targets (14). Similar mechanisms have been proposed for mammalian *de novo* DNMTs, which are supported by recent data showing piwi RNA-directed DNA methylation in germ cells (15) and a short non-coding RNA is necessary for some DNMT3b-mediated methylation through the formation of a sequence-specific triplex structure (16). Antisense RNA to the *EF1a* promoter causes transcriptional silencing in cells by inducing DNA methylation (17). Additionally, RNA transcripts are essential for genomic imprinting and X-chromosome inactivation, both of which occur via DNA methylation (18). This includes *Tsix* RNA, which regulates X-inactivation and binds to DNMT3A *in vivo* but neither DNMT3B nor DNMT1 (19). Further evidence is shown by DNMTs binding various single-stranded (ss) forms of RNA and DNA, in some cases at an allosteric site (20–25). However, how RNA modulates the catalytic properties of the *de novo* DNMTs is not known.

We probed the interactions between the human *de novo* DNMT3A and three RNA transcripts previously shown to either associate with DNMT3A or change promoter-specific methylation. These include a siRNA duplex to the NUP153 mRNA (20), a ssRNA antisense to the *EF1a* promoter (*EFS1*) (26), and a single-stranded antisense RNA to the *E-cadherin* (*CHD*) promoter (27). We find these three RNA molecules bind DNMT3A in two different fashions. *NUP153* duplex RNA and ss*EF1a* RNA bind allosterically outside the catalytic domain, causing no measurable change in DNMT3A activity. The *CHD* RNA can bind to the catalytic domain and is a potent inhibitor of DNMT3A, and its modulation of DNMT3A activity is dependent on the RNA structure.

MATERIALS AND METHODS

Materials

The DNA used as substrates, poly-dIdC (~1900 bp; Sigma-Aldrich) and GCbox2 (GGGAATTCAAGGGG CGGGGCAATGTTAGGG) duplex were purchased from Midland Certified Reagent Company. Annealing was confirmed by 12% polyacrylamide gels. The recognition site

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Table 1. RNA sequences used in this study

RNA name	Sequence	Description
CHD	5′ GGGGUGA**CG**GGGGACAGGCGGGGCUG 3′	Antisense *E-cadherin* promoter
EFS1	5′ GAGUUUACCC**CGCG**CCACCUU 3′	Antisense *EF1a* promoter
Nup153 duplex	5′ ACCAAAUAAAACUGGCAAAdTdT 3′	
siRNA	3′ dTdTUGGUUUAUUUUGACCGUUU 5′	Nuclear pore complex mRNA
Nup153-as	5′ ACCAAAUAAAACUGGCAAAdTdT 3′	
Nup153-s	5′ UUUGCCAGUUUUAUUUGGUTdTd 3′	

for DNMT3A is underlined. The human promoter substrates were described previously (28). RNA oligonucleotides are listed in Table 1. *CHD* RNA (antisense *E-cadherin* promoter) was purchased from IDT and HPLC purified. *EFS1* (antisense *EF1a* promoter) and *NUP153* antisense and sense were purchased from Dharmacon, desalted and ethanol precipitated. Concentrations are given in number of molecules. RNA purity was visualized by 12% denaturing (12 M Urea) PAGE gel and duplex formation was confirmed by 12% native PAGE.

RNA preparation

RNA was prepared by adding 1 mM $MgCl_2$, then heating to 85°C for 5 min and slow cooling for 5 h unless otherwise noted (heating slow cooled). Otherwise the RNA was fast cooled by placed directly on ice after the heating step or unheated RNA solutions were thawed by allowing them to equilibrate to room temperature before being used. In methylation and binding assays, the $MgCl_2$ was diluted to 50 μM. The RNA after preparation was visualized on a 12% native PAGE gel in 0.5× TBE. The gel was run at 4°C, for 10 h, visualized by SYBR Gold (Invitrogen) and imaged on a Storm 840 phosphorimager.

Protein expression and purification

The plasmids used for *E. coli* recombinant protein expression include pET28-hDNMT3ACopt, pET28-DNMT3A2Copt and pET28-hDNMT3A_catalytic_domain. Plasmid construction, expression and purification were previously described (28).

Native gel mobility RNA shift assays

Full-length DNMT3A, DNMT3A2 and catalytic domain and M.HhaI (concentration as indicated), were incubated at room temperature for 15 min with 100 nM RNA. Binding occurred in reaction buffer with 10 μM *S*-Adenosyl-L-homocysteine (AdoHcy). Samples were run on native 6% polyacryamide gels in 0.5× TBE pH 7.8 at 250 V for 30 min, stained with SYBR Gold and data were analyzed using ImageJ (29). K_D values where determined from a single exponential using Prism V4.0 (GraphPad). Error bars represent the mean standard deviation of at least two replicates.

Methylation assays

Methylation assays were carried out in RNase free reaction buffer with 50 mM Tris–Cl, 1 mM EDTA, 1 mM DTT, 0.2 mg/ml BSA, 20 mM NaCl and 2 μM AdoMet at pH 7.8. Reactions were initiated by addition of pre-mixed DNA and RNA, and then quenched after 1 h. Details of the assay are the same as those previously described (28). IC_{50} assays used enzyme at 30 nM, DNA was either poly-dIdC at 5 μM or human promoters 10 μM, and RNA concentrations were varied as indicated in the figure legends. The RNAse control used 5 mg/ml of RNAse A pre-incubated with DNA and RNA for 5 min at 37°C before carrying out the methylation assay.

Fraction Inhibition was calculated by 1 − (product formed with RNA/product formed without RNA). The data were fit to a non-linear regression (one phase decay), where half life indicates IC_{50} using Prism. Error bars represent the mean standard deviation of at least three replicates. IC_{50} values for the RNA on each promoter were determined by measuring the amount product formed at varying RNA concentrations.

Mechanism of inhibition studies used full-length DNMT3A and the catalytic domain at 30 nM; poly-dIdC and *CHD* RNA were used at concentrations indicated in the figure legends. The data were fit using Sigma Plot 6.1 enzyme kinetics modular 1.0, by modeling a single substrate single inhibitor for competitive, non-competitive, uncompetitive and mixed type. Modeling using partial inhibition approaches resulted in the same R^2 as the full-type inhibition. The models were ranked by R^2 value. Error bars represent the mean standard deviation of at least three replicates. Data are graphically represented in Lineweaver–Burk form using Sigma Plot 6.1 enzyme kinetics modular 1.0.

Processivity assays

Processivity assays were carried out as previously described (28) with DNMT3A at 50 nM. Reactions were initiated by the addition of substrate DNA, poly-dIdC (5 μM). A total of 200 nM *CHD* RNA was added after 21 min. The control is concurrent addition of RNA and DNA at the start of the reaction. Data were fit to a non-linear regression (one phase decay). Data were fit separately before the experimental reaction had *CHD* RNA added (time 0–21 min) and after the *CHD* RNA was added (21–260 min), using Prism. Error bars represent the mean standard deviation of at least three replicates. The number of turnovers is calculated by the amount of production formation (nM) divided by the amount of catalytic active sites (nM) total protein concentration times percent active sites (18%) as previously described (22).

M.HhaI methylation IC_{50} assays were carried out like DNMT3A assays, but with M.HhaI (30 nM), AdoMet (5.5 μM) and reactions were run for 20 min. M.HhaI expression and purification are described in Matje *et al.* (30).

RESULTS

The three RNA sequences studied in this study were previously shown to directly or indirectly associate with one or more of the DNMT enzymes or have been implicated in the epigenetic regulation of specific genes (Table 1). The antisense but not the sense strand of RNA to the human *EF1a* promoter (*EFS1*) binds DNMT3A in cells, whereas DNMT1 showed no such binding (26). The *EF1a* RNA also caused transcriptional silencing in cells by inducing DNA methylation (17). The *CHD* RNA was implicated in DNA methylation changes localized to the *E-cadherin* promoter (27); although this study was retracted (31), the mitigating circumstances suggest that the body of work was correct. Indeed, the *CHD* RNA was successfully used to direct transcriptional gene silencing in subsequent work (32). The siRNA duplex to the *NUP153* mRNA binds to mouse DNMT3A and DNMT3B but not to DNMT1 or DNMT2 (20). Thus, two of the RNA molecules tested in this study regulate transcriptional gene silencing in cells but the mechanism for regulation is not known. We tested the binding of these RNA to DNMT3A and test if these RNA molecules can modulate the activity of DNMT3A.

The secondary structures of the ssRNAs were characterized by melting curve analysis and native PAGE (Figure 1A). Three RNA preparation protocols were used for all RNA samples and were found to generate identical products for ss*NUP153* and ss*EF1a* RNA molecules as seen by native PAGE. In contrast, the method of sample preparation dictates *CHD* RNA structure and aggregation (Figure 1A). Figure 1A shows the difference between unheated (lane 3), heated to 85°C then slow cooled (lane 1) or heated to 85°C followed by snap cooling on ice (lane 2). The unheated sample (lane 3) shows several forms: a highly aggregated (*a*) and a multimeric or secondary form of RNA (*b* and *c*). When the RNA was heated to 85°C followed by fast cooling on ice, the majority of the RNA is in the single-stranded unstructured form (lane 2). Heating the RNA to 85°C followed by slow cooling results in multiple bands (lane 1). Much of the RNA is located higher in the gel than the single-stranded form, with a band indicated as *b,* and smearing at *c* and some single-stranded unstructured form. There is also a loss in aggregation at the top of the gel (*a*), which occurred in the unheated sample. We found that the forms *CHD* RNA adopts under different preparation conditions have significant impact on the mechanisms and degree of inhibition.

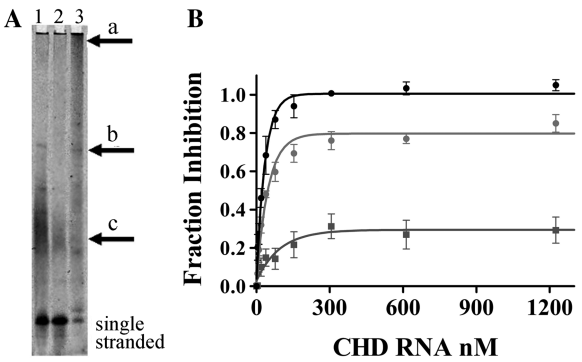

Figure 1. Conformational and functional characterization of the *CHD* RNA. The *CHD* RNA takes on secondary structure that controls DNMT3A inhibition. (**A**) Inhibition curve for the *CHD* RNA on DNMT3A. Arrow bars represent standard deviation from three reactions. Black circle = *CHD* RNA unheated; grey circle = *CHD* RNA heated slowly cooled; grey square = *CHD* RNA heated fast cooled. (**B**) Native-PAGE gel of *CHD* RNA after different treatments, same amount of RNA loaded for each lane. Lane1: RNA heated slow cooled, 2: heated fast cooled and 3: unheated, *a* points to aggregated RNA still in the well, *b* and *c* are higher order structures and *d* is single-stranded unstructured.

We sought to examine the effect of *CHD* RNA on DNMT3A methylation activity using filter binding assays to measure changes in the amount of tritiated methyl groups transferred from cofactor to poly-dIdC DNA by the enzyme (28). All three preparation conditions of the *CHD* RNA caused inhibition on full-length DNMT3A (Figure 1B). Unheated *CHD* RNA shows the greatest inhibition with an IC_{50} of 24 ± 4.5 nM and 100% inhibition. The slow cooled *CHD* RNA has an IC_{50} of 33 ± 6.6 nM with 89% inhibition and the snap cooled sample showed the least amount of inhibition with 31% inhibition and an IC_{50} of 67 ± 31 nM (Supplementary Table S1). The maximal inhibition by *CHD* RNA on DNMT3A activity was observed with the unheated and slow cooled RNA correlating with these samples having mostly secondary bands in the native gel (Figure 1A). All subsequent *CHD* RNA experiments used slowly cooled RNA (Figure 1A).

CHD RNA is a potent inhibitor of DNMT3A. The inhibition seen by the *CHD* RNA occurs on the full-length DNMT3A (Figure 2A) with an IC_{50} of 40 ± 6.6 nM. We tested if the N-terminal domains mediate this inhibition, such as the PWWP domain, which was previously shown to bind DNA (22). DNMT3A2 is a truncated isoform expressed in early development (33), while the catalytic domain of DNMT3A (cDNMT3A) has been demonstrated to have kinetic properties that are essentially identical to the full length (22). The IC_{50} of *CHD* RNA on DNMT3A2 was found to be 24 ± 6.5 nM (Figure 2B) and 12 ± 1.4 nM against cDNMT3A (Figure 2C and D and Supplementary Table S2).

We sought to determine if the *CHD* RNA-mediated inhibition of DNMT3A was sequence dependent via comparison to single-stranded and duplex *NUP153* RNA and *EF1a* ssRNA. These RNAs were also prepared by slow cooling but additional experiments were done that showed preparation did not change the results for other RNA sequences tested. *EF1a* RNA was tested up to 2000 nM with no inhibition observed (Figure 2E). The sense, antisense and duplex *NUP153* RNA also showed inhibition (Figure 2F and G). No changes in enzyme activity with *EF1a* or *NUP153* (antisense, sense or duplex) RNA were observed with DNMT3A2 or the DNMT3A catalytic domain (data not shown).

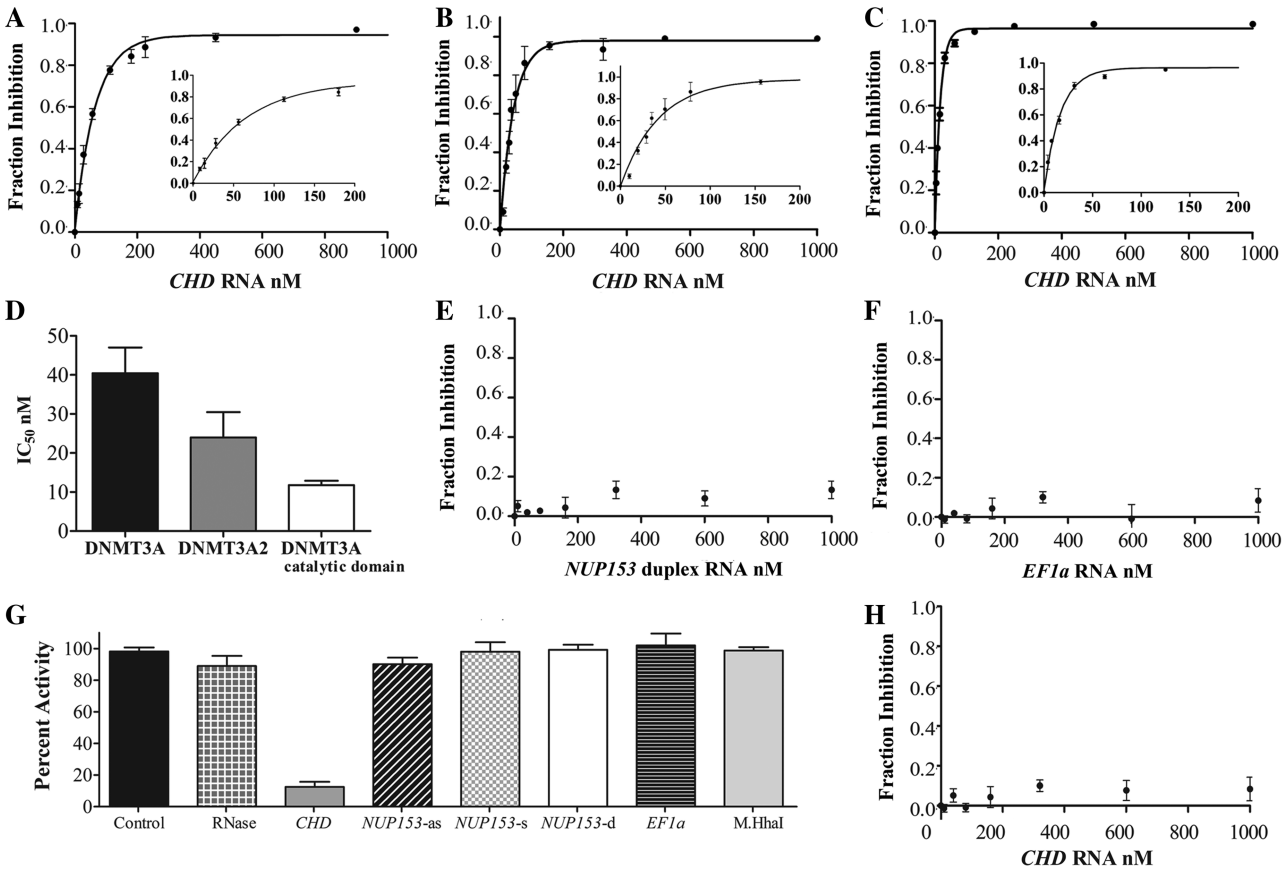

Figure 2. Inhibition by the ss*CHD* RNA. The modulation of DNMT3A activity was tested with four single-stranded and one double-stranded RNA, and only the CHD RNA altered DNMT3A activity. No inhibition was seen on the bacterial homolog. Inhibition curves for *CHD* RNA with the (**A**) full-length DNMT3A, (**B**) *DNMT3A2* or (**C**) catalytic domain. (**D**) *CHD* RNA IC_{50} values for DNMT3A truncations. Inhibition curves for *EFS1* RNA (**E**) and duplex *NUP153* (**F**) on full-length DNMT3A. (**G**) The extent of DNMT3A and M.HhaI inhibition with different RNA molecules including RNase treatment of *CHD* RNA prior to DNMT3A activity assay. RNA was varied from 0 nM to 2000 nM with largest affect on activity given in the bar graph. Control has no RNA added. RNA and DNA (5 μM) were pre-mixed and combined with enzyme (50 nM) and AdoMet (2 μM) to start the reactions. (**H**) *CHD* RNA had no affect on M.HhaI activity.

To confirm the inhibition observed with *CHD* RNA is specific to DNMT3A, this RNA was tested against M.HhaI, a bacterial homolog with high structural conservation in the catalytic domain. *CHD* RNA does not inhibit M. HhaI tested up to 2000 nM, 150-fold greater than the DNMT3A IC_{50} (Figure 2H). To confirm that the *CHD* RNA causes the potent inhibition of DNMT3A, we treated the RNA and DNA (substrate) with RNase for 5 min prior to assaying for methylation activity. This pre-treatment resulted in complete loss of the *CHD* RNA-mediated inhibition (Figure 2G).

We confirmed the duplex *NUP153* RNA binds full-length DNMT3A, as previously shown by Jeffery and Nakielny (20). The binding affinity of the RNA to DNMT3A was determined to be 520 ± 69 nM (Figure 3A). The ss*EF1a* RNA showed very similar results as the *NUP153* RNA, binding to the full-length DNMT3A with a K_D of 690 ± 89 nM (Figure 3B), but not binding to the catalytic domain. Minimal to no RNA binding was observed for the DNMT3A catalytic domain. However, the truncated isoform DNMT3A2 showed similar binding as the full-length protein (Figure 3C). We also tested RNA binding to the bacterial homolog M.HhaI up to four times the K_D of *NUP153* and *EF1a* with minimal to no binding for either RNA (Figure 3C). *EF1a* and *NUP153* RNA bind the enzymes but do not cause inhibition like the *CHD* RNA.

To determine if the *CHD* RNA-mediated inhibition is dependent on the nature of the DNA substrate, we tested the inhibitory effect on methylation of seven human promoters (28). RNA inhibition was observed to varying degrees with all human promoters. The extent of inhibition ranged from 60% to 91% and the IC_{50} values ranged from 22 ± 4 nM to 68 ± 22 nM (Figure 4A–D, Supplementary Table S3 and Supplementary Figure S1). We observed that the IC_{50} values is inversely related to the human promoters K_m for DNMT3A (Figure 4D), this suggests that the substrate and RNA are binding to the same form of the enzyme. The 3-fold variation in RNA inhibition potency observed with different DNA substrates suggests that at least some of the inhibition involves a complex between the enzyme, DNA and RNA (see below).

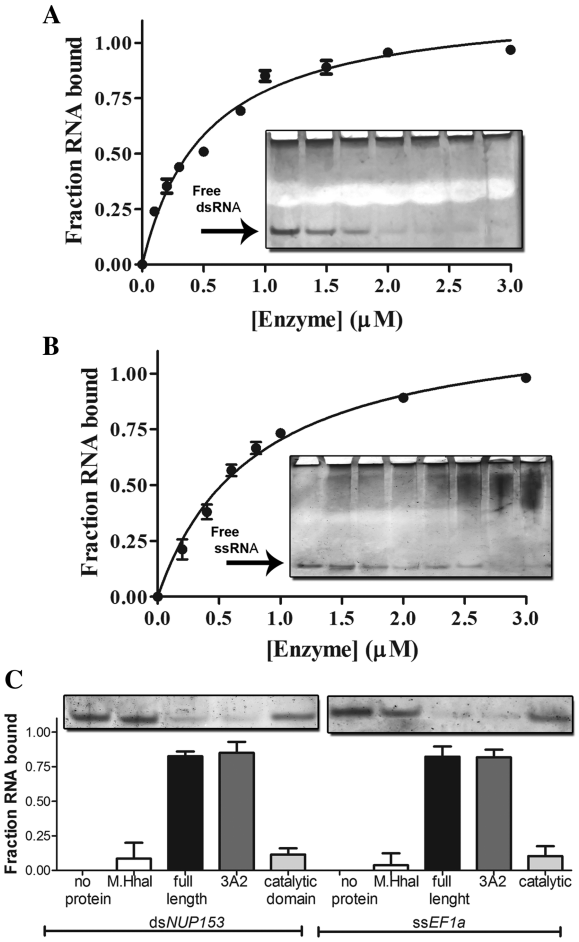

Figure 3. RNA binds to DNMT3A allosterically. RNA-binding curve for DNMT3A full-length with duplex *NUP153* (**A**). or ss*EF1a*, (**B**) error bars are standard error for at least two independent samples *insert* is Native PAGE gel showing the loss of a RNA (100 nM) band as an indicator of DNMT3A binding (varied from 0.2 μM–2 μM). (**C**). The amount of RNA binding to DNMT3A full-length, truncations and M.HhaI at 1 μM, RNA (100 nM), native PAGE of the unbound RNA. RNA type as indicated, data from three independent gel shift samples.

Methylation assays were carried out to probe the mechanism of inhibition by varying the substrate concentration at multiple RNA concentrations. The results are shown in Figure 5. The inhibition data on the full-length DNMT3A best fit a non-competitive or mixed type model ($R^2 = 0.933$; Figure 5 and Supplementary Table S4), however, the fit to a competitive model was not that different ($R^2 = 0.933$). The catalytic domain showed competitive and mixed type ($R^2 = 0.951$) as the best-fit models (Supplementary Figure 2 and Supplementary Table S4). Since mixed type inhibition involves an extra variable, non-competitive model best fits the data for the full-length DNMT3A and competitive for the catalytic domain. The non-competitive model best fits the data for the full-length DNMT3A and a competitive model best fits the catalytic domain data. Uncompetitive inhibition was the worst fit for both enzymes. Although the difference in fit between each model was not great enough to conclusively determine the mechanism of inhibition, that data suggest the RNA interacts with the same form of the enzyme as DNA, in addition to binding the binary form (enzyme–DNA complex).

Since conventional modeling of the mechanism was inconclusive, we used another approach to determine if RNA and DNA (substrate) bind to the same form of the enzyme by testing if the RNA can inhibit the enzyme once bound to DNA. Our previous demonstration that DNMT3A acts processively on substrates with multiple sites of methylation provides the basis for this approach (28). An initial control was carried out by adding the *CHD* RNA and DNA substrate at the same time, which gave the expected 80% inhibition. When RNA is added 25 min into the reaction no inhibition was seen for 45 min, at which point a 20% decrease in inhibition is observed. A similar level of inhibition is seen when DNA lacking CpG sites is added as the chase (28), suggesting that the *CHD* RNA does not inhibit the enzyme when bound to DNA (Figure 6). These results are consistent with the RNA-mediated inhibition occurring largely as a result of the enzyme dissociating from the DNA and then binding the RNA. However, consideration of all of the inhibition data suggest that DNMT3A inhibition occurs through multiple complexes, as discussed below.

DISCUSSION

Although compelling evidence supports direct and indirect roles of RNA in the regulation of DNA methylation (15,16), we know very little about how RNA–DNMT interactions alter the function of these enzymes. Of the four ssRNA sequences we studied, only the *CHD* RNA altered the function of DNMT3A (Figure 3). The potency and extent of inhibition is very dependent on the conformation of the RNA (Figure 2), further suggesting that the RNA–DNMT interface is quite specific. The *CHD* RNA did not inhibit the highly structurally homologous bacterial DNMT M.HhaI (34), providing further evidence of a unique RNA–DNMT3A interaction (Figure 3H). Two other RNA molecules did bind DNMT3A, the *NUP153* duplex and the ss*EF1a*, with no measurable catalytic changes to DNMT3A, suggesting there may be several mechanisms of interaction with DNMT3A alone. Cellular data also show multiple types of RNA can direct transcriptional gene silencing through alterations in DNA methylation (16,17,32,35). Our data suggest the events observed in the cells may occur through a direct interaction of RNA to DNMT3A.

DNMT3A binds *CHD* RNA with similar affinity as duplex DNA, whereas small molecule inhibitors show much weaker binding (36). The full-length DNMT3A data best fit a model invoking *CHD* RNA binding to at least one form of the enzyme already bound to the DNA substrate (e.g. non-competitive or mixed type inhibition). This form of allosteric inhibition is similar to previously characterized inhibition of DNMT1 by ssDNA (24). The catalytic domain results are best fit to a competitive inhibition model, which is inconsistent with the full-length data, so we utilized the fact that DNMT3A catalyzes

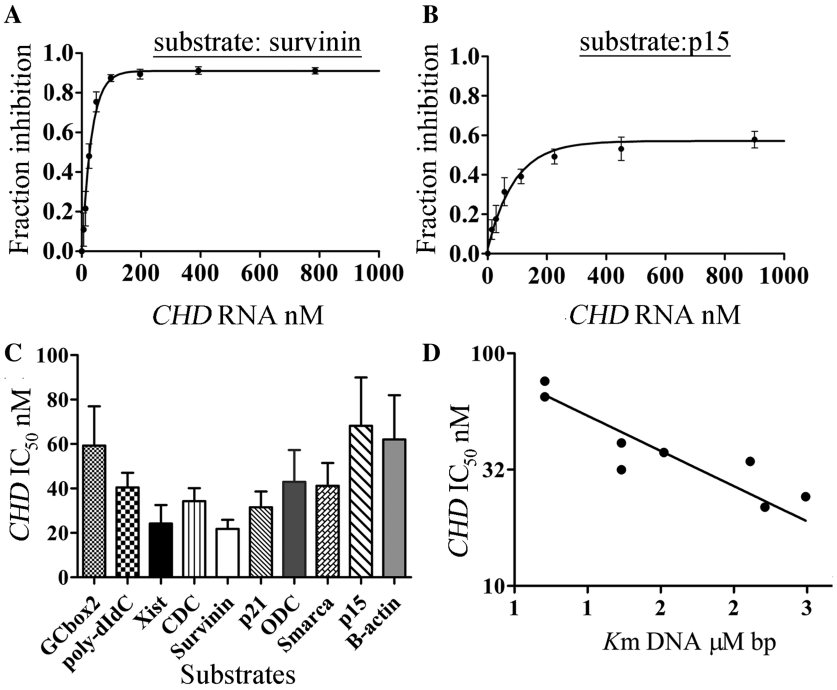

Figure 4. The potency of RNA inhibition on DNMT3A is dependent on DNA substrate. Inhibition curves on the full-length DNMT3A (50 nM) with *CHD* RNA, with different DNA substrates (10 μM), (**A**) *Survinin* human promoter, (**B**) *p15* human promoter and (**C**) IC_{50} values for *CHD* RNA with different substrates, inhibition curves are in Supplementary Figure S1. (**D**) Inverse relationship of the substrate K_m to the IC_{50} values from the *CHD* RNA on DNMT3A, log–log linear regression $R^2 = 0.82$.

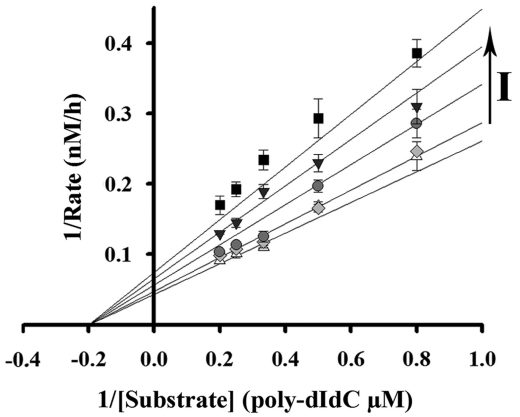

Figure 5. Inhibition Model for *CHD RNA* on full-length DNMT3A. The difference between models was not substantial, but the data best fit a non-competitive or mixed type model. Data shown fit to a non-competitive model $R^2 = 0.933$ (mixed type = $R^2 = 0.933$, competitive $R^2 = 0.928$, uncompetitive $R^2 = 0.92$). *CHD* RNA inhibitor 0, 10, 30, 50 and 70 nM. Inhibition model for *CHD RNA* on the catalytic domain of DNMT3A is shown in Supplementary Figure S2.

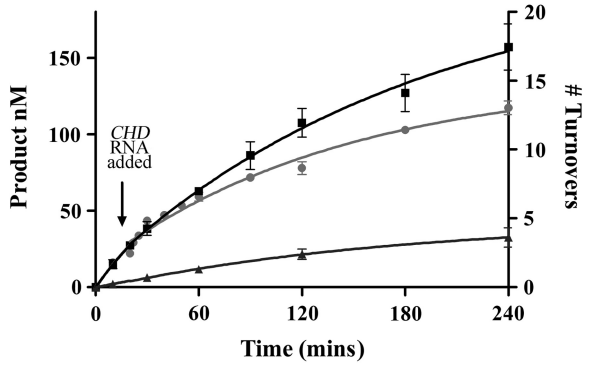

Figure 6. The *CHD* RNA does not inhibit the enzyme when bound to DNA. DNMT3A was previously shown to catalyze multiple turnovers on a polymeric substrate, so RNA was added after DNMT3A bound to the polymeric substrate, to test if the enzyme is inhibited when bound to DNA. RNA-mediated inhibition occurs largely as a result of the enzyme dissociating from the DNA and then binding the RNA. *CHD* RNA (200 nM) was added 25 min after the full-length DNMT3A (50 nM) was combined with the DNA (poly-dIdC at 5 μM). Filled square = DNA, open circle = DNA then RNA added at 25 min, filled triangle = DNA and RNA added at the start of reaction. Arrow indicates when *CHD* RNA was added.

multiple turnovers on a polymeric substrate (28) to test if the *CHD* RNA can inhibit the enzyme once bound to DNA. The lack of immediate inhibition upon addition of the RNA (Figure 6) shows that the dissociation of DNMT3A from its DNA substrate is required for *CHD* RNA inhibition.

Thus, inhibition by *CHD* RNA during catalysis on poly-dIdC is largely or exclusively mediated by the RNA binding to the same form of the enzyme, which binds the DNA. The most straightforward interpretation is that this involves the same active site, although this is not formally required. The catalytic domain of DNMT3A forms oligomeric complexes with itself (37), where all monomers are most likely not active. The *CHD* RNA

may bind to an inactive subunit and or bind where it alters the stability of such a complex. For example, RNA binding may disrupt oligomerization, which may not occur once the enzyme is bound to DNA. The somewhat poor fitting of the inhibition data with the full-length enzyme may be due to the *CHD* RNA binding to both the catalytic site and an allosteric site where the other RNA molecules are binding.

The *NUP153* and *EF1a* RNA also bound DNMT3A but no changes in activity were observed. These two RNAs, unlike the *CHD*, only bound to the full-length and DNMT3A2 and not to the catalytic domain alone, indicating allosteric binding outside the catalytic domain. The binding affinity was also weaker than the *CHD* or DNA by about 8-fold, also indicating a different binding site. The PWWP domain of DNMT3A and DNMT3B has previously been shown to bind nucleic acids and this domain is needed for heterochromatin association (22,38,39). Recent studies of several chromatin modification complexes revealed that thousands of RNAs including antisense ncRNAs bind to chromatin modifying complexes (12,40–43). Histone modifying enzymes bind RNA to associate with chromatin or other binding partners. The RNA acts as a scaffold bridging two independent complexes together, but no changes in catalysis has been observed (44). The RNA molecules that bind DNMT3A suggest a similar scaffolding mechanism may be occurring.

Regulatory implications of RNA–DNMT3A interactions

We showed that the RNA-mediated inhibition of DNMT3A is highly sequence-specific, involves the catalytic domain, and involves the RNA binding to the same form of DNMT3A which binds the DNA substrate as well as to the DNMT3A:DNA complex. What is the potential relevance of these observations to the regulation of DNMT3A function *in vivo*? RNA may regulate DNMT3A *in vivo* but with distinct functional consequences as a result of occurring in a more complex context involving other proteins or factors. DNMT3A is well known to interact with numerous partner proteins (6–9), and the impact of RNA on DNMT3A in such settings could alter the function of the RNA.

Inhibitory roles for RNA *in vivo* are uncommon but not without precedent (45). DNMT3A inhibition may serve to limit enzymatic activity at particular loci until the enzyme is either activated or directed to a particular genomic position. RNA-mediated inhibition of DNMT3A is prevented once the enzyme initiates methylation (Figure 6), which may reconcile the inhibition with localized activity. This situation is supported by our observation that DNMT3A inhibition varies with different DNA substrates. Our *in vitro* observations may provide a mechanism for the recent demonstration that knockdown of two antisense ssRNAs derived from CpG islands induce methylation at the corresponding promoters (46).

Alternatively, our studies were designed to reveal the functional impact of RNA molecules on DNMT3A, whereas the primary role of the RNA may be as a scaffold for DNMT3A and its partner proteins (16). This model is consistent with some RNA sequences binding DNMT3A without functional impact. RNA scaffolding is also the mechanism used by chromatin modifying complexes and transcription factors to direct site specific binding (47). The extensive transcription of the human genome (48–50) may provide RNAs, which either directly module DNMT3A function, and/or provide a scaffold to localize the enzyme, leading to proximal methylation (16). We suggest that the potent and sequence-specific *in vitro* modulation of DNMT3A function by RNA supports the *in vivo* mechanisms in which RNA is postulated to regulate transcription (32).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–4 and Supplementary Figures 1 and 2.

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