Methylation of Xilf3 by Xprmt1b alters its DNA, but not RNA, binding activity

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Methylation of Xilf3 by Xprmt1b Alters Its DNA, but Not RNA, Binding Activity[†]

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ABSTRACT: Modification of proteins by methylation has emerged as a key regulatory mechanism in many cellular processes, including gene control. Eighty to ninety percent of the arginine methylation in the cell is performed by the protein arginine methyl transferase PRMT1. ILF3, a protein involved in gene regulation at several levels, has been shown to be a substrate and regulator of PRMT1 in mammals. Here we show that the *Xenopus* orthologue of ILF3 (Xilf3) is methylated in vivo, and, at least in vitro, this methylation is carried out by Xprmt1b. The in vitro methylation of Xilf3 inhibits its ability to bind to DNA while leaving RNA binding activity unaltered. Consistent with these activities having a role in vivo, the DNA binding activity of the Xilf3-containing CBTF complex and the transcription of its target gene, *Xgata2*, are both decreased by overexpression of Xprmt1b in embryos. However, in contrast to other RNA binding proteins, a changing degree of methylation does not alter the subcellular localization of Xilf3. Several other proteins involved in gene regulation can bind both RNA and DNA; these data demonstrate a mechanism by which such binding activities may be controlled independently.

ILF3[1] was originally identified as a component of a dimeric transcription regulator that bound to the IL2 enhancer and could regulate that gene in vitro (*1, 2*). Its NF90 variant has recently been shown to bind to this region in a complex with Ku80 in vivo (*3*). ILF3 was subsequently identified in a number of different contexts, and the associated data have shown that it has roles in both transcriptional (*4, 5*) and post-transcriptional (*6–8*) gene regulation. These distinct functions may be associated with the protein diversity generated by alternative splicing of the products of the *ILF3* locus (*9*).

ILF3 has two dsRNA binding domains (dsRBDs), a nuclear localization signal, and two other regions that could be important for nucleic acid binding at its C-terminus, an RGG motif and a GQSY domain (*10*). The RNA binding domains can also bind to DNA, in particular if it is A-form DNA (*11*). A null mutation of *ILF3* in mice is lethal neonatally due to misregulation of a number of muscle-specific transcription factors that are thought to be controlled post-transcriptionally by ILF3 (*8*). During development of *Xenopus* embryos, Xilf3 (previously called 4F, p122, or CBTF[122] but renamed to conform with *Xenopus* nomenclature recommendations), the orthologue of the mammalian ILF3 protein, is found as part of a multisubunit transcription factor (CBTF) that controls the transcription of the *Xgata2* gene both in oocytes and at the gastrula stage of development (*12, 13*). Xgata2 is implicated in dorsal-ventral patterning of developing *Xenopus* embryos, and consistent with the role of Xilf3 in Xgata2 transcription, expression of dominant interfering forms of Xilf3 causes defects in dorsal-ventral patterning (*11*). The importance of the ILF3 protein in a variety of cellular roles and during development has led to the investigation of its regulation.

The ILF3 protein is phosphorylated in vivo at the G2−M transition of the cell cycle, and phosphorylation at mitosis has been shown not to affect the RNA binding activity of ILF3 (*10*), although phosphorylation is coincident with its movement from the nucleus to the cytoplasm. Xilf3 is also a phosphoprotein, and mimicking phosphorylation of specific residues can affect its nuclear translocation (*14, 15*). The cytoplasm to nucleus translocation of Xilf3 occurs during development at the midblastula transition (MBT) when zygotic transcription begins and maternal transcripts are degraded. Mutations that prevent RNA binding by ILF3 cause it to be nuclear prior to the MBT as does injection of RNase into the developing embryo, showing that RNA binding acts to anchor Xilf3 in the cytoplasm of the early embryo (*16*).

In addition to phosphorylation, ILF3 has also been shown to be modified by methylation (*17*), like several other RNA binding proteins (*18–25*). In a study by Tang and co-workers, a yeast two-hybrid screen searching for proteins that bind to PRMT1 identified the RGG rich region of ILF3. Immunochemistry showed that these two proteins colocalize in HeLa cells. Additionally, immunoprecipitation of HeLa cell lysates using anti-ILF3 pulled down PRMT1 enzyme activity, and a PRMT1−GST fusion protein also bound ILF3 when incubated with these same lysates. A number of PRMT1

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[1] Abbreviations: ILF3, interleukin enhancer binding factor 3; PRMT1, protein arginine methyltransferase 1; IL2, interleukin 2.

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binding proteins have been shown to regulate its methyltransferase activity, and ILF3 also activates it (*17*).

In *Xenopus*, Xprmt1b has a critical role during development; it is induced by Ca^{2+} and is subsequently necessary for neural commitment. For example, its overexpression upregulates neural genes such as Xzic3 directly in isolated animal pole explants or whole embryos, and a morpholino oligonucleotide directed against Xprmt1b reduces or blocks the expression of a number of neural markers in the whole embryo (*26*). Xprmt1b mRNA is expressed maternally and in the inner (sensorial) ectoderm at the gastrula stage; subsequently, it is mainly in the brain and neural tube (*26*). Since this expression pattern is very similar to that of Xilf3 (*13*) and because of the interaction found between the mammalian orthologues of these proteins, we decided to test whether Xprmt1b regulates Xilf3 in *Xenopus* embryos.

We present data that show the modification of ILF3 by PRMT1 to be conserved in *Xenopus* and methylation has functional significance both in vitro and in vivo. Our results indicate that methylation of Xilf3 regulates its DNA, but not RNA, binding activity, providing a potential switching mechanism for the transcriptional and post-transcriptional functions of this protein.

MATERIALS AND METHODS

Embryos were prepared and cultured as previously described (*27*); they were staged according to the method of Nieuwkoop (*28*).

Protein Preparation and Nucleic Acid Binding Assay. The dsRBD and dsRBD+RGG regions of Xilf3 were subcloned into pGex2T (Amersham Pharmacia Biotech). The GST fusion proteins were expressed in *Escherichia coli* and purified by glutathione-Sepharose and DNA−cellulose column chromatography as previously described (*16*). The purified proteins were assayed and quantified by SDS−PAGE and UV spectroscopy using the calculated extinction coefficient of each protein. Xprmt1b was prepared as described previously (*26*). The filter binding assays used to analyze DNA and RNA binding were performed using probes corresponding to the −66 to −31 sequence of the *Xenopus GATA-2* promoter as either dsRNA or DNA. The dsRNA and DNA binding assays were performed side by side using three separate preparations of proteins according to the method of Stockley (*29*).

Methylation Assay. To allow analysis by SDS−PAGE, Xprmt1b was used bound to glutathione-Sepharose resin to reduce the reaction volume. Xprmt1b was stored in glycerol and washed with IPH buffer [50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, and 0.5% NP40] prior to use. The volumes of enzyme and protein were chosen empirically (1:1, 1:2) as the stoichiometry of the reaction is unknown. Xprmt1b, the purified Xilf3, and 1 μL of [^{3}H]-*S*-adenosylmethionine (methyl donor) were incubated at 30 °C for 30 min. The same reaction was conducted with nonlabeled SAM. SDS loading buffer was added, and the samples were analyzed on parallel SDS−PAGE gels. The gel in which the nonlabeled reaction mixtures were assessed was Coomassie stained, and the other gel was fixed, dried, and exposed on a phosphorimager tritium screen.

Immunoprecipitation and Western Blotting. One hundred embryos were homogenized in 400 μL of 1XRIPA [1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), and protease inhibitor cocktail (Roche)]. After freon extraction [1,1,2-trichlorotrifluoroethane (Sigma)], the lysate was divided into two 200 μL aliquots and 2.5 μL of either polyclonal rabbit anti-Xilf3 antibody (provided by B. Bass, University of Utah, Salt Lake City, UT) or normal rabbit serum was added to the tubes. Tubes were gently agitated for 2 h at 4 °C, after which they were centrifuged at 14000*g* for 10 min. The supernatant was mixed with 0.02 g of preswollen protein A Sepharose (Sigma). Following a further incubation for 2 h at 4 °C, the resin was washed eight times in 500 μL of 1XRIPA before being resuspended in 1× SDS loading buffer. Immunoprecipitated proteins were assayed by SDS−PAGE and Western blotting.

Chromatin-Associated Immunoprecipitation. The method used was essentially that of Sachs et al. (*30*). Embryos were incubated in formaldehyde, prior to chromatin preparation and shearing by sonication to an average length of approximately 750 bp. The DNA was purified by isopycnic centrifugation on CsCl gradients using a Beckman ultracentrifuge. DNA was incubated with the relevant antibodies or with normal rabbit serum (NRS). Antibody-bound complexes were precipitated with Protein A Sepharose and washed, and the DNA was released by proteinase K digestion. The sequence content of the input and bound material was analyzed by qPCR using Taqman. The probe and primer sequences are available upon request.

Analysis of mRNA Levels by Real-Time RT-PCR. cDNA was synthesized from RNA extracted from dissected explants of injected embryos cultured until stage 11 using the method of Steinbach and Rupp (*31*). RNA expression was assayed as cDNA by PCR conducted on an ABI 7900HT sequence detection system using TAQMAN fluorescently labeled probes. Data were analyzed using the ΔΔCt method (ABI) using ornithine decarboxylase (ODC) as a control gene and uninjected explants as the control tissue. The probe and primer sequences are available upon request.

Electrophoretic Mobility Shift Assays of Endogenous CBTF. DNA oligonucleotides (Invitrogen) were annealed to form duplexes and end-labeled with T4 polynucleotide kinase (NEB) using [γ-^{33}P]ATP. The proteins were incubated with the nucleic acid probe for 15 min on ice in EMSA buffer (*32*) in the presence of 500 ng of poly(dI-dC). Either wild-type or mutant nonlabeled competitor was added at a 50-fold excess to two of the reaction mixtures to allow identification of the specific DNA−protein complexes. After incubation, the DNA and DNA−protein complexes were separated on a 4% native polyacrylamide gel in 0.25× TBE. The gels were dried and visualized using a phosphorimager.

Immunohistochemistry. To observe endogenous Xilf3, embryos were fixed in MEMFA, embedded in PEDS wax, and cut into 10 μm sections on a Leica RM2165 microtome. Dewaxed sections were blocked for 30 min with PBT and 10% non-immune, heat-inactivated goat serum. The block was replaced with a 1:1000 dilution of the anti-Xilf3 serum (a gift from B. Bass) in the same buffer and incubated for 1 h. Three 5 min washes in PBT were followed by a 1 h incubation with goat anti-rabbit HRP-conjugated antibody (1:1000). Three final washes in PBT preceded color development with Fast-DAB (Sigma). To express the exogenous Xprmt1b or VLD, 2 ng of the appropriate synthetic RNA

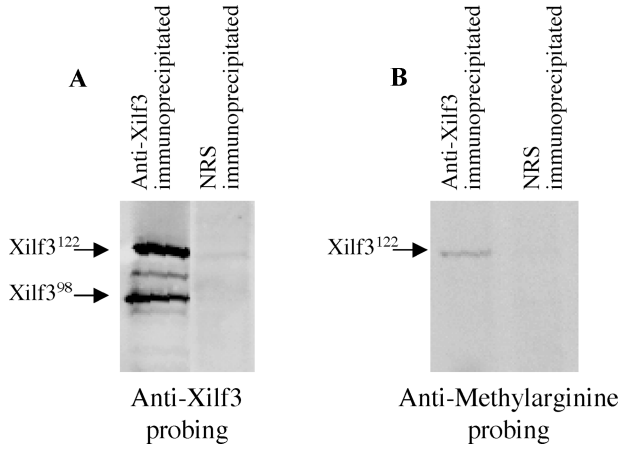

FIGURE 1: Xilf3 is methylated in vivo. Xilf3 was immunoprecipitated from 100 embryos at stage 8 with anti-Xilf3 antibody or normal rabbit serum. Three duplicate gels of the samples were run for Western blot probing with either anti-Xilf3 (A) or anti-methylarginine (B) antibodies, and the third gel was assayed by Coomassie brillant blue staining (not shown).

was injected into embryos at the two-cell stage, and they were allowed to develop to the stages shown. Sections were visualized using DIC optics on a Nikon E800 eclipse microscope and images captured using a Nikon DN100 camera.

RESULTS

Xilf3 Is Methylated Pre-MBT in Vivo and Is a Substrate of Xprmt1b in Vitro. In mammals, ILF3 is methylated by PRMT1. We first examined whether Xilf3, the *Xenopus* orthologue of ILF3, was also methylated in vivo. Probing of immunoprecipitated Xilf3 using an anti-methylarginine antibody (Abcam) on a Western blot revealed a band corresponding to Xilf3, as identified by a control probing with anti-Xilf3 antibody on a duplicate membrane (Figure 1). We next tested the ability of Xprmt1b to methylate Xilf3 in vitro. Xprmt1b and two fragments of Xilf3 were expressed as GST fusions in *E. coli* and purified. The Xilf3 fragments corresponded to amino acids 326−608 (28.5 kDa) and 302−656 (41 kDa), although with the addition of the GST fusion the total molecular masses were approximately 60 and 70 kDa, respectively. These recombinant proteins were designated Xilf3(28.5) and Xilf3(41), respectively. Both fragments include the NLS and the dsRBDs and have been shown to bind DNA and RNA. The longer fragment also includes the RGG motif (Figure 2A). The ability of either Xilf3 fragment to act as a substrate for Xprmt1b was analyzed by a standard methylation assay with [^{3}H]-*S*-adenosylmethionine as the methyl donor. The products of the reaction were separated by SDS−PAGE and labeled proteins visualized. Only the longer Xilf3 fragment that contained the RGG motif was methylated (Figure 2C); we calculate two to three arginines were methylated per molecule of Xilf3(41). Methylation of other RNA binding proteins has been shown to modulate their RNA binding activity (reviewed in ref *33*); we therefore tested whether the nucleic acid binding activity of Xilf3 was regulated by methylation.

Methylation Decreases the DNA Binding Activity of Xilf3. To test whether the nucleic acid binding activity of Xilf3 was regulated by methylation, we prepared Xilf3(41) and either methylated it in a standard reaction or performed a mock methylation from which SAM was omitted. The success of the methylation reaction was assayed by analyzing an aliquot of the products by SDS−PAGE and phosphorimager visualization alongside the binding assay. The filter binding assays showed that DNA binding was inhibited significantly by methylation, whereas dsRNA binding was unaffected by methylation (Figure 3). We then tested whether the inhibition of Xilf3 DNA binding by methylation might be significant in vivo.

Xgata2 Is a Direct Target of Xilf3. To test whether the methylation of Xilf3 affects transcription in vivo, a direct target gene of Xilf3 needed to be identified. We have previously obtained in vitro biochemical evidence that Xilf3 is a subunit of CBTF which controls *Xgata2* transcription in gastrula stage embryos (*13*). To test directly whether Xilf3 is bound to the *Xgata2* promoter in embryos, we modified the *Xenopus* chromatin immunoprecipitation (ChIP) method of Sachs and co-workers (*30*) so that it could be used in gastrula stage embryos. Antibodies recognizing Xilf3 and hyperacetylated histone H4 (as a marker of an active promoter) were used to immunoprecipitate chromatin at stage 13, when the *Xgata2* gene is transcribed in a large proportion of cells in the embryo. A high level of the *Xgata2* promoter was immunoselected, but the magnitude of the signal was greatly reduced at stage 23 when Xgata2 mRNA can be detected in far fewer cells (*34*) (Figure 4). Normal rabbit serum did not precipitate this sequence and provided a control for the specificity of the antisera used in the ChIP. Two amplicons were used as negative controls to check the specificity of the immunoprecipitation, one from the downstream transcribed region of the *Xgata2* gene [it appears that histone acetylation at *Xgata2* is promoter proximal as with most genes (*35, 36*)] and a second from the thyroid hormone receptor β-promoter, which is not expressed until metamorphosis (*30, 37*). Neither of these DNA sequences was immunoprecipitated by Xilf3 or hyperacetylated histone H4 antiserum. These data show that Xilf3 is bound to the *Xgata2* promoter when the gene is active and together with functional evidence (*11*) show that Xilf3 is a direct regulator of *Xgata2*.

Overexpression of Exogenous Xprmt1b Increases the Level of Xilf3 Methylation in Vivo but Reduces both the Level of Binding of the Xilf3-Containing Complex CBTF to the Xgata2 Promoter Sequence and Xgata2 mRNA Levels in Animal Cap Explants. Since it is straightforward to overexpress proteins in *Xenopus* embryos, we took advantage of this experimental system to test whether increasing Xprmt1b activity in embryos can alter the activity of Xilf3 and the complex of which it is a component, CBTF. Embryos were injected into the animal pole with synthetic RNA encoding Xprmt1b, cultured until stage 10.5, and harvested. We first confirmed that overexpression of Xprmt1b led to an increased level of methylation of Xilf3 by immunoprecipitation and Western blot probing with an anti-methylarginine antibody (Figure 5A). We next used total protein extract from injected embryos in electrophoretic mobility shift assays (EMSAs) using an oligonucleotide duplex corresponding to the 36 critical bases of the *Xgata2* promoter (sequence shown in Figure 5B) that binds the Xilf3-containing complex CBTF. Several complexes are present on the developed image; that corresponding to the specific CBTF probe interaction was identified by use of either wild-type or mutant unlabeled

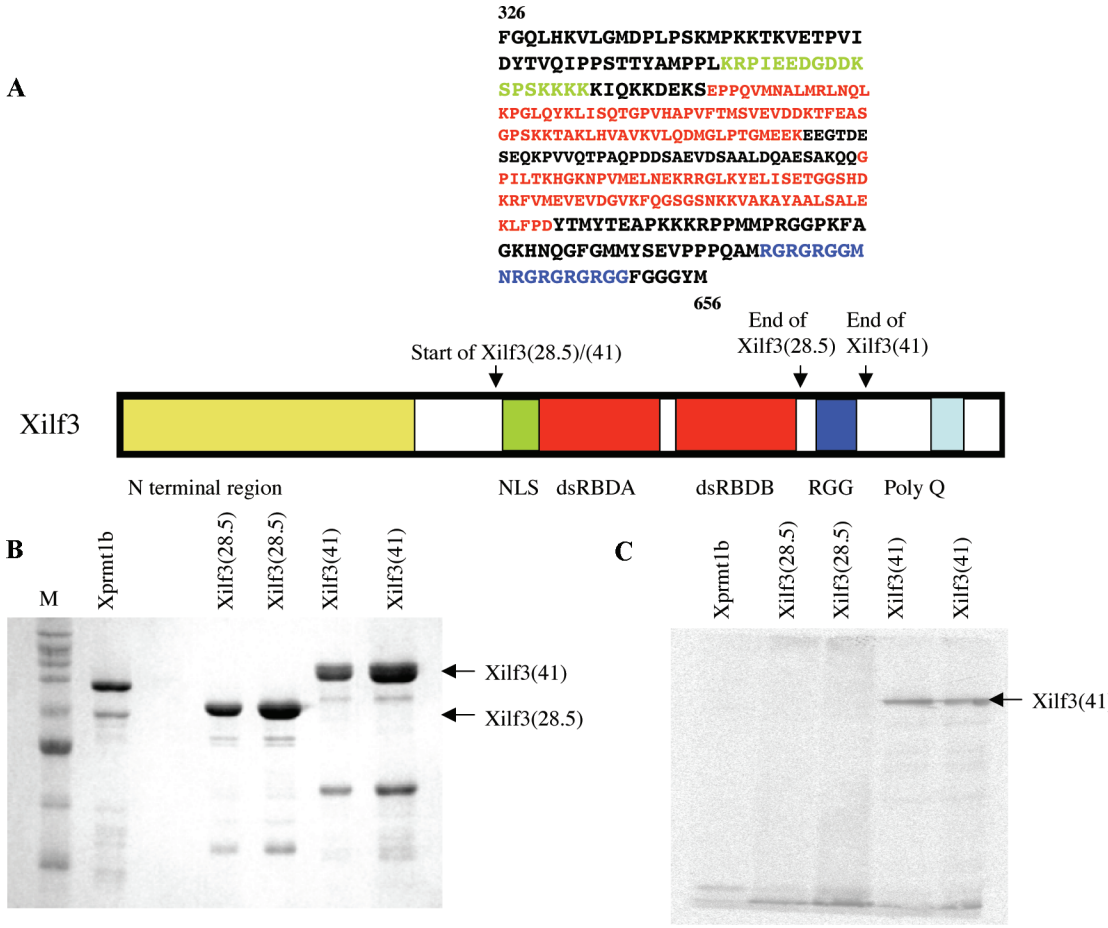

FIGURE 2: Xilf3 is methylated at its RGG rich domain in vitro by the methyltransferase Xprmt1b. A schematic of full-length Xilf3 is shown in panel A, and the amino acid sequences of the relevant regions are shown above, numbered with respect to the full-length protein. The NLS is colored green; dsRBDs are colored red, and the RGG sequence is colored blue. The truncated Xilf3 constructs, Xilf3(28.5) and Xilf3(41), were expressed in *E. coli* as GST fusions and affinity purified like Xprmt1b was. Either 5 or 10 μL of Xilf3(28.5) and Xilf3(41) was incubated with Xprmt1b, in the presence of [^{3}H]SAM and then resolved on a 10% SDS−PAGE gel in parallel with the duplicated nonradioactive loading of the purified proteins. (B) The unlabeled gel was Coomassie stained, and proteins were sized using a 10 kDa protein marker (lane M). The labeled gel was fixed, dried, and exposed to a phosphorimager screen. The exposed image (C) shows methylation of Xilf3(41), which contains the RGG rich domain of Xilf3 missing from Xilf3(28.5), by Xprmt1b.

competitor oligonucleotide duplex in two control reactions. The complex that was affected by the wild-type cold competitor, but unaffected by mutant sequences, represents a specific interaction. The specific complex (Figure 5B, arrow) decreases in intensity with increasing amounts of Xprmt1b expressed, indicating a decreasing affinity of CBTF-containing Xilf3 for the *Xgata2* promoter sequence in embryos.

To test if the reduction in affinity for the *Xgata2* promoter led to a reduction in the level of expression of *Xgata* in vivo, we again injected Xprmt1b, or GFP mRNA as a control, into embryos. Animal pole explants were then prepared from the embryos at stage 8 and cultured until siblings reached stage 14. mRNA levels were then measured using qRT-PCR using ODC as an internal control (Figure 5C). Previously published data have shown that expression of Xprmt1b neuralizes ectoderm (*26*); therefore, chordin and Xbmp4 mRNA levels were analyzed to ensure that the injected Xprmt1b was active. The increase in the level of chordin and the decrease in the level of Xbmp4 mRNAs confirmed that this was the case. *Xgata2* expression was repressed in Xprmt1b-expressing animal pole explants; this is consistent with methylation of Xilf3 inhibiting its DNA binding and the level of *Xgata2* transcription decreasing as a result.

Altering Xprmt1b Activity Does Not Affect Xilf3 Subcellular Localization. For a subset of RNA binding proteins, methylation has been shown to alter subcellular localization (*18*, *39*, *40*). We therefore tested whether altering Xprmt1b activity in embryos by overexpression of Xprmt1b or its dominant interfering form, VLD (*38*), affected Xilf3 subcellular localization. Embryos were treated with synthetic RNA encoding either GFP as a control, Xprmt1b, or VLD. The embryos were allowed to develop to either blastula or early gastrula stages and fixed. The embryos were then sectioned and Xilf3 visualized by antibody staining (Figure 6). None of the embryos (n = 20 for each treatment) showed alterations in localization of Xilf3, which remained distributed throughout the cytoplasm prior to the MBT and mainly nuclear after it. Evidence that the RNA injections were effective in changing Xprmt1b activity came from the analysis of Xbmp4 and Xchordin mRNA levels (Figure 4).

DISCUSSION

Our main finding, that Xilf3 has its DNA binding activity changed by methylation while its RNA binding remains unaltered, has implications for an expanding group of proteins that can bind to both RNA and DNA and are

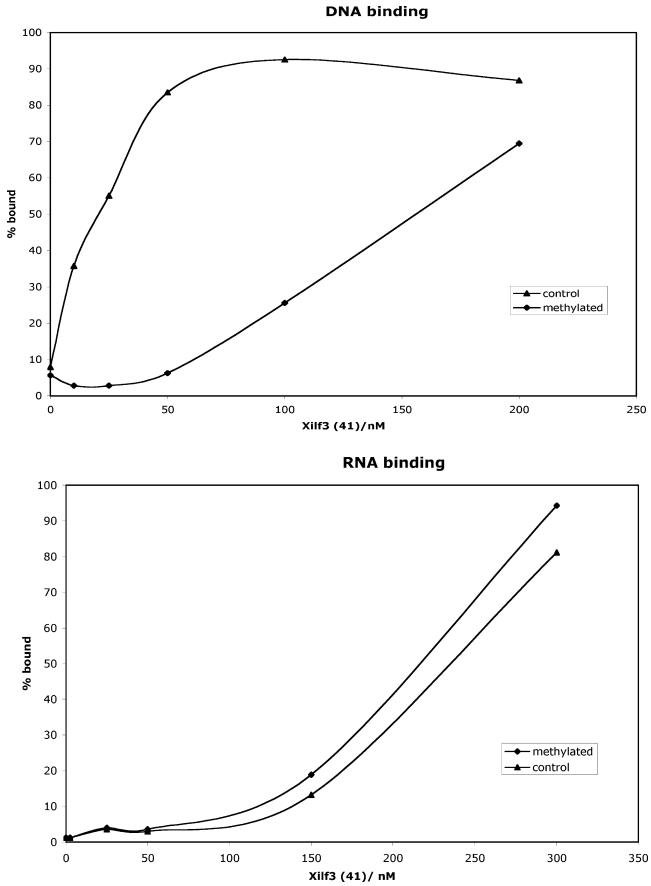

FIGURE 3: Methylation of Xilf3 decreases its DNA binding activity without affecting RNA binding in vitro. Truncated Xilf3 [Xilf3(41)] was expressed in *E. coli* and affinity purified like Xprmt1b was. The Xilf3 preparation was divided into two and each half incubated with Xprmt1b in the presence (methylated) or absence (control) of SAM. The efficiency of the methylation was monitored by taking a small aliquot of the reaction mixture and spiking it with [^{3}H]SAM as described in the legend of Figure 1. The control or methylated Xilf3 was then incubated at the concentrations shown with ^{32}P-labeled dsRNA or dsDNA 40-mer oligonucleotides. The amount of protein−nucleic acid complex was then monitored using a filter binding assay, and amounts of radiolabeled protein−nucleic acid complex retained on the filter were measured on a Perkin-Elmer 1450 microbeta scintillation counter and plotted as the percent bound. The percent bound was defined by two control samples; 0% was the level of radioactive retention to the filter from a sample with no protein, and 100% retention was determined by adding a sample identical to the first control to a presoaked filter but without filtering prior to counting. The dsRNA and DNA binding assays were performed side by side using three separate preparations of proteins, and the same binding curve was observed.

involved in gene regulation at a number of levels. Examples of these include RNA helicase A, which is an RNA binding protein and transcriptional coactivator (*41, 42*); hnRNP C1/C2, an RNA binding protein that is also a sequence-specific DNA binding protein (*43*); and ILF2, which has some sequence homology to ILF3 and regulates both translation and transcription (*44, 45*). The fact that a post-translational modification can specifically alter one nucleic binding activity implies that this type of protein may, while capable of binding both DNA and RNA, be directed to bind only one of these under a particular set of conditions. Methylation of other RNA binding proteins has been shown to alter their affinity for RNA; HnRNP A1 has a lower affinity when its RGG domain is methylated (*46*), and the level of Rev binding to RNA is reduced when Rev is in its methylated state (*47*).

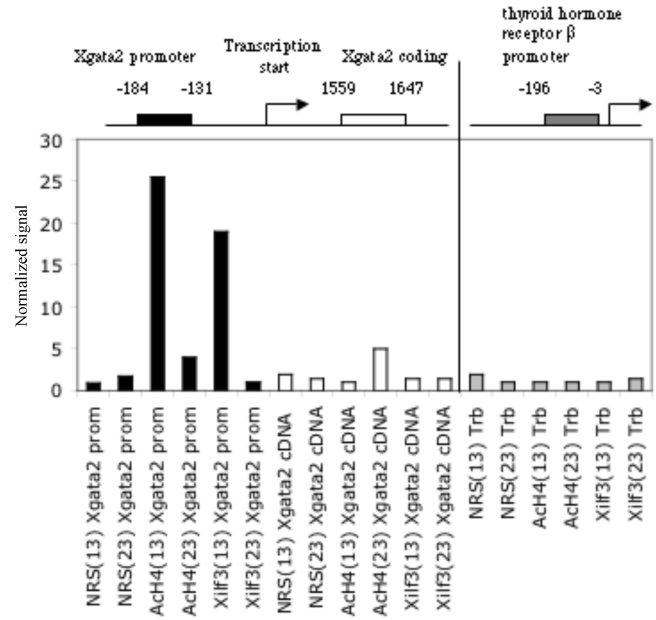

FIGURE 4: Xgata2 promoter has Xilf3 and acetylated histone H4 bound when it is active. The relative sequence content of immunoprecipitated material per input is shown for six ChIPs analyzed at three amplicons using qPCR: Xgata2 promoter (black), Xgata2 cDNA (white), and Trb (gray). Nuclei prepared from 100 stage 13 (*Xgata2* highly active) or 23 (*Xgata2* barely active) embryos were incubated in formaldehyde, and the prepared chromatin was sheared by sonication to an average length of approximately 750 bp and purified by isopycnic centrifugation on CsCl gradients. Chromatin equivalent to 15 embryos was immunoprecipitated with normal rabbit serum (NRS) or antiserum recognizing hyperacetylated histone H4 (AcH4) or Xilf3 (CBTF). The sequence content of the input and bound material was analyzed by qPCR using Taqman probes corresponding to the thyroid hormone receptor (Trb) or *Xgata2* (Xgata2 Prom) promoters or to exon 4 of the *Xgata2* gene (Xgata2 cDNA). The bound per input signal was normalized to 1 for the lowest ratio for each ChIP/amplicon set; triplicates varied by <10%. Shown above the graph is a schematic of the placement of the amplicons with numbering relative to the start of transcription.

By contrast, Hrp 1p shows no changed RNA binding when methylated (*48*), and Xilf3 seems to fall into this latter category. Consistent with this observation, deleting the RGG motif in Xilf3 causes a slight increase in its RNA binding affinity (*14*), suggesting that this motif may contribute solely to DNA binding. A decreased level of DNA binding upon methylation of a protein is not unique; MBD2 is similarly regulated (*49*).

Our data support an important role for the regulation of Xilf3 DNA binding by methylation in vivo. The 122 kDa isoform of Xilf3 is methylated in vivo in contrast to the 98 kDa isoform, confirming that the RGG domain is the site of methylation in vivo as it is in vitro, since it contains the only arginine residues in the region that differ between these two isoforms. The change in *Xgata2* expression that we observed upon overexpression of Xprmt1b in embryos may occur via a number of mechanisms. It is possible that Xprmt1b is dorsalizing the ectoderm as shown previously (*26*) and that *Xgata2*, which is ventrally expressed (*34, 50*), is downregulated as a consequence. Alternatively, since Xbmp4 and Xgata2 act in a positive feedback loop (*51, 52*) and *Xbmp4* is downregulated by Xprmt1b expression, this may decrease Xgata2 mRNA levels.

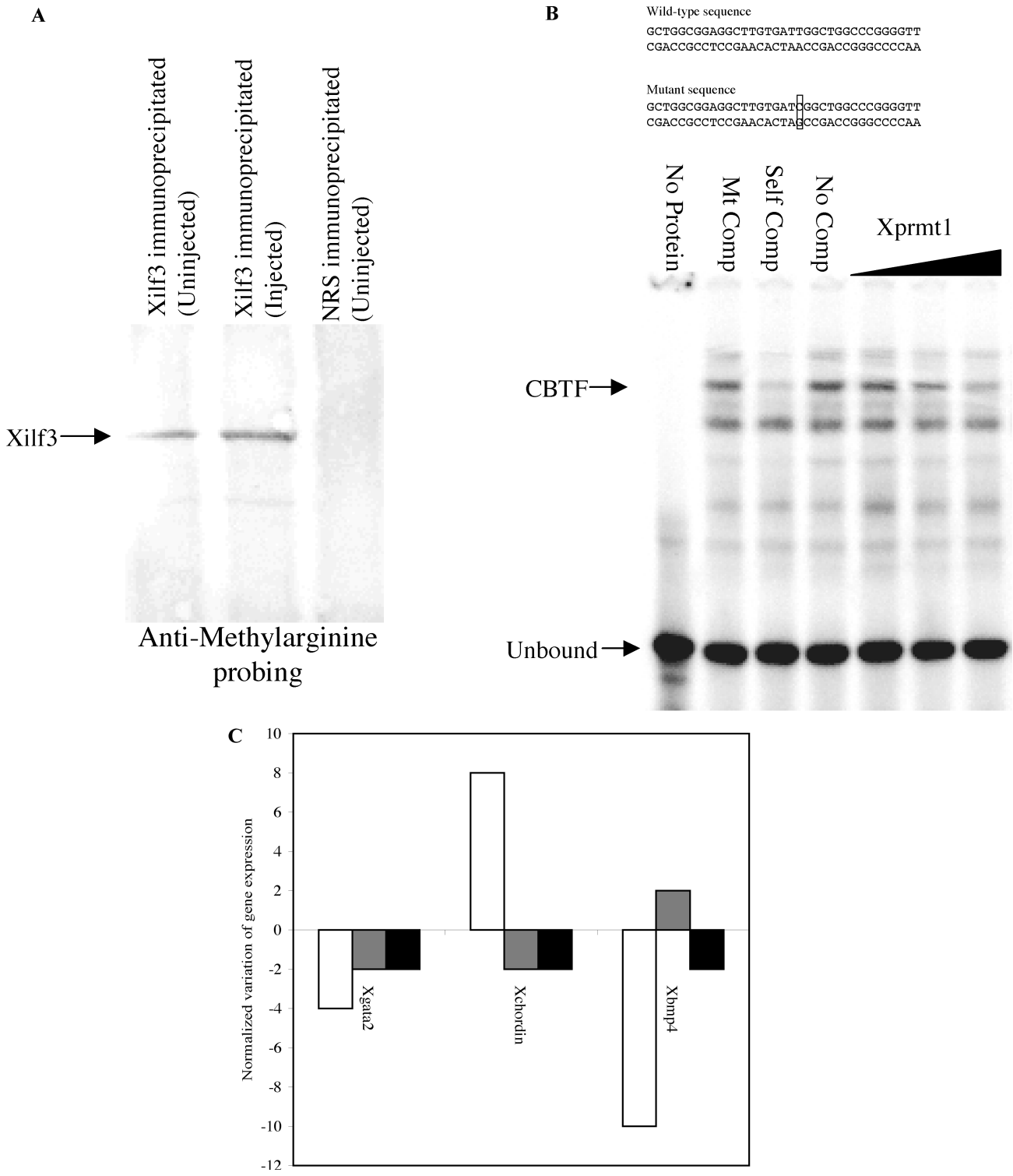

FIGURE 5: Overexpression of exogenous Xprmt1b increases the level of Xilf3 methylation in vivo but reduces both the level of binding of the Xilf3-containing complex CBTF to the *Xgata2* promoter sequence and Xgata2 mRNA levels in animal cap explants. Two nanograms of synthetic RNA encoding Xprmt1b was injected into two cell embryos, and the embryos were cultured until stage 10.5. Xilf3 was immunoprecipitated from both injected and uninjected embryos, alongside a control normal rabbit serum (NRS) immunoprecipitation. Two duplicate gels of the samples were run for Western blot probing with either anti-methylarginine (A) or anti-Xilf3 (not shown). (B) Two cell embryos were treated with 0.5, 1, or 2 ng of synthetic RNA encoding Xprmt1b, and a control set of embryos was treated with 2 ng of GFP-encoding RNA. The embryos were cultured until stage 10.5 before being harvested and assayed for endogenous CBTF binding to a radiolabeled DNA sequence corresponding to 36 bases of the *Xgata2* promoter. The GFP-treated sample was used in three control reactions; in two of these reactions, either unlabeled wild-type or mutant oligonucleotide was added at a 50:1 excess. Samples were separated on a 4% polyacrylamide gel and exposed to a phosphorimager screen. (C) Quantitative RT-PCR (qRT-PCR) was performed on RNA extracted from animal cap explants from embryos expressing Xprmt1b (white), its dominant negative form VLD (gray), or an engrailed domain as a control (black). The difference in gene expression triggered by VLD mRNA is insignificant since it is similar to the control. Together with the increase in the level of Xchordin expression, the depletion of xBMP4 and Xgata2 mRNA levels shows a global dorsalization of the injected animal caps. The increase (positive numbers) or decrease (negative numbers) in the level of expression of the genes was normalized to the housekeeping gene ODC and to uninjected embryos, and values are the means of two independent experiments each amplified in triplicate, each triplicate varied by less than 5%.

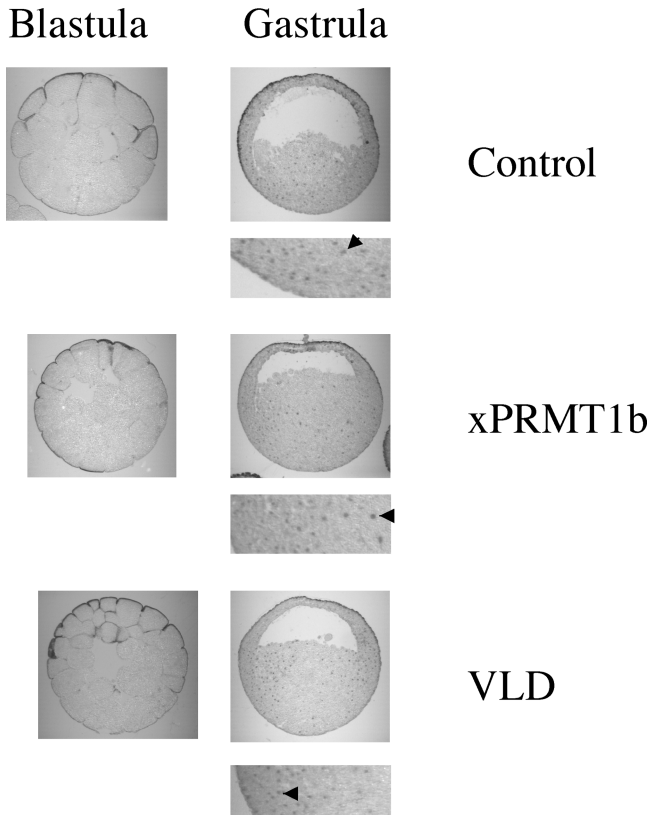

FIGURE 6: Overexpression of exogenous Xprmt1b does not change the subcellular localization of Xilf3. Embryos were treated at the two-cell stage with synthetic RNA encoding GFP (as control), Xprmt1b, or the dominant interfering version of this protein, VLD. They were allowed to develop to stage 7 (blastula) or 10 (gastrula) fixed and paraffin sections prepared. Xilf3 in sectioned material was visualized using anti-Xilf3 antiserum and DAB. The staining is cytoplasmic in pre-MBT embryos and nuclear (arrow, lower close-up panels) in post-MBT embryos regardless of the protein that was expressed. That the proteins were expressed successfully was shown by mRNA analysis for Xbmp4 and Xchordin.

Our data, however, support a direct effect on *Xgata2* transcription via methylation of a critical transcription factor. Methylation of Xilf3 in vitro decreases its DNA binding activity, and methylation in vivo inhibits the DNA binding of the Xilf3-containing CBTF complex. The decrease in the Xgata2 mRNA level observed upon Xprmt1b overexpression is therefore expected, since CBTF binding is critical for *Xgata2* transcription (*11*). Our data provide a simple and attractive model for the mechanism by which Xprmt1b regulates *Xgata2*, and the subsequent decrease in the level of Xbmp4 (and consequent dorsalization) may be downstream of this direct effect. Alternatively, Xprmt1b may directly inhibit expression of both *Xgata2* and *Xbmp4* to dorsalize the ectoderm.

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