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xDnmt1 regulates transcriptional silencing in pre-MBT *Xenopus* embryos independently of its catalytic function

Donncha S. Dunican[1,2], Alexey Ruzov[1,3], Jamie A. Hackett[1] and Richard R. Meehan[1,2,*]

We previously reported that the maintenance cytosine methyltransferase xDnmt1 is essential for gene silencing in early *Xenopus laevis* embryos. In the present study, we show that silencing is independent of its catalytic function and that xDnmt1 possesses an intrinsic transcription repression function. We show that reduction of xDnmt1p by morpholino (xDMO) injection prematurely activates gene expression without global changes in DNA methylation before the mid-blastula transition (MBT). Repression of xDnmt1p target genes can be reimposed in xDMO morphants with an mRNA encoding a catalytically inactive form of human DNMT1. Moreover, target gene promoter analysis indicates that silencing is not reliant on dynamic changes in DNA methylation. We demonstrate that xDnmt1 can suppress transcription activator function and can be specifically localised to non-methylated target promoters. These data imply that xDnmt1 has a major silencer role in early *Xenopus* development before the MBT as a direct transcription repressor protein.

KEY WORDS: DNA methylation, MBT, *Xenopus*

INTRODUCTION

DNMT1 is a multi-domain protein with an N-terminal regulatory domain and a C-terminal catalytic domain (Goll and Bestor, 2005). The enzymatic function of DNMT1 is necessary to maintain and perpetuate DNA methylation patterns at CpGs laid down by de novo methyltransferases in response to developmental cues. DNA methylation in mammals is essential for transcriptional silencing of transposons, regulation of many imprinted genes and the maintenance of X-inactivation in female somatic cells (Goll and Bestor, 2005). Methylated CpG pairs can repress transcription of adjacent genes either by directly interfering with nuclear factor site recognition or, indirectly by binding methyl-CpG specific binding proteins (Klose and Bird, 2006). Altered patterns of gene expression (including single copy genes, imprinted genes and transposons) occurs in hypomethylated (*dnmt1*) mutant mouse embryonic fibroblasts (Jackson-Grusby et al., 2001). Nonetheless, many tissue-specific gene promoters are hypo-methylated and are not expressed in early mouse or *Xenopus* embryos (Walsh and Bestor, 1999; Stancheva et al., 2002), implying that additional silencing mechanisms may be operative. A screen of *dnmt1*$^{n/n}$ fibroblast cells also noted that a high proportion of mis-expressed genes are transcribed from CpG-island promoters that are constitutively unmethylated (Lande-Diner et al., 2007).

A decrease in DNMT1 levels results in early embryonic lethality in mouse, frog and zebrafish, probably owing to multiple defects, including activation of a cell death pathway (Li et al., 1992; Stancheva and Meehan, 2000; Jackson-Grusby et al., 2001; Stancheva et al., 2001; Rai et al., 2006). Recently a mutant mouse with a catalytically inactive form of *Dnmt1* has been generated that exhibits a developmental arrest phenotype that is very similar to those observed for targeted deletion mutants (Takebayashi et al., 2007). This suggests that the catalytic function of DNMT1 is very important in early mouse embryogenesis, although undifferentiated embryonic stem cells are relatively unaffected by loss of DNMT1 activity (Jackson-Grusby et al., 2001; Tsumura et al., 2006). By contrast, complete inactivation of DNMT1 in human cancer cells leads to activation of a G2/M checkpoint and mitotic catastrophe with minimal changes in DNA methylation levels (Chen et al., 2007). Taken together these studies suggest the reported phenotypes of DNMT1 depletion in early development and cell lines may reflect the loss of both its enzymatic and non-enzymatic functions.

Although there is no evidence of global demethylation, imprinting, or inactivation of sex-specific chromosomes in *Xenopus laevis*, we have shown previously that xDnmt1 has an essential function in maintaining gene silencing prior to zygotic gene activation at the mid-blastula transition (MBT) in early amphibian development (Stancheva and Meehan, 2000). However, it was not clear from this study whether maintenance of gene silencing prior to the MBT in *Xenopus* depended on the enzymatic or non-enzymatic functions of xDnmt1. Here, we show that the silencing function of xDnmt1 in early amphibian development is independent of its methyltransferase activity. We report that a partial reduction in xDnmt1p levels by morpholino (xDMO) injection into *Xenopus laevis* embryos results in premature zygotic gene activation without a concomitant decrease in DNA methylation levels, either globally or at specific loci. Rescue experiments with an mRNA encoding a catalytically inactive form of human Dnmt1 (DNMT1) strongly suggest that DNA methylation is not used as a general silencer of gene expression in *Xenopus* embryos. Our data support a model in which xDnmt1 can regulate embryonic gene silencing directly and independently of its catalytic function.

MATERIALS AND METHODS

Oligonucleotides and morpholinos

The sequences of the primers and morpholinos used in this study are shown in Table 1.

Embryo manipulations

Xenopus embryos were handled as described (Ruzov et al., 2004). Morpholino oligonucleotides against xDnmt1 (xDMO) were designed and synthesised by GeneTools. Human rescue mRNAs

[1]Human Genetics Unit, MRC, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK. [2]Genes and Development group, School of Biomedical Sciences, The University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK. [3]Institute of Gene Biology, Russian Academy of Sciences, Vavilova 34/5, Moscow, 119334, Russian Federation.

*Author for correspondence (e-mail: richard.meehan@hgu.mrc.ac.uk)

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were synthesised from wild-type or mutant (C1226Y) DNMT1 plasmids (gift from Michael Rountree) using the T3/T7 Capscribe kit (Boehringer).

TNT
cDNA encoding full-length xDnmt1 was used as a template in coupled *in vitro* transcription-translation (TNT, Promega) reactions performed in the absence or presence of xDMO followed by PAGE.

RT-PCR
RT-PCR was performed as published (Ruzov et al., 2004).

In situ analysis
In situ protocols were performed using standard methods.

Southern blotting
Southern blotting was performed as described (Stancheva and Meehan, 2000). xSatellite I (xSatI) probe was generated by PCR.

Bisulfite sequencing
The bisulfite sequencing protocol has been described previously (Stancheva et al., 2002).

Promoter cloning
xOct91 and *xOct25* promoter regions were cloned from *Xenopus laevis* genomic DNA using the DNA Walking Kit (SeeGene, Korea). *xOct60* promoter was cloned by synteny PCR and the xOct91 promoter region (–463 to –12) was cloned into *Sac*I/*Bgl*II sites of pGL3-Luc basic.

Western blotting
Embryonic extracts were isolated using RIPA buffer and xDnmt1 levels were detected by immunoblotting with α-xDnmt1 antibody 3C6 (Shi et al., 2001). Mouse cell extracts were prepared and the following antibodies were used: α-T7 (T7-xSp1) (Novagen); α-human DNMT1 (NEB); and αPCNA (Abcam). Embryonic histone extracts were prepared by acid extraction and blotted with the following antisera: panAcH4 (Cell Signalling, 9441S), H3K9Ac (Abcam, AB4441), H4K5Ac (Upstate, 06-759), PanMethKH3 (Abcam, AB7315), H3K4me3 (Abcam, AB8580), H4K20me3 (Abcam, AB9053), H3K9me3 (Abcam, AB8898 and H3 (Abcam, AB1791).

GST pull down
Binding reactions for DNA GST pull downs were prepared as for EMSA (Ruzov et al., 2004). CpGpos oligonucleotide probes were used (Voo et al., 2000). Reactions were incubated for 10 minutes on a shaker at 30°C, washed four times with PBS, treated with Proteinase K, extracted and analysed using PAGE.

Transient transfections and reporter assays
Human 293T cells and mouse N2A cells were cultured using standard methods. Constructs for reporter assays were transfected into Neuro2A cells using established methods (Invitrogen). Assays were carried out independently in quadruplicate.

ChIP analysis
Xenopus A6 cells were transfected with xDnmt1-GFP, pCMVxDnmt1 or without plasmid DNA. CHIP was performed with a GFP antibody (Abcam).

RESULTS

xDnmt1p reduction causes premature gene activation

In previous work we used an antisense RNA (AS) strategy to knockdown xDnmt1p transiently in early embryos; it resulted in DNA hypomethylation and premature gene activation (Stancheva and Meehan, 2000). Here, we use highly stable (xDMO) morpholinos, which inhibit xDnmt1 mRNA translation in vitro and in vivo (Fig. 1A), and result in a phenotype that is indistinguishable from antisense RNA depletion. The xDMO morphants develop normally up to MBT but at gastrulation exhibit an extended open blastopore, which by neurulation results in the appearance of dead shedding white cells on the surface of a high proportion (80%) of embryos (arrows in Fig. 1B and insert) and a failure to form a neural tube. By tadpole stage, only 2% of the xDMO morphants are phenotypically normal compared with 91% for controls (see Fig. 3). This is intriguing as antisense injection results in almost complete depletion of xDnmt1 in stage 8 pre-MBT embryos (Stancheva and Meehan, 2000), whereas xDMO injection results in a 40-50% reduction of xDnmt1 protein (Fig. 1A). Despite this difference, the phenotypes of the AS and xDMO embryos are virtually indistinguishable.

Array screens in our laboratory (D.S.D. and R.R.M., unpublished) indicate that up to 25% of genes in these experiments are mis-expressed in stage 8 xDMO morphants. We used RT-PCR to verify the expression of these putative methyl-CpG dependent target genes in pre-MBT (stage 7-8) embryos (wild type and xDMO morphants). All tested genes were mis-expressed in xDMO morphants (*xCycD1*, *xSox17β*, *xMix1*, *xp68*, *xDep*, *xOct91* and *xID2*) relative to histone H4 and *xOct60* expression (Fig. 1C; see Fig. S1 in the supplementary material). Whole-mount RNA in situ hybridisation revealed that ectopic transcripts are present throughout the animal pole of xDMO stage 8 morphants (Fig. 1D). A control shows equal expression of the maternal oocyte-specific gene *xOct60* between the two embryo sets. We conclude that the xDnmt1p reduction in xDMO morphants is sufficient for premature gene activation before MBT, the induction of apoptosis and phenotypic defects that result in reduced survival rates. More importantly, premature gene activation is a general feature of xDMO embryos, implying an essential global role for xDnmt1p in embryonic gene repression.

xDMO morphants retain normal DNA methylation at repeat and unique sequences

To determine whether global methylation levels were altered in xDMO morphants, we tested the dispersed satellite I repeat (xSatI) by Southern blotting, which is methylated at its two *Hpa*II (CCGG) sites through development (Stancheva et al., 2002). Genomic DNA from both wild-type and xDMO siblings showed a comparable resistance to *Hpa*II digestion either by itself or in double digestion with *Hin*dIII (Fig. 2A). We used bisulphite sequencing analysis to precisely map CpG methylation at xSatI sequences in wild-type and xDMO genomic DNA. No hypomethylated CpGs were observed in xDMO DNA relative to the wild type (Fig. 2B; boxed numbers indicate % methylation at each CpG). As xSatI is distributed through the *Xenopus* genome, this suggests there are no genome-wide changes in DNA methylation in xDMO morphants.

Subsequent to finding normal methylation patterns in xDMO repeat DNA, the next issue was to evaluate the methylation profile of xDMO target genes (*xOct91* and *xCycD1*, Fig. 2C). This analysis showed that the pattern of methylation at *xOct91* and *xCycD1* promoters and upstream regions in stage 8 xDMO morphants was identical to stage 8 wild type (Fig. 2D). *xOct91* and *xCycD1* are zygotically activated during normal development after MBT (see Fig. S2A in the supplementary material) so we compared bisulfite maps when they are either transcriptionally silent (stage 8) or active (stage 10). There was no significant difference in CpG methylation at the *xOct91* and *xCycD1* loci between the inactive and active stages (data not shown), indicating that DNA methylation does not play a direct role in regulating their expression during normal development. We made similar observations for the *xOct25* and xSox17β promoter regions (data not shown). Together with the xSatI methylation analysis, this led us to conclude that DNA methylation is not directly regulating the expression of the *xOct91* and *xCycD1* loci in xDMO morphants and by extension other genes

that are misexpressed in stage 8 xDMO embryos. Our data imply that premature gene activation during *Xenopus* embryogenesis is governed by a mechanism independent of DNA modification.

Several studies have identified crosstalk between the DNMT1 proteins and histone modifying enzymes (Fuks, 2005). One possibility is that premature transcription in xDMO morphants may be due to global alterations in histone modification states as a consequence of xDnmt1p depletion. We tested this possibility by direct comparisons of histone mark abundance levels by immunoblotting. These experiments revealed no significant differences for various histone acetylation and histone methylation marks globally in early (stage 8) or later (stage 15) xDMO morphants (Fig. 2E). In stage 8 embryos, most histone marks are low to undetectable and only accrue as development proceeds, particularly in the case of H3K4me3 (see Fig. S2 in the supplementary material) and H4K20me3. However, these experiments cannot completely rule out subtle histone mark changes at specific gene promoters. Our data imply that neither global

Table 1. Primer and morpholino sequences used

Morpholino	Sequence
(xDMO) and xDnmt1a	GGACAGGCGTGAAACAGACTCGGC
xDnmt1b	GAACAGGCGTGAGACACACTCGGC
Control	CGCTCAGCTCCTCCATGTCTGCCGC

RT-PCR primer	Sequence
xId2u	ctcctgtacaatatgaatgattg
xId2l	acaagatgctgatgtctgtg
xp68u	ttgatgaagcagacagaatg
xp68l	cgttacacacatcaacaatc
xOct60u	ccatattgtacagccaaacctc
xOct60l	gttccagtcaaaggaagcag
xTrip7u	tcatcaaaacccgaacctc
xTrip7l	caattcattctatctccgcac
xCycd1u	atttcaagtgcgtccagaag
xCycd1l	ggaattgtcggtgtaaatgc
xSox17Bu	gtcatggtaggagagaac
xSox17Bl	tctgtttagccatcactgg
xMix1u	cctaatagttcctcacatc
xMix1l	ttgaagtggtagatacagg
xDepu	agagcggaatggcaactgt
xDepl	caacgtccacagcctcaga
xH4u	cgggataacattcagggt
xH4l	tccatggcggtaactgtc
xOct91u	cagatggcagcggacag
xOct91l	caactggttggcagaatcc
xOct25u	taatggagagatgcttgatg
xOct25l	ttctctatgttcgtcctcc
xBf2u	cgaagagaccgatatcgatg
xBf2l	ctgcaggatggacatggtg
xGapdhu	tgccattctcagccttaac*
xGapdhl	acggatttggctgtattgg*
xOdcu	gtcaatgatggagtgtatggatc*
xOdcl	tccattccgctctcctgagcac*
xPtenu	taccaggaggatggattcg
xPtenl	ggttgtggtcttcaaacgg
xMatu	gcttctgtgccagttacg
xMatl	tctgccagaatagtccgc

Promoter cloning primer	Sequence
xOct25tsp1	ccaccagcactgaccttgtcacgag
xOct25tsp2	gtccaagtccttgaggtgcagg
xOct25tsp3	cactctgccatccttgcaacgg
xOct91tsp1	ctgatgttcccctatacagctc
xOct91tsp2	gagctcttgctcaaatcctacc
xOct91tsp3	ctaagttgctcggtgttcgctc
xOct25-oct60_U	ccacaaggttacatggcagctcc
xOct25-oct60_L	tggggaaggaggtttggctgtac

Bisulfite data primer	Sequence
xOct25 Bis1	attgatgtataataaaggagg
xOct25 Bis2	tttgtatgttatatagagtg
xOct25 Bis3	gttttggtttattatttattagg
xOct25 Bis4	cacttcactttacaacaactacc
xOct25 Bis5	ctttacaacacttcactttac
xOct25 Bis6	cttaacaaatctttacaacac
xOct25 Bis7	gggttaggttgttttgagttg
xOct25 Bis8	agagatggggattagatttg
xOct25 Bis9	tttgagttgagggtaaatag

Bisulfite data primer	Sequence
xOct25 Bis10	cctaaaaccaccaacactaac
xOct25 Bis11	caatcaaatcaactaaaacc
xOct25 Bis12	taatatccaaaaacttacaatc
xOct91 Bis1	gatatttatgaagttttattg
xOct91 Bis2	ttatgaagttttattggtgag
xOct91 Bis3	gttttttatatgttaatgag
xOct91 Bis4	acccaattaataaaacttac
xOct91 Bis5	ctccaaataacataattctc
xOct91 Bis6	tttcctctactccaaataac
xOct91 Bis7	gaggtgttttattggatatg
xOct91 Bis8	gttttattggatatgtatag
xOct91 Bis9	atagattaatagtttaaatag
xOct91 Bis10	ccctatacaactcttactc
xOct91 Bis11	tatacataatctaatattcc
xOct91 Bis12	atctattaattatacataatc
xOct60 Bis1	aaatttttaagggtagaggttg
xOct60 Bis2	ggtagaggttgtatttttttaaag
xOct60 Bis3	aaagaggggttggttttttg
xOct60 Bis4	ttaactaaaaatacccaataac
xOct60 Bis5	atccttttaactaaaaataccc
xOct60 Bis6	tacccaaatcatccttttaac
xOct60 Bis7	aattggttaatgagagagaag
xOct60 Bis8	tgagagagaagattaattagtg
xOct60 Bis9	aagattaattagtgggtttatg
xOct60 Bis10	ccaactcttccaacccaaaacc
xOct60 Bis11	aaactaccaactcttccaaccc
xOct60 Bis12	aaacccaaactaccaactcttc
xCycd1 Bis1	ttagtttgatatttgtgtttg
xCycd1 Bis2	ggtttaatttaagttttatag
xCycd1 Bis3	tttattttattggttaattgtatg
xCycd1 Bis4	actaaacacccaacatacac
xCycd1 Bis5	aaacacccaacatacacc
xCycd1 Bis6	taaaataaaacttcaacctac
xCycd1 Bis7	ttatgaatggaggggggtg
xCycd1 Bis8	gaggggtgggtgtag
xCycd1 Bis9	caccaacctccaacatcc
xCycd1 Bis10	cccaatacccaaaaaaacac
xSatl Bis1	gttaatattaatttgaggtttag
xSatl Bis2	gtttgaatagtttagttggtag
xSatl Bis3	aaatactaaataaaaaaaccc
xSatl Bis4	ttcaaactaatactaaacaaac

Miscellaneous primers	Sequence
xSat1u	catttgagaagctgactagc
xSat1l	agaaccactgtcctcattaatc

ChIP primers	Sequence
O25CHIP1	ccgcagttggaggggtggc
O25CHIP2	gtctcaaagaggccaatgaatg
O25CHIP3	ttgaggtgcaggaaagcaac
O25CHIP4	gtaatcactctgccatccttg
CycD1CHIP5	caagtggaagaagagctggc
CycD1CHIP6	gtgggtgcagaggctccttc
CycD1CHIP7	gccctcctgatggtgtccac
CycD1CHIP8	gatgaggttcctgtccagatg
xlTubChipU	tgaaacaggagcaggaaagc
xlTubChipL	gctctgggtggaataactgc

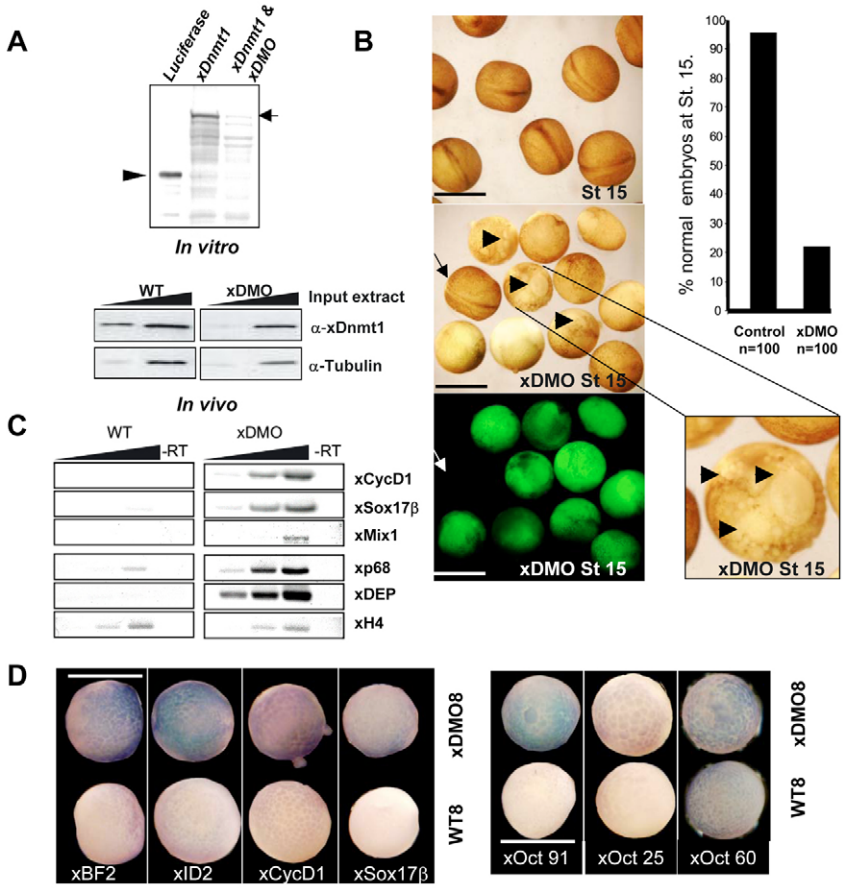

Fig. 1. xDMO embryos have reduced xDnmt1 levels, are abnormal and mis-express genes. (**A**) Top panel: in vitro inhibition of xDnmt1 translation (black arrowhead) using xDMO (compare lanes 2 and 3). Bottom panel: in vivo inhibition of xDnmt1 translation in pre-MBT (stage 7-8) embryos (compare wild-type and xDMO extracts). Tubulin is used as a loading control. (**B**) Left panel: phenotypes of stage 15 embryos. Morphant xDMO embryos exhibit apoptotic lesions (arrowheads and enlargement) and lack neural folds (black arrow) compared with control stage 15 embryos. xDMO embryos contain fluorescein, unlike the control embryo (compare arrowed embryos). Right panel: comparison of percentage (*n*=100) of successfully neurulating embryos for wild type and xDMO. (**C**) xDMO embryos mis-express a range of transcripts. Wild-type and xDMO RNA was assayed by RT-PCR over a 10-fold dilution range (0.1, 0.3 and 1 μl cDNA for each sample indicated by the black triangles). H4 is a loading control. (**D**) In situ analysis reveals ectopic expression of the indicated xDMO targets throughout the animal pole (compare wild-type and xDMO panels). The maternally expressed gene *xOct60* is not mis-expressed. Scale bars: 1 mm in B,D. Animal pole views are shown.

changes in DNA methylation or specific histone modifications are associated with activation of xDMO target genes in stage 8 embryos, but rather that high levels of xDnmt1p that are present in early *Xenopus* embryos are essential for their repression (Shi et al., 2001).

Dnmt1p catalytic activity is not essential for repression

To explore the possibility that xDnmt1 may have a non-enzymatic role in gene silencing, we carried out a series of rescue experiments with wild-type and mutant forms of human DNMT1 (Fig. 3A). Two-cell embryos were injected with xDMO alone or in combination with either wild type or mutant human DNMT1 (hDNMT1^{C1226Y}) mRNA (which xDMO does not bind), and fixed for whole-mount RNA in situ analysis. *xBF2* and *xOct25* were ectopically activated (in agreement with our array and RT-PCR screens) in xDMO morphants, but the presence of either wild-type DNMT1 or hDNMT1^{C1226Y} mRNA (1 ng) significantly reduced the extent of activation by up to 50% as measured by densitometry (Fig. 3B; see Fig. S3B in the supplementary material). In two further series of experiments, both types of hDNMT1 mRNAs increased the frequency of phenotypically normal embryos at stage 15 (neurulation) two-fold (from 20% to more than 40%), indicating the specificity of the morpholino, but more importantly that the catalytic function of human DNMT1 is not required to rescue the xDMO morphant phenotype and re-impose gene silencing (Fig. 3C,D). A high proportion of rescued embryos do not show evidence of developmental delay and form neural folds (Fig. 3D, black arrows) equivalent to those seen in wild-type neurula embryos. Unlike xDMO morphants, the rescued embryos continue to develop and can form tadpoles at a high frequency (Fig. 3E). The major conclusion from these developmental studies is that a catalytically inactive human mRNA can partially restore the normal transcriptional program and phenotype in *Xenopus* embryos, thereby underlining a physiologically relevant non-enzymatic role for xDnmt1p in gene repression.

xDnmt1p is a transcriptional repressor and can be localised to target gene promoters

In light of the above data, we sought to explore potential mechanisms for DNMT1-mediated repression. The N-terminal non-catalytic region of mammalian DNMT1 is an effective transcription repressor when artificially recruited to a promoter (Fuks, 2005), and knockdown of DNMT1 in transformed cells specifically activates expression of two genes, independently of DNA methylation (Milutinovic et al., 2004). We hypothesised that if xDnmt1, like its mammalian counterparts, contained regions that can directly bind non-methylated DNA, this would enable it to act as a general repressor of transcription during early *Xenopus* development (Chuang et al., 1996; Suetake et al., 2006). We tested three candidate regions (Fig. 4A, black bars G1-G3) of xDnmt1 as GST fusion proteins for DNA binding activity in vitro using a pull down assay. Under the stringent conditions employed, all three GST fusion proteins bound the CpGpos oligonucleotide (Voo et al., 2000) as shown in Fig. 4A. Additional experiments suggest that xDnmt1 binding has relaxed sequence specificity (data not shown).

We tested whether xDnmt1 can repress the activation of a minimum promoter that has four copies of the xSp1-binding site driving luciferase expression (p4xSp1-Lucif) (Kockar et al., 2001). Relative induction by xSp1 is reduced by 55% in the presence of

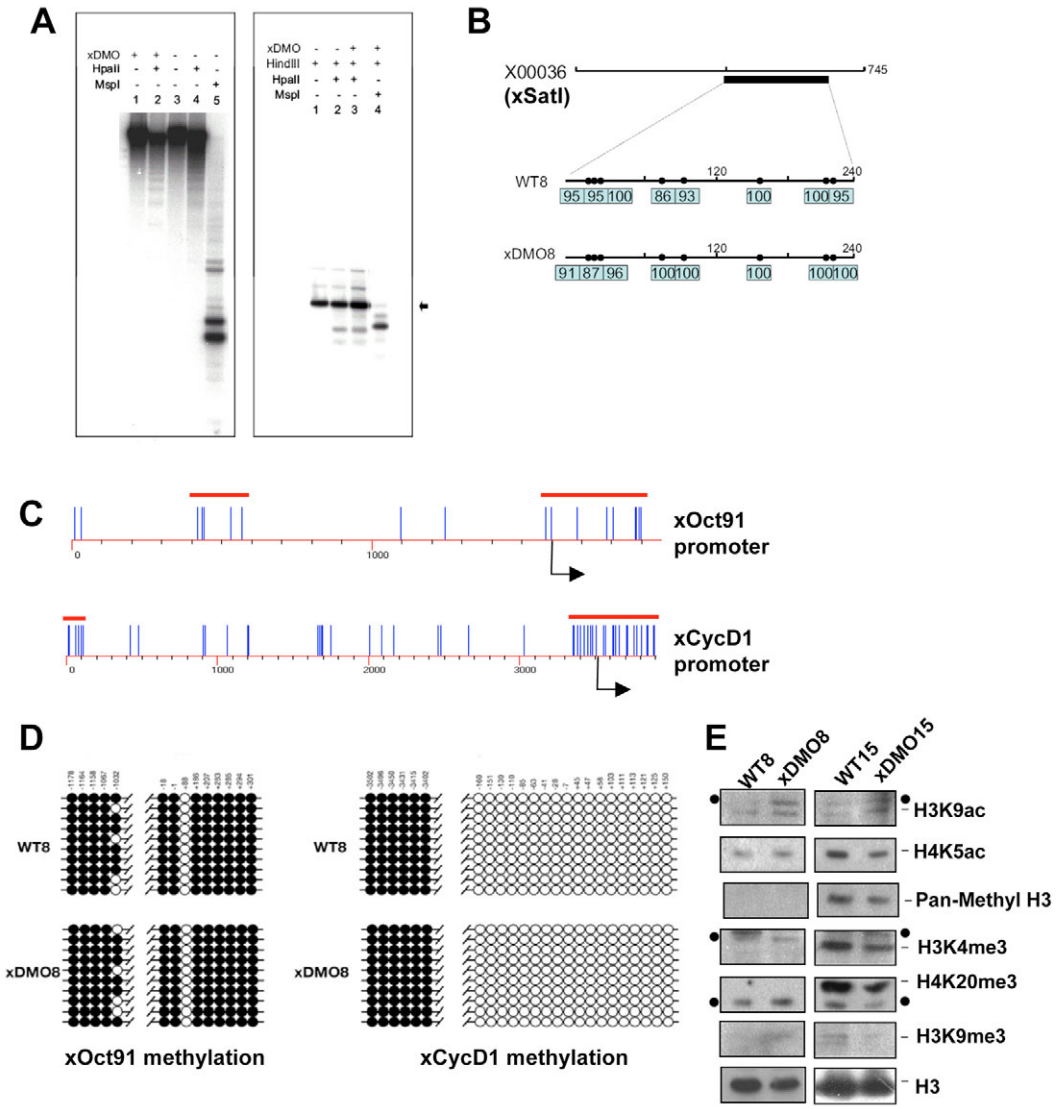

Fig. 2. No changes in DNA methylation at repeat or single copy sequences in xDMO morphants. (**A**) xDMO DNA is heavily methylated at xSatI *Hpa*II sites (compare lanes 2 and 4). *Hpa*II is a methyl-sensitive restriction enzyme and *Msp*I is the methylation insensitive (CCGG) counterpart (lane 5). Right panel: *Hin*dIII was used to generate the 750 bp xSatI monomer (black arrow); double digestion with *Hin*dIII and *Hpa*II showed no difference in monomer methylation in the wild-type and xDMO samples (lanes 2-3). (**B**) Bisulphite sequencing (clones *n*=40) shows no significant difference in CpG methylation between wild-type and xDMO genomes at xSatI sequences. Boxed numbers are percentage CpG methylation; black circles indicate CpG distribution in xSatI. (**C**) CpG distribution in cloned promoters of *xOct91* and *xCycD1*. Blue bars, CpG; black arrows, transcription start sites; red bars, regions sequenced. (**D**) Bisulfite analysis (sequences *n*=40, ten representative clones are shown) was used to determine the methylation status of *xOct91* (left) and *xCycD1* (right) promoters and upstream regions. Numbers above each CpG indicate genomic position relative to transcription start. Filled circles, methylated CpGs; empty circles, non-methylated CpGs. (**E**) Immunoblot analysis of wild-type and xDMO histones shows no significant change in various histone modification marks between histone WT and xDMO extracts at stages 8 and 15. Histone modifications are low to absent at stage 8 and accrue by stage 15. Black dots indicate non-specific bands.

xDnmt1 and both the catalytically active and inactive forms of human DNMT1 (Fig. 4B), which are expressed equally in transient transfection assays (Fig. 4C, left panel). Analysis of the raw luciferase data suggests that as the Dnmt1 dose is increased, p4xSp1 activation is repressed up to 10-fold without affecting cell numbers (data not shown). However, the expression of the co-transfected luciferase (*Renilla*) reporter is concomitantly repressed by fivefold and results in a normalised value of a twofold (50%) reduction. Western blot analysis shows that there is no observable difference in xSp1 levels between cells transfected with a 10-fold difference of the xDnmt1 expression plasmid (Fig. 4C, right panel). Our data suggest that untethered DNMT1 can act as a general repressor of promoters when it is abundant, and that its catalytic activity is dispensable for this function.

To reflect the *xOct91* repression scenario in early development, we measured the effect of Dnmt1 overexpression on *xOct91* promoter activity. Each form of DNMT1 repressed the activity of the *xOct91* reporter construct by ~50% or more relative to the empty vector control (Fig. 4D). The repressive capacity of hDNMT1^{C1226Y} strongly indicates that the mechanism of inhibition of these transgenes is independent of DNA methylation. In addition, repression by DNMT1s is not relieved by an HDAC inhibitor (TSA) and requires full-length

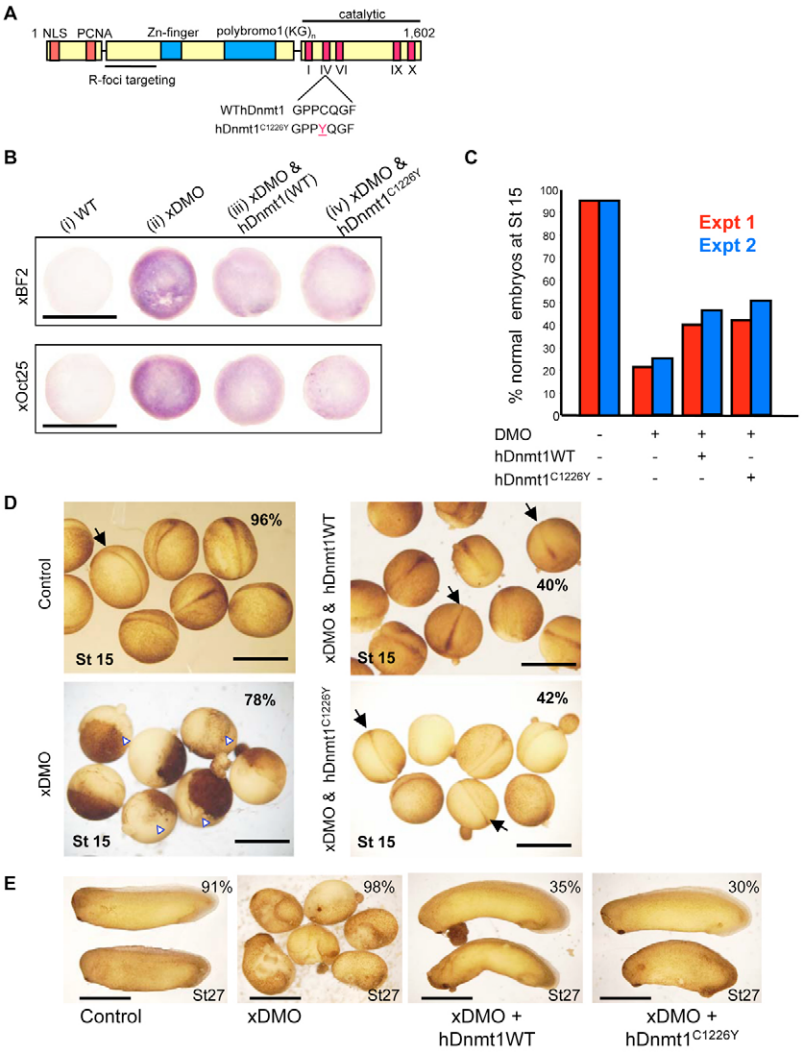

Fig. 3. A catalytically inactive form of human DNMT1 can restore repression and rescue xDMO morphants. (**A**) Functional domains present in human DNMT1 and an inactivating point mutation of hDNMT1 (hDNMT1^{C1226Y}) (Jair et al., 2006). (**B**) Catalytic function of human DNMT1 is not required to rescue *xOct25* and *xBF2* gene repression. Compare rescue in situ intensities (iii and iv) with signals in control (i) and xDMO morphants (ii). Animal pole views are shown. (**C**) hDNMT1WT and hDNMT1^{C1226Y} restore normal *Xenopus* neurulation with comparable efficiency. Experiment 1 (n=100) is in red and experiment 2 (n=60) is in blue. Compare number of normally neurulating embryos in xDMO only (~20%) with rescued injected embryos (>40%). (**D**) Rescued embryos are similar in phenotype to wild-type siblings. Neurulating embryos are shown. There are apoptotic cells and open blastopores (white arrowheads) in xDMO embryos (bottom left). Such lesions are absent in rescued embryos (right panels), which are similar to control injected embryos (top left) (black arrows indicate neural folds). (**E**) Phenotypes of late stage (stage 27) embryos. Only 2% (i.e. 98% of embryos fail) of xDMO morphants develop to late tadpole stage (stage 27) compared with 35% and 30% for wild-type (hDNMT1WT) and mutant (hDNMT1^{C1226Y}) rescued embryos, which develop normally. Scale bars: 1 mm.

forms of the protein for efficient silencing and xDMO rescue (J.A.H., A.R. and R.R.M., unpublished). We conclude that DNMT1 can act as a general repressor of non-methylated promoters and that its catalytic activity is not required for this function.

As a putative repressor of gene expression and possessing DNA affinity, we hypothesised that xDnmt1 should bind the loci it regulates. We were unable to perform ChIP assays against endogenous xDnmt1 using monoclonal antibodies as they were sensitive to formaldehyde crosslinking (D.S.D., unpublished). We performed ChIP with *Xenopus laevis* A6 cells that were transfected with a GFP-xDnmt1 expression construct and an anti-GFP polyclonal antibody. This analysis localised GFP-xDnmt1 to the *xOct25* and *xCycD1* non-methylated promoters in A6 cells (Fig. 4E,F) but not to an intronic region of the constitutively active *Xenopus* α-Tubulin gene. Enrichment of GFP-xDnmt1 at non-methylated, non-expressed loci (xDMO target genes) is consistent with a model in which xDnmt1p can bind and repress gene expression independently of DNA methylation during development.

DISCUSSION

Our experiments and recent reports both suggest that DNMT1 has an essential role in early amphibian and mouse development (Takebayashi et al., 2007). Mutant mice expressing a DNMT1 point-mutant protein lacking catalytic activity (DNMT1-C1229S) fail to develop, arrest after gastrulation (E9.5) with a near-complete loss of DNA methylation and mis-express normally methylated genes with phenotypes very similar to those of the *dnmt1$^{c/c}$* mutant (Lei et al., 1996; Takebayashi et al., 2007). This indicates that the catalytic function of *Dnmt1* is required to support early mouse embryogenesis, probably owing to the many developmental processes, including X-chromosome inactivation, suppression of retrotransposon activity, imprinting and regulation of germ-cell specific gene expression, that are dependent on DNA methylation (Goll and Bestor, 2005; Maatouk et al., 2006). By contrast, *Xenopus laevis* lacks imprinting and processes equivalent to X-inactivation, which may underlie its non-dependence on DNA methylation in early development. Additionally primordial germ cell development is specified by different mechanisms in amphibians and mammals (Crother et al., 2007). Our work suggests that the intrinsic repression function of xDnmt1p has been used to maintain gene silencing in pre-MBT embryos until co-ordinated and precise zygotic activation occurs at multiple gene loci. This period of gene silencing over 11 cell divisions is not observed during mouse embryogenesis (Meehan et al., 2005) and implies distinct regulatory mechanisms for zygotic gene activation are used between mice and frogs.

Zygotic activation of gene expression during early *Xenopus* development is mainly dictated by maternal inheritance of repressor and activator components (Veenstra, 2002). A two-cell *Xenopus*

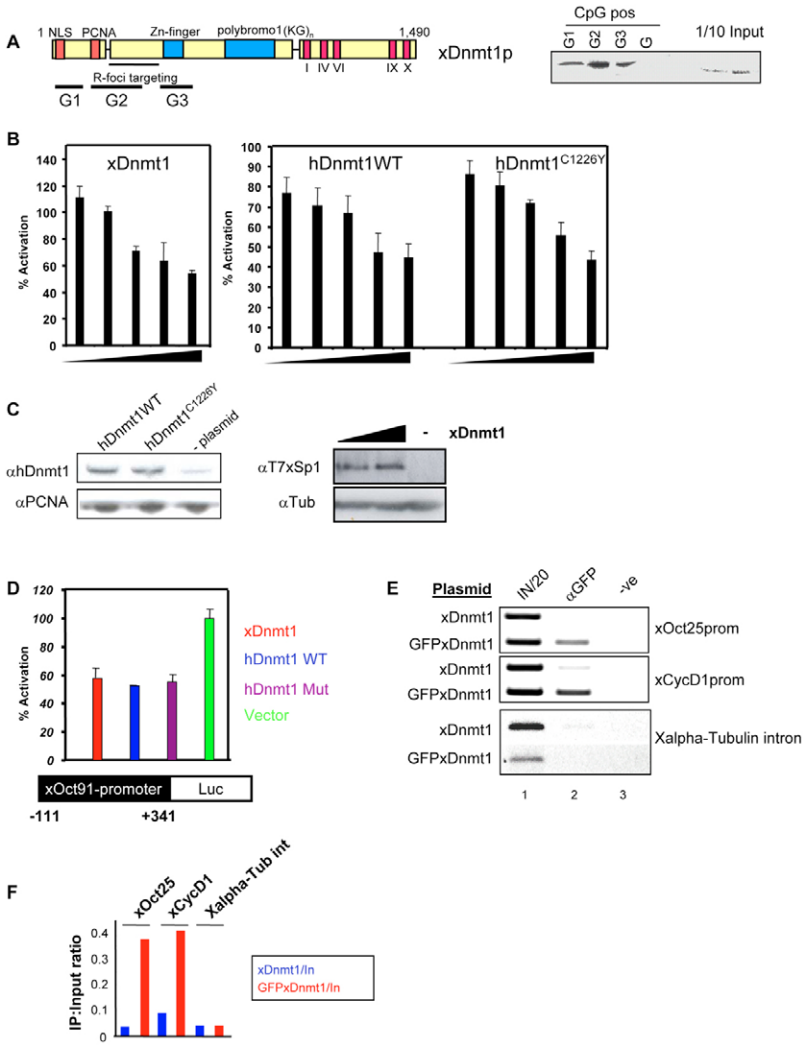

Fig. 4. Dnmt1 represses activation of non-methylated transgenes in vivo and xDnmt1 localises to non-methylated target genes. (**A**) N-terminal xDnmt1 fusions bind dsDNA oligos. The domain structure of xDnmt1 (top). xDnmt1-Gst fusions domains G1-G3 (black bars). CpGpos oligonucleotides were used in pull-down assays with the three Gst fusions and Gst only. All three constructs bound CpGpos compared with the Gst protein (G) lane. (**B**) In vivo assays to test the repression activities of xDnmt1, hDNMT1WT and hDNMT1^{C1226Y}. Exogenous xSp1 was used to activate the Sp1-Luc reporter in the presence of increasing amounts of Dnmt1 [black triangles; 0.125-2 μg plasmid DNA per transfection]. xSp1 activation of the reporter alone was assigned 100%. Data were obtained from nine independent assays and normalised to TK-Renilla. (**C**) Left panel: both forms of human DNMT1 (hDNMT1WT and hDNMT1^{C1226Y}) are expressed equally after transfection into 293T cells relative to PCNA and endogenous hDNMT1 (–plasmid). Right panel: N2A cells were transfected with T7xSp1 against low or high xDnmt1 levels (black bar), cell extracts were blotted with α-T7. αTubulin was used as a loading control. (**D**) An *xOct91* promoter (–111 to +343) reporter construct is repressed by co-transfection with 500 ng of xDnmt1, hDNMT1WT and hDNMT1^{C1226Y} but not by the empty vector control. (**E**) Chromatin IP (ChIP) analysis shows recruitment of GFP-xDnmt1 to the non-methylated *xOct25* and *xCycD1* promoters, but not an α-tubulin intron in A6 cells. Note the enrichment of GFP-xDnmt1 at both promoters using α-GFP (lane 2) but not the control xDnmt1 lacking GFP (panel xDnmt1). Lane 1, input (1/20 used in IP); –ve, no antibody control. (**F**) Bar chart shows fold enrichment of GFP-xDnmt1 at the *xOct25* (eightfold) and *xCycD1* (4.5-fold) promoters compared with a non-tagged control and with αTubulin.

embryo contains up to 100 ng of histones and 10 ng of xDnmt1 (Shi et al., 2001; Veenstra, 2002), which together impose dominant repression of transcription. At MBT, virtually all of the free histone pool has been depleted and we hypothesize that transcription repression is now sensitive to xDnmt1 protein levels. Radioactive labelling experiments and successful depletion by antisense RNA and morpholinos suggest that continuous translation is required to maintain high xDnmt1p levels in oocytes and embryos (Kimura et al., 1999). The rate of transcription per cell increases ~200-fold when xDnmt1p levels are reduced to approximately 2 pg/cell in normal embryos (Newport and Kirschner, 1982a; Hashimoto et al., 2003). In xDMO morphants, a 40-50% reduction in xDnmt1p before the MBT is sufficient to allow the low level of general transcription machinery components that are present, such as RNA Pol I, Pol II and TBP, to dramatically activate a generalised pattern of gene expression approximately two cell cycles earlier than normal. However, this pattern of gene activation does not equate with a normal transcriptional activation profile as the spectrum of genes that are mis-expressed in xDMO morphants (and antisense-depleted embryos) is much greater, as measured by cDNA library screens and array analysis (D.S.D. and R.R.M., unpublished). This suggests that the transcriptional competence of normal late blastula/early gastrula embryos is crucially dependent on the non-catalytic and catalytic silencing function of xDnmt1. In general, transcription at MBT occurs before histone modifications, such as H3K4M3 (Fig. 2E) or H4 acetylation, accumulate (Almouzni et al., 1994). This may explain why we do not observe an accumulation of histone modifications in pre-MBT xDMO morphants. It is possible that changes in histone modifications during *Xenopus* development are linked with a dynamic alteration in the organization of different chromatin domains that occurs after the MBT when gene-specific subdomains are set up (Vassetzky et al., 2000).

By depleting xDnmt1p in stage 8 embryos, we propose that this interferes with its non-catalytic repression function, which is crucially dependent on its high abundance in early *Xenopus* embryos. Some developmentally decisive genes, such as *xBra*, remain silenced unless DNA hypomethylation also occurs (see Fig. S4 in the supplementary material). In this instance, we suggest that *xBra* must represent a minor class of genes that are regulated directly by DNA methylation, as the extent of transcription activation in antisense and xDMO stage 8 embryos is essentially equivalent (data not shown). A generalised interpretation of the data is that repression by xDnmt1 in vivo is bimodal. In early development, xDnmt1 functions as a maintenance methyltransferase to perpetuate patterns of DNA methylation globally and at distinct loci (*xBra*). At these loci, silencing is dependent on the catalytic function of Dnmt1 and potential interpretation of the methyl-CpG mark. However, our data suggest that xDnmt1p may also act as a direct titratable repressor component at multiple loci that has been previously hypothesised to be present in *Xenopus* embryos (Newport and Kirschner, 1982b).

It is possible that mammalian DNMT1 also has multiple silencing functions because screens of mis-expressed genes in *Dnmt1*$^{-/-}$, *Trp53*$^{-/-}$ MEFs indicate that a high proportion (up to 80%) of mis-expressed genes have CpG island promoters, which would be predicted to be methylation free at all times (Jackson-Grusby et al., 2001; Lande-Diner et al., 2007). Similar to our xDMO targets, it is highly possible that these genes are inhibited through direct action of the Dnmt1 protein at promoters, hinting at conservation of non-methyl dependent functions. Recent work demonstrates that complete inactivation of DNMT1 function in human cancer cells results in cell death (Chen et al., 2007), but this decrease in viability occurs with minimal changes in global DNA methylation. This observation supports the hypothesis that DNMT1 possesses essential functions independent of its role as a maintenance methyltransferase, and links its absence with activation of a cellular checkpoint response. Unlike the situation in tumour cells (Spada et al., 2007), limited reduction in xDnmt1p levels appears to be sufficient to activate a cell death program in *Xenopus* embryos. The pathway that mediates activation of apoptosis in DNMT1-deficient cells is dependent on TRP53 function, but the activating signal has yet to be identified (Jackson-Grusby et al., 2001; Stancheva et al., 2001). It has also been observed that undifferentiated *Dnmt1*$^{c/c}$ ES cells have a growth advantage compared with wild-type controls, but this advantage was lost in the presence of a normal or mutant (C1229S) DNMT1 mini-genes (Damelin and Bestor, 2007). These observations support multifunctional non-enzymatic roles for DNMT1 in development, cellular differentiation and cancer.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/7/1295/DC1

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