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MBD4 and MLH1 are required for apoptotic induction in xDNMT1-depleted embryos

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Loss of the of the maintenance methyltransferase xDNMT1 during *Xenopus* development results in premature transcription and activation of a p53-dependent apoptotic program that accounts for embryo lethality. Here, we show that activation of the apoptotic response is signalled through the methyl-CpG binding protein xMBD4 and the mismatch repair pathway protein xMLH1. Depletion of xMBD4 or xMLH1 increases the survival rate of xDNMT1-depleted embryos, whereas overexpression of these proteins in embryos induces programmed cell death at the onset of gastrulation. MBD4 interacts directly with both DNMT1 and MLH1, leading to recruitment of the latter to heterochromatic sites that are coincident with DNMT1 localisation. Time-lapse microscopy of micro-irradiated mammalian cells shows that MLH1/MBD4 (like DNMT1) can accumulate at DNA damage sites. We propose that xMBD4/xMLH1 participates in a novel G2 checkpoint that is responsive to xDNMT1p levels in developing embryos and cells.

KEY WORDS: DNMT1, MBD4, MLH1, Apoptosis, *Xenopus*

INTRODUCTION

Epigenetic regulatory mechanisms involve heritable alterations in chromatin structure that govern the transcription profile and genome stability of a cell during animal development or in different disease states (Meehan et al., 2005). It is well established that modification of DNA at CpGs by the addition of a methyl group at the 5 position of cytosine affects gene expression by suppressing the function of gene regulatory elements, including promoters, enhancers, insulators and enhancer blockers (Goll and Bestor, 2005). Dynamic changes in DNA methylation patterns during development or in cancer cells contribute directly to altered transcription expression profiles (Gius et al., 2004; Hershko et al., 2003). Targeted mutation of the gene encoding the maintenance methyltransferase DNMT1 in mice results in DNA hypomethylation, transcription activation and embryo lethality that is associated with activation of apoptosis (Jackson-Grusby et al., 2001; Lei et al., 1996; Li et al., 1992; Takebayashi et al., 2007). Equally, overexpression of DNMT1 is incompatible with mouse and amphibian development (Biniszkiewicz et al., 2002; Stancheva and Meehan, 2000). Homozygous mutant *Dnmt1*$^{-/-}$ mouse embryos can be identified as early as 8.5 days postcoitum (dpc), and by 9.5 dpc they exhibit severe developmental delay, lack visible somites and display a distorted neural tube (Lei et al., 1996). This contrasts with *Dnmt1*$^{-/-}$ ES cells, which proliferate normally in culture unless they are induced to differentiate, although they do exhibit high rates of microsatellite instability (Guo et al., 2004; Jackson-Grusby et al., 2001). Conditional deletion of DNMT1 in mouse embryonic fibroblasts (MEFs) results in DNA demethylation and a uniform p53-dependent cell death (Jackson-Grusby et al., 2001). However, MEFs with a hypomorphic mutant *Dnmt1*$^{n/n}$ can proliferate in culture at normal exponential rates and do not undergo senescence even after 100 generations in the absence of p53 function (Lande-Diner et al., 2007). Inactivation of p53 prolongs the proliferative capacity of *Dnmt1* null fibroblasts (Jackson-Grusby et al., 2001).

Despite obvious differences in biology and early developmental strategies, the phenotype of *xDnmt1*-deficient *Xenopus laevis* embryos shows remarkable similarity to the mouse *Dnmt1*$^{-/-}$ phenotype, as indicated by the presence of axial defects, failure to form a neural tube, and improper patterning of the somites (Stancheva and Meehan, 2000). In frogs, severe depletion of maternally expressed xDNMT1p by antisense (AS) RNA results in the premature transcription of developmentally essential genes and p53-mediated apoptosis (Stancheva and Meehan, 2000; Stancheva et al., 2001). One generalisation from the analysis of DNMT1 depletion or inhibition in embryos is that it is not essential for the survival of embryonic cells during early cleavage stages (Meehan and Stancheva, 2001). Instead the phenotypical consequences of disrupting DNMT1 function only become apparent during and after gastrulation when p53-dependent apoptosis is initiated (Stancheva et al., 2001). A partial reduction (between 35-50% depletion) in xDNMT1p levels, which is not enough to result in global changes in DNA methylation levels, is sufficient for activation of a cell death program in *Xenopus laevis* embryos (Dunican et al., 2008). These observations support the hypothesis that DNMT1 possesses essential functions that are independent of its role as a maintenance methyltransferase and links its absence with activation of a cellular checkpoint response (Brown and Robertson, 2007).

The initiating signals for apoptosis in DNMT1-deficient embryos are unknown, one hypothesis is that hypomethylation leads to the release of chromosomal proteins that act as sensors of DNA methylation levels and induce p53 activation (Stancheva et al., 2001). Several protein candidates can be considered as sensors, including methyl-CpG binding proteins (MBD), which bind to chromatin by a methylation-dependent mechanism (Klose and Bird, 2006). However this model in its crude form would not be compatible with the dynamic changes in DNA methylation patterns

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that occur during development (Morgan et al., 2005). Special cases in which normal alterations in methyl-CpG levels would be unable to trigger apoptosis would need to be in place. Another possibility is that the signal originates with xDNMT1p itself, perhaps through its association with the replication machinery or DNA repair pathways (Guo et al., 2004; Leonhardt et al., 1992; Vertino et al., 2002). Our recent observation that the xDNMT1-deficient phenotype is independent of alterations in DNA methylation levels would support this latter possibility (Dunican et al., 2008).

The functional properties of the methyl-CpG binding protein MBD4 (MED1) suggest it as a possible candidate mediator of the apoptotic response in xDNMT1-depleted embryos. Like other MBD proteins, it has been shown to function as a methylation-dependent transcription repressor, but it is also a thymine glycosylase and interacts with the mis-match repair (MMR) protein MLH1 (Bellacosa et al., 1999; Hendrich et al., 1999; Kondo et al., 2005; Parsons, 2003). MBD4 thus, like DNMT1, potentially links epigenetic modification mechanisms with the MMR pathway (Parsons, 2003; Guo et al., 2004). An important function of MMR proteins is to sense DNA damage and to mediate the decision to repair the lesion or to induce apoptosis (Palii et al., 2008). The levels of several MMR proteins are reduced in *Mbd4*$^{-/-}$ mouse embryonic fibroblasts, which can account for the diminished apoptotic response of these cells to DNA-damaging agents (Cortellino et al., 2003; Sansom et al., 2003). In wild-type cells, DNA-damage recognition by MMR factors is sufficient to trigger cell cycle arrest and apoptosis through direct interaction with signalling kinases such as ATM, ATR, CHK1 and CHK2, which ultimately activates p53 (Luo et al., 2004). In line with this observation, it has been shown that overexpression of MLH1 induces apoptosis in cell lines probably through its interaction with ATM (Luo et al., 2004; Zhang et al., 1999).

Here we show that inhibition of xMBD4 function in early *Xenopus* embryos reduces the phenotypical severity of xDNMT1 depletion, whereas microinjection of mouse *Mbd4* mRNA induces an apoptotic response that disrupts early development. In UVA laser micro-irradiated cells, MBD4, MLH1 and DNMT1 can be colocalised at sites of DNA damage. Our results are compatible with a model in which MBD4 functions as an apoptotic-signalling molecule through its direct interactions with DNMT1 and MLH1.

MATERIALS AND METHODS

Embryos and microinjections

Xenopus embryos were manipulated according to standard procedures (Stancheva and Meehan, 2000). Two-cell embryos were injected with antisense morpholino oligonucleotides (10 ng for DNMT1 and Kaiso, 5-10 ng for MBD4, MLH1 and p53, per embryo) or with RNA (0.2-1 ng for wild-type mMBD4 and xMLH1 per embryo) synthesised by the mMessage Machine kit (Ambion). The ability of morpholino oligonucleotides to suppress translation was checked by the TNT Quick Coupled Transcription/Translation Kit (Promega) using 1 μg of plasmid DNA and 4 μg of morpholino (Fig. S6 in the supplementary material). The following morpholinos were used: xDNMT1 (xDMO1), GGACAGGCGT-GAAACAGACTCGGC; xMBD4 (xM4MO), TCCGGCACAGGAGC-AGCCATGTTTG; xMLH1 (xMLH1MO), CAGCCGCCGAATAACT-CCCGCCATT; xKaiso (xKMO), GATCAGCTTTTTTGTCTCCATGTCT; xp53 (xp53MO), TGCCGGTCTCAGAGGAAGGTTCCAT.

DNA isolation and Southern blots

DNA from wild-type and microinjected embryos was isolated and 10 μg of each sample were digested to completion with *Hpa*II or *Msp*I (MBI-Fermentas) and electrophoresed in a 1% native agarose gel. DNA digests were transferred to nylon membranes (Osmonics) and hybridised according to the manufacturer's protocols with a labelled 750-bp *Xenopus* satellite I probe (Stancheva and Meehan, 2000; Stancheva et al., 2001).

Plasmids and constructs

Xenopus laevis MBD4 (AW641890), mouse MBD4 (BE335048 and BE457540) and *Xenopus* DNMT1 (CA790804) clones were used along with the *Xenopus* MLH1 clone DKFZp724LO463Q3. The full-length (477 amino acid) protein, the glycosylase domain (307-477 amino acids) and the methyl-CpG binding domain (55-133 amino acids) of xMBD4 were cloned into the *Bam*HI/*Sal*I sites of pGEX-4T-1. The RFP-PCNA construct has been described (Mortusewicz et al., 2005). The GFP-MLH1 expression vector was constructed by cloning MLH1 cDNA into the pEGFP-C1 vector. A red variant of the GFP-MLH1 expression vector was made by replacing GFP with mCherry using *Nhe*I and *Bsr*GI. The full-length T7-tagged xMBD4 expression construct was made by cloning into the *Xba*I-*Bam*HI sites of pCGT7 vector (Cazalla et al., 2005). The hDNMT1-FLAG construct was obtained from W. Nelson (Agoston et al., 2005). The GFP-MBD4 construct was obtained from A. Bird and mutated to make the catalytically dead version GFP-MBD4^{D534A} (Hendrich and Bird, 1998; Rai et al., 2008). The GFP-xDNMT1 fusion was made by cloning full-lenth xDNMT1 into pEGFP-C1. The MLH1-GFP construct was a gift from V. Cryns (Chen et al., 2004). xDNMT1-GST fusions were made by cloning regions corresponding to 1-130 amino acids, 124-380 amino acids and 505-600 amino acids into the pGEX-4T-1 vector (GE Lifesciences).

Whole-mount TUNEL assay

Published procedures for the whole-mount TUNEL staining of embryos were followed (Hensey and Gautier, 1997).

Protein interaction assays

GST-tagged fusions were expressed in the ER2566 *E. coli* strain, purified using glutathione sepharose 4B and dialysed. GST pull-downs were performed according to standard protocols using in vitro translated and ^{35}S-Met-labelled proteins. Samples were subjected to SDS-PAGE, transferred to 3MM paper, dried and visualised by phosphorimaging. HeLa cells were used for immunoprecipitation experiments. DNA constructs were transfected with Lipofectamine 2000 (Invitrogene). Cells were analysed 24 hours after transfection. Immunoprecipitation was performed using the Catch and Release v2.0 system (Upstate). Anti-T7-tagged antibody (Novagen), anti-FLAG (Upstate) and monoclonal anti-GFP (CRUK) antibodies were used.

Cell culture, transfection, FACs analysis, UVA laser microirradiation and live imaging

Subcellular localisation studies using GFP and RFP fusion proteins were done according to standard procedures, using *p53*$^{-/-}$ MEFs as recipients (Lande-Diner et al., 2007). Cells were analysed 24 hours after transfection. Positive signals were visualised with Alexa Fluor secondary antibodies. Slides were examined using a Zeiss Axioplan II fluorescence microscope with Plan-Neofluar objectives, a 100 W mercury source (Carl Zeiss, UK) and a Chroma #84000 quadruple band-pass filter set (Chroma Technology, Rockingham, VT, USA) with the excitation filters installed in a motorised filter wheel (Ludl Electronic Products, USA). Grey-scale images were captured with a Hamamatsu Orca AG CCD camera [Hamamatsu Photonics (UK)] Instruments Pentamax). Image capture and analysis were performed using in-house scripts written for IPLab Spectrum (Scanalytics Corp, Fairfax, VA, USA). Transfection of expression plasmids, microirradiation and live cell imaging was as described (Mortusewicz et al., 2005; Mortusewicz et al., 2007). For apoptosis analysis, primary mouse embryonic fibroblasts or HCT116 cells were transfected using lipofectamine, stained with PE Annexin V (BD Biosciences) 8-10 hours after transfection, sorted on BD FACSAria II SORP sorter and analysed using BD FACs Diva software. The percentages of annexin-positive cells in populations of GFP-positive and GFP-negative cells were identified.

Rat and mouse DNMT1 analysis

Rat DNMT1 Myc-tagged constructs were kindly provided by Kunio Shiota (Kimura and Shiota, 2003). Mouse DNMT1-GST fusions were obtained from Sara Nakielny (Jeffery and Nakielny, 2004). Immunostaining was performed according to standard techniques using *p53*$^{-/-}$ mouse embryonic fibroblasts (MEFs) as recipients (Lande-Diner et al., 2007). Cells were

analysed 24 hours after transfection. A mouse monoclonal anti-T7 tag antibody (Novagen) was used. Positive signals were visualised with Alexa Fluor secondary antibodies. Slides were examined as described above.

RESULTS

xMBD4/xMLH1 mediate the apoptotic response in xDMO morphants

Recently, we demonstrated that injection of a morpholino against xDNMT1 (xDMO) into two-cell embryos results in premature zygotic transcription before the mid-blastula transition (MBT) and phenotypical abnormalities that mimic the effect of AS *xDnmt1* RNA injection (Dunican et al., 2008; Stancheva et al., 2001). However, the xDMO morphants do not exhibit any general changes in DNA methylation levels (Fig. 1A) (Dunican et al., 2008). Despite this, they undergo apoptosis, as evidenced by the appearance of cellular shedding from the embryo surface and by TUNEL positivity (Fig. 1B,C; Fig. 3B), features that we have previously characterised for *xDnmt1* AS RNA-injected embryos (Stancheva et al., 2001). This suggests that global hypomethylation of DNA is not the trigger for apoptosis and that an alternative pathway is responsible for initiating programmed cell death when xDNMT1p levels are reduced. We focused on the possible involvement of xMBD4 in initiating apoptosis, as its murine counterpart has been implicated as a central molecule in signalling pathways that are activated in response to DNA damage and (like DNMT1) it is linked with the MMR pathway (Cortellino et al., 2003; Parsons, 2003; Sansom et al., 2003; Bellacosa et al., 1999; Hendrich et al., 1999).

We first tested by morpholino injection whether depletion of xMBD4 or its binding partner xMLH1 could alleviate the effect of xDNMT1 depletion. Fig. 2 shows a panel of control and injected embryos (Fig. 2A-F) at equivalent stage 12-13. In comparison to the wild-type embryos, the xDMO morphants show a high proportion of abnormal embryos (70%), many of which display characteristic white patches of shedding cells that are indicative of apoptotic death (Fig. 2B). Higher magnification versions of Fig. 2A-F are available for clarity (see Fig. S1 in the supplementary material). A very small proportion (2%) of the xDMO morphants survive to develop to stage 30 (Fig. 2J,L), but in comparison to wild-type controls have phenotypical defects that are indicative of abnormal development, including spina bifida (compare Fig. 2J and Fig. 2K). Co-injection of xDMO with either the xMBD4 (xM4MO) or the xMLH1 (xMLH1MO) morpholinos relieves the severity of the phenotype (Fig. 2C,D). There are fewer obviously apoptotic embryos and a higher proportion (65% and 60%, respectively) have a normal appearance at the gastrula/neurula stage (Fig. 2G,H,L). Indeed, many of these double-injected embryos go on to form a high percentage (20% and 13%, respectively) of normal stage 30 embryos when compared with xDMO morphants (2%; Fig. 2L).

We had shown previously that targeted degradation of p53 levels by human papilloma virus E6 protein (HPV-18E6) can rescue the apoptotic response in AS RNA-depleted embryos (Stancheva et al., 2001). As an additional control we also co-injected with xDMO a morpholino against xp53 (xp53MO), which as predicted, rescued the apoptotic phenotype induced by xDMO injection and led to a high survival rate at gastrulation/neurulation (80%) and tadpole stage (75%; Fig. 2E,L). However, none of the surviving xDMO/xp53MO morphants had a normal appearance, and they exhibited a reduction of trunk and posterior regions with relatively small head structures by stage 30 (Fig. 2I). This is probably because, in addition to its role in DNA-damage signalling pathways, xp53 is required for proper mesoderm specification during *Xenopus*

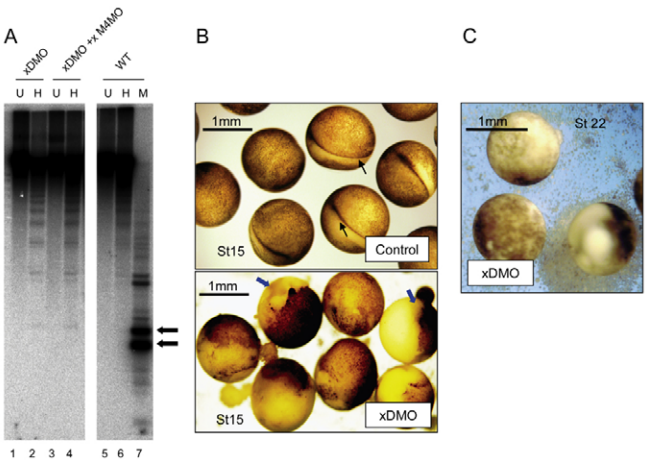

Fig. 1. xDMO morphants are not hypomethylated and exhibit an apoptotic phenotype. (**A**) Southern blot analysis with the xSATI repeat sequence demonstrating no hypomethylation at CCGG sites in xDMO morphants (lanes 1 and 2). No enzyme (U), *Hpa*II (H) and *Msp*I (M) restriction enzyme digests were performed on the indicated samples. *Msp*I is a methylation insensitive restriction enzyme, whereas *Hpa*II is methylation sensitive (C5mCGG). The digestion pattern of *Hpa*II is different to that of *Msp*I in wild-type (WT) embryos, the latter gives rise to fragments of 690 and 430 bp (arrows), indicating complete digestion. The *Hpa*II pattern is the same in WT, xDMO and embryos injected with both xDMO and xM4MO (anti-xMBD4) morpholinos, indicating high levels of DNA methylation at these repeats in all three samples. (**B**) Phenotypes of wild type (WT) and xDNMT1 morpholino (xDMO) embryos at stage 15. The xDMO morphants exhibit apoptotic lesions (blue arrows) and a lack of neural folds (black arrows in WT) compared with uninjected wild-type siblings or control morpholino-injected embryos. (**C**) Post-stage 15 xDMO morphants exhibiting developmental arrest and shedding cells.

development (Cordenonsi et al., 2003; Takebayashi-Suzuki et al., 2003). As an additional control, we also co-injected an xKaiso morpholino (xKMO) with xDMO, which is also required for pre-MBT silencing (Ruzov et al., 2004). Unlike xM4MO, co-injection with xKMO enhances cell death by gastrulation/neurulation with no survivors at this stage (Fig. 2F). We conclude that either xMBD4 or xMLH1 is specifically necessary for the activation of the apoptotic pathway that results from xDNMT1 depletion.

Overexpression of xMBD4 or xMLH1 results in apoptosis

Beause depletion of either xMBD4 or xMLH1 ameliorates the apoptotic response that results from a decrease in xDNMT1 levels, we hypothesised that overexpression of either protein would induce the cell death pathway. To test this possibility, we microinjected mRNAs (1 ng) for murine MBD4 (mMBD4) and MLH1 (mMLH1) into two-cell embryos, allowing them to develop until equivalent stage 12-13, and assayed them for apoptosis with the TUNEL assay. In comparison to wild-type embryos, the mMBD4 and mMLH1 mRNA induced developmental arrest as exogastrulae and resulted in hyper TUNEL-positive staining (Fig. 3A-D). For comparison, the xDMO morphants showed less intense TUNEL staining and also an exogastrulae phenotype at the equivalent stage (Fig. 3B). The effect of mMBD4 overexpression is dose dependent, at a lower amount (200 pg) the embryos went on to develop to tadpole stages but with axial defects (see Fig. S2A,B in the supplementary material); none of the high dose-injected embryos developed beyond gastrulation

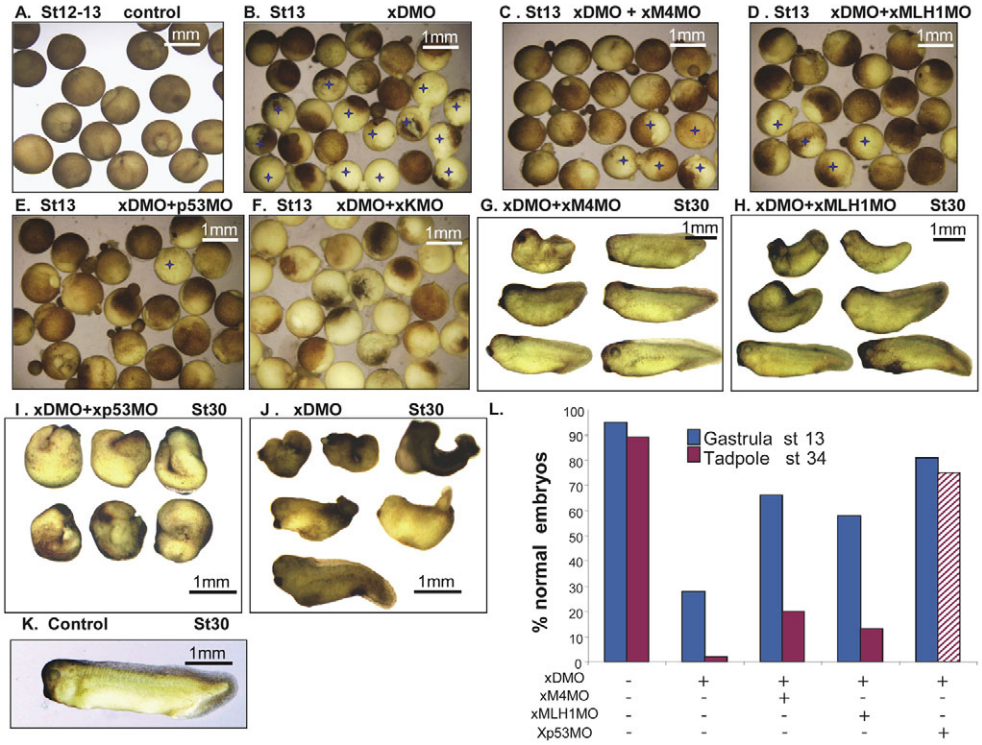

Fig. 2. The cell death phenotype of xDMO morphants can be alleviated by co-injection with either xMBD4, xMLH1 or xp53 morpholinos. (**A**,**K**) Uninjected control embryos at stages 12-13 and stage 30 (tadpole), respectively. (**B**,**J**) xDMO morphants at stage 13 and stage 30, respectively. Dying (apoptotic) embryos are indicated with a '+' in B. (**C**,**G**) Embryos co-injected with xDMO and an anti-xMBD4 morpholino (xM4MO) at stage 13 and stage 30, respectively. The mortality rate is reduced at stage 13 compared with that of embryos injected with xDMO only (see graph in L). (**D**,**H**) Embryos co-injected with xDMO and an anti-xMLH1 morpholino (xMLH1MO) at stage 13 and stage 30, respectively. The mortality rate is reduced at stage 13 compared with that of embryos injected with xDMO only (see graph in L) and the number of apoptotic embryos is reduced. (**E**,**I**) Embryos co-injected with xDMO and an anti-xp53 morpholino (xp53MO) at stage 13 and stage 30, respectively. The mortality rate is reduced at stage 13 and stage 30 compared with that of embryos injected with xDMO only (see graph in L), but the surviving stage 30 embryos have an abnormal appearance. (**F**) Embryos co-injected with xDMO and an xKaiso morpholino (xKMO) at stage 13 exhibit enhanced embryo mortality. (**L**) The percentages of normal embryos and embryos with developmental defects at stage 13 and stage 34 following injection with xDMO alone or co-injection with xM4MO, xMLH1MO or xp53MO.

(Fig. 3E,I). Injection of an equivalent dose of β-galactosidase mRNA had no adverse effect on development (see Fig. S3 in the supplementary material).

As additional proof that mMBD4 overexpression results in the activation of a caspase-dependent pathway, we cultured the control and mMBD4 mRNA-injected embryos in the presence of 20 μM Z-DEVD-FMK (a caspase inhibitor), a concentration that does not affect the development of control embryos (data not shown). This ensured that a high proportion of the mMBD4 mRNA-injected embryos (55%) developed to tadpole stage, although many of them had axial defects that were similar to those observed for the low-dose injection (Fig. 3E-G; see also Fig. S2A,B in the supplementary material). In addition, the effect of mMBD4 overexpression (1 ng mRNA) could be suppressed by the presence of either xp53MO or xMLH1MO, which resulted in 27% and 18% survival, respectively, by stage 30 (Fig. 3I). Furthermore, the presence of xM4MO reduced the lethality of the mMBD4 mRNA injection, which suggests that endogenous xMBD4 is recruited in the apoptotic response pathway that is activated upon mMBD4 mRNA injection (Fig. 3H).

In agreement with these data, overexpression of either GFP-MLH1 or GFP-MBD4 in transiently transfected primary mouse embryonic fibroblasts (MEFs) resulted in activation of an apoptotic response 2-4 (for MLH1) or 2-3 (for MBD4) times above that caused by GFP alone (Fig. 3J). The glycosylase catalytic activity of MBD4 is not required to activate apoptosis, as an inactive mutant, GFP-MBD4^{D534A}, also induced apoptosis in MEFs (Fig. 3J). This suggests that the response is not activated through the DNA damage that is potentially mediated by the glycosylase activity of MBD4. By contrast, overexpression of GFP-MBD4 did not induce apoptosis in HCT116 cells, which lack functional MLH1 (Papadopoulos et al., 1994), whereas overexpression of GFP-MLH1 did induce apoptosis in these cells (Fig. 3K). We conclude that MBD4 can signal an apoptotic response in combination with MLH1, although it is not clear how xDNMT1p depletion acts as a trigger for apoptotic activation through xMBD4/xMLH1 (see model in Fig. S2C in the supplementary material).

xMBD4 interacts with xDNMT1

Because hypomethylation is not required for the apoptotic response, yet depletion of xDNMT1 is and the absence of xMBD4 or xMLH1 inhibits apoptosis, we hypothesised that the xMBD4 protein interacts directly with xDNMT1. To test this idea, we co-expressed tagged forms of either human DNMT1 (FLAG) or xDNMT1 (GFP) with xMBD4 (T7) in HeLa cells and tested their interaction by immunoprecipitation (IP) and subsequent western blotting. IP of xMBD4 precipitates *Xenopus* and human DNMT1 (Fig. 4A; data not shown). In the reverse experiment, IP of xDNMT1 or human DNMT1 brings down xMBD4 (Fig. 4B). To test for a direct

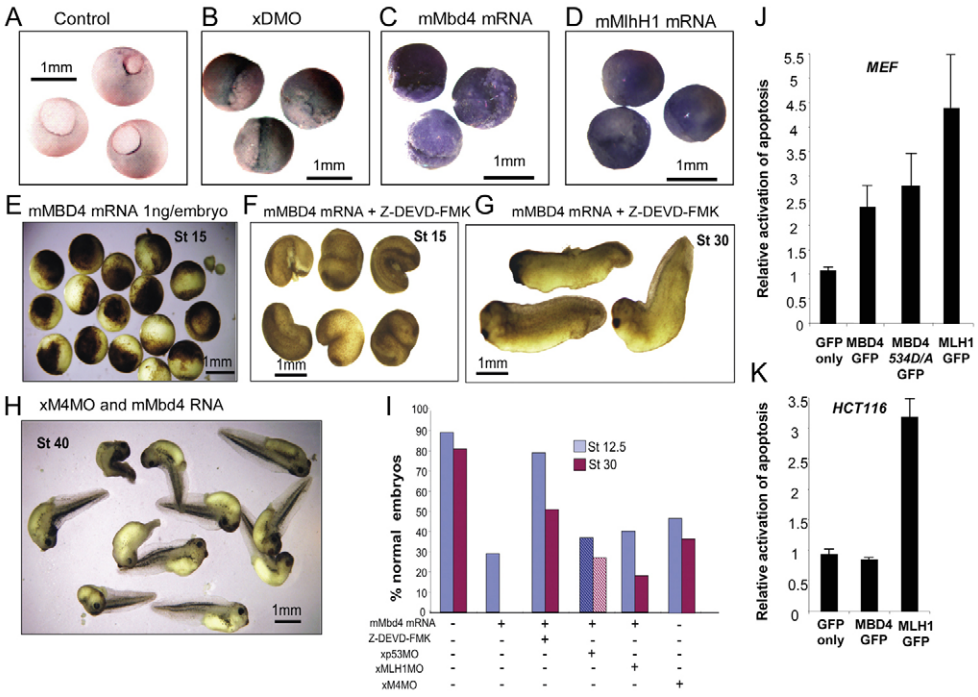

Fig. 3. The apoptotic phenotype of MBD4-injected embryos can be inhibited with Z-DEVD-FMK, xM4MO, xMLH1MO and xp53MO. (**A-D**) TUNEL staining (blue) indicates activation of apoptosis, compared with control embryos, in xDMO stage 12 morphants and embryos microinjected with either MBD4 (mMBD4) or MLH1 (mMLH1) from mouse. (**E**) Phenotype of embryos injected with 1 ng of mMBD4 mRNA at stage 15 (neurulation). (**F**) Phenotype of embryos injected with 1 ng of mMBD4 mRNA and incubated with the caspase inhibitor Z-DEVD-FMK at stage 15. (**G**) Phenotype of embryos injected with 1 ng of mMBD4 mRNA and incubated with the caspase inhibitor Z-DEVD-FMK at stage 30 (tadpole). (**H**) Phenotype of embryos injected with 1 ng of mMBD4 mRNA and xM4MO at stage 30. (**I**) Percentages of normal embryos and embryos with developmental defects at stage 13 and stage 34 overexpressing mMBD4 alone or in the presence of Z-DEVD-FMK, xMLH1MO or xp53MO. (**J**,**K**) MEFs (J) or HCT116 cells (K) were transiently transfected with GFP-MBD4, GFP-MBD4^{D534A}, GFP-MLH1 or GFP only, stained with PE annexin V and analysed by FACS. The ratios between the percentages of apoptotic cells in GFP-positive versus GFP-negative cells are presented. Overexpression of GFP-MBD4, GFP-MBD4^{D534A} and GFP-MLH1 leads to the activation of apoptosis in MEFs, at least 2.5 times more than that caused by GFP only. By contrast, the MBD4-GFP fusion does not induce apoptosis in MLH1-deficient HCT116 cells.

interaction, we made a GST fusion of xMBD4 and investigated whether it could pull down in vitro-translated xDNMT1. As a positive control, we also tested the interaction between mouse MLH1 and xMBD4 (Bellacosa et al., 1999); unsurprisingly, xMBD4 interacts with MLH1 via its C-terminal glycosylase domain (Fig. 4C), whereas the N-terminal MBD domain of MBD4 mediates its interaction with xDNMT1 (Fig. 4C). We also mapped the MBD4-interaction domain on DNMT1 from mouse, rat and *Xenopus* using a combination of IP and GST pull-down experiments (see Fig. S4A-C in the supplementary material). We could define a minimum domain of approximately 70 amino acids in the N-terminal targeting sequence (TS) region of DNMT1 that accounts for the MBD4 interaction (Fig. S4D in the supplementary material); interestingly, this overlaps but is not the same as a region in rat DNMT1 that interacts with MECP2 (Kimura and Shiota, 2003).

Previous work has shown that MBD4 localisation to heterochromatin in mouse cells is methylation dependent (Hendrich and Bird, 1998). We could also demonstrate by immunofluorescence that epitope-tagged versions of xMBD4 localise at methylated heterochromatic foci in *p53*$^{-/-}$ mouse embryonic fibroblast (MEF) cells (to avoid inducing the apoptotic response), whereas MLH1 does not (Fig. 4D; Fig. 5A). However, in the presence of xMBD4, MLH1 can be recruited to heterochromatin (Fig. 4E). DNMT1 associates with chromatin during the G2 and M phases, and this association is mediated by a specific targeting sequence that shows a strong preference for constitutive but not facultative heterochromatin (Easwaran et al., 2004). Interestingly, DNMT1 can colocalise with xMBD4 at heterochromatic foci (Fig. 5A). By contrast, MLH1 is not preferentially localised to constitutive heterochromatin in the presence of overexpressed DNMT1, although there is some overlap when cells are in G1 and S phases (Fig. 5B). Because MBD4 interacts with both MLH1 and DNMT1 by independent domains, it is possible that xMBD4 can act as a recruiter of MLH1 to chromatin sites that are coincident with methylated DNA and DNMT1 during the cell cycle. This possibility is borne out in triple transfection experiments in which GFP-tagged DNMT1 can be colocalised with Cherry-tagged MLH1 in the presence of xMBD4 (Fig. 5C).

MBD4 and MLH1 accumulate at irradiated sites

Recent work suggests that DNMT1 participates in the MMR pathway and can be recruited to sites of DNA damage by PCNA (Guo et al., 2004; Mortusewicz et al., 2005). PCNA encircles double-stranded DNA as a trimer, forming a sliding clamp that tethers proteins involved in DNA replication and repair. We analysed the recruitment kinetics of either GFP-MLH1 or GFP-MBD4 in the presence of RFP-PCNA in HeLa cells (which do not have prominent heterochromatic foci) sensitised by BrdU incorporation after inducing localised DNA damage with a UVA laser micro-irradiation system (Mortusewicz et al., 2005). Under these conditions, various types of DNA lesion are generated leading to the recruitment of PCNA. As might be expected, this also led to the recruitment of GFP-MLH1 to damaged sites, but its accumulation lagged behind that of PCNA (Fig. 6A; see also

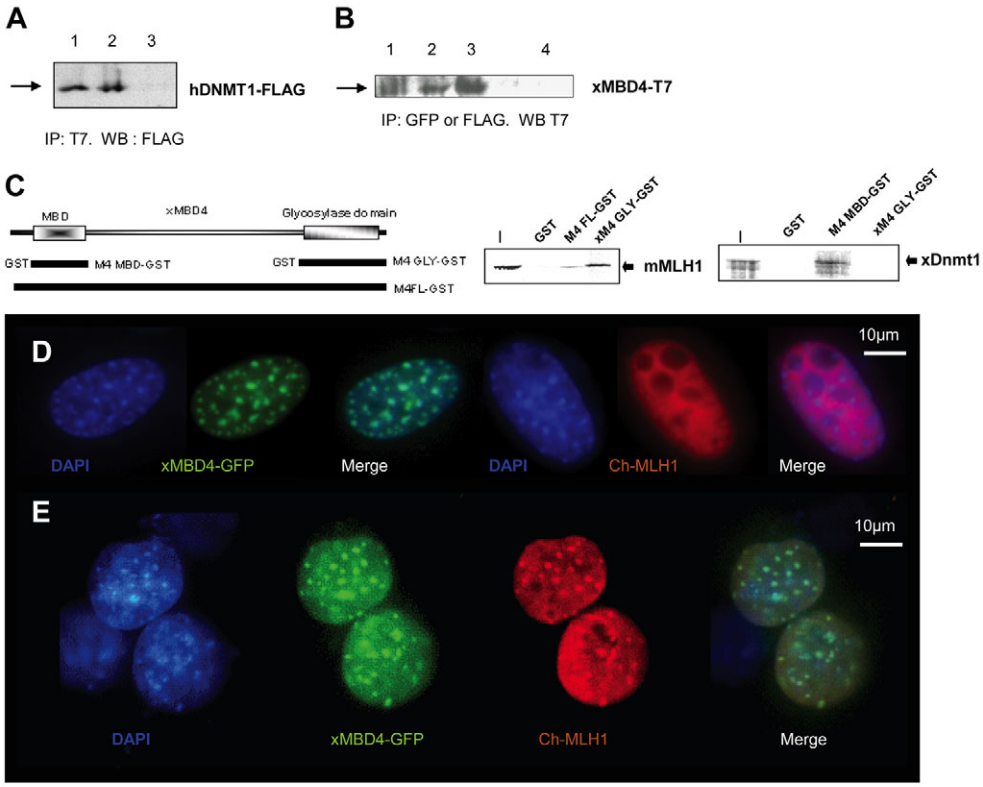

Fig. 4. xMBD4 interacts directly with xDNMT1 and can also recruit MLH1 to methylated DNA present at constitutive heterochromatin. (**A**) hDNMT1-FLAG injected by itself or with T7-tagged xMBD4 (T7xMBD4) was expressed in HeLa cells. Cell extracts were prepared and immunoprecipitated with an anti-T7 monoclonal antibody (mAb) and subsequently western blotted with an anti-FLAG mAb. Lane 1; hDNMT1-FLAG input; lane 2, T7xMBD4 and hDNMT1-FLAG IP; lane 3, hDNMT1-FLAG only immunoprecipitation (IP). An arrow indicates the hDNMT1-FLAG. (**B**) A reciprocal IP was done between T7xMBD4 and either hDNMT1-FLAG or GFP-xDNMT1. Cell extracts were prepared and immunoprecipitated with either an anti-GFP or anti-FLAG mAb and subsequently western blotted with an anti-T7 mAb. Lane 1, T7xMBD4 input; lane 2, T7xMBD4 and GFP-xDNMT1 IP; lane 3, T7xMBD4 and hDNMT1-Flag IP; lane 4, T7xMBD4 only IP (anti-GFP). An arrow indicates the T7xMBD4 protein. (**C**, left) Schematic of xMBD4 indicating its methyl-CpG binding (MBD) and glycosylase domains. The GST-fusion proteins used in pull-down assays are shown underneath; FL, full length. (Right) Plasmids encoding either mouse MLH1 (mMLH1) or xDNMT1 were transcribed and translated in vitro, and incubated with GST only or with the indicated GST-fusion proteins. After extensive washing, ^{35}S-labelled bound proteins were resolved on SDS-PAGE, dried and exposed. Notice the glycosylase region of xMBD4 interacts with mMLH1, and the MBD domain interacts with xDNMT1. (**D**) Localisation of either MBD4-GFP or Cherry-MLH1 in the nucleus of mouse *p53*$^{-/-}$ MEFs. MBD4 (like endogenous mMBD4) is localised at methylated heterochromatin, DAPI bright spots. By contrast, MLH1 shows general nuclear staining (like endogenous MLH1) (Schroering et al., 2007). (**E**) Co-transfection of MBD4-GFP and Cherry-MLH1 into mouse *p53*$^{-/-}$ MEFs results in the recruitment of Cherry-MLH1 to methylated heterochromatin, as indicated by DAP1 bright spots (blue). The merged images for each transfection (D,E) are also shown.

Movies 1-4 in the supplementary material). Likewise GFP-MBD4 was recruited to PCNA foci, but its retention in the presence of PCNA was less stable than that of MLH1 (Fig. 6B; see Movies 1-4 in the supplementary material). However, when GFP-MBD4 and Cherry-MLH1 were co-transfected, both molecules were recruited to micro-irradiated sites and maintained there during the 5-minute observation period (Fig. 6C). In addition, RFP-DNMT1 and GFP-MBD4 could localise to sites of DNA damage (Fig. 6D). These data suggest that the interaction of these partners on chromatin is influenced by DNA damage and that, while present at these lesions, it is possible that MBD4 and DNMT1 can participate in the decision to either repair the DNA damage (including the DNA methylation profile) or activate a cell death program.

A catalytically inactive form of DNMT1 rescues the cell death phenotype in AS RNA-injected embryos

The interaction between xMBD4/xMLH1 and xDNMT1 suggests a model whereby loss of xDNMT1p releases or activates xMBD4 to initiate the apoptotic program via xp53. It also suggests that catalytically dead forms of DNMT1 might suppress the apoptotic pathway, as this role appears to be independent of changes in DNA methylation (Fig. 1) (Dunican et al., 2008). As shown previously, AS RNA-injected embryos are severely depleted in xDNMT1 protein levels during early stages (stage 5-10), resulting in their DNA becoming hypomethylated, and they exhibit high rates of embryo lethality by stage 30 even though xDNMT1p levels are restored by stage 11 (Stancheva and Meehan, 2000; Stancheva et al., 2001) (Fig. 7A). Substitution of cysteine 1226 with tyrosine in the catalytic site of human DNMT1 (hDNMT1^{C1226Y}) results in an enzyme with no methyltransferase activity (Jair et al., 2006). The presence of either the wild-type or mutant hDNMT1 mRNA substantially rescues the phenotypical deficits associated with transient depletion of xDNMT1 before the MBT, with the appearance of between 20-25% phenotypically normal embryos at stage 30 (Fig. 7B,C) compared with the low number (4%) of abnormal survivors observed following AS RNA-only injection. This suggests that hDNMT1^{C1226Y} can suppress activation of the apoptotic pathway in AS RNA-injected embryos, and the

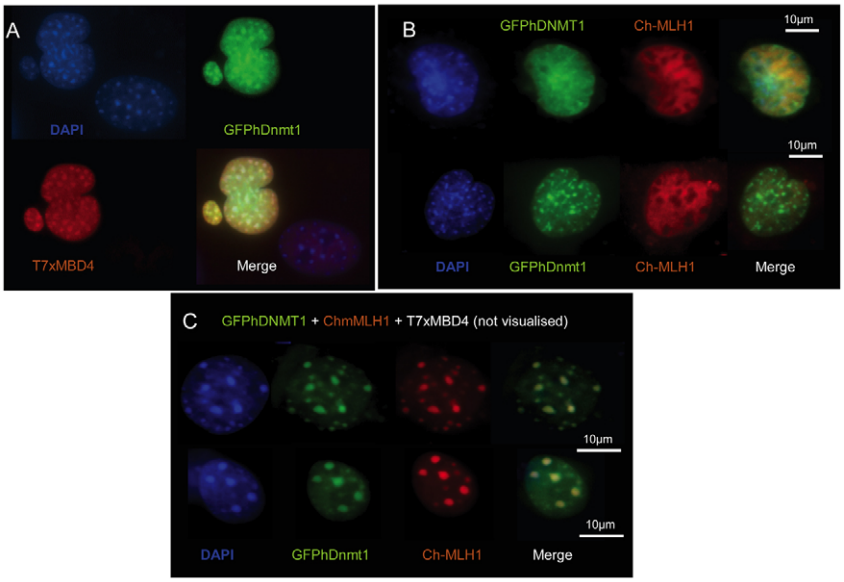

Fig. 5. MBD4 can target MLH1 to heterochromatic sites that also contain DNMT1. (**A**) Co-transfection of GFP-hDNMT1 and T7xMBD4 into *p53*$^{-/-}$ MEFs results in their colocalisation at methylated heterochromatin, as indicated by the DAP1 bright spots (blue). (**B**) Co-transfection of GFP-hDNMT1 and Cherry-MLH1 (Ch-MLH1) into *p53*$^{-/-}$ MEFs does not lead to the recruitment of Cherry-MLH1 to methylated heterochromatin, as indicated by the DAP1 bright spots. The nuclear sub-localisation of DNMT1 in cells is cell cycle dependent and two major patterns are observed. DNMT1 is either localised at late-replicating constitutive heterochromatin (lower panel) or is more evenly distributed (upper panel). There is some overlap (as indicated by the merged signal) of MLH1 with DNMT1 in the upper panel, but not when DNMT1 is localised at methylated heterochromatin (in late S phase or G2; lower panel). (**C**) By contrast, a triple transfection with GFP-hDNMT1,Cherry-MLH1 and T7xMBD4 (not visualised) results in colocalisation of GFP-hDNMT1 and Cherry-MLH1 at methylated centromeric heterochromatin. MLH1 is resident at this region in all transfectants, whereas hDNMT1 is either localised at late-replicating constitutive heterochromatin or is more generally localised (data not shown).

subsequent phenotypical abnormalities. It also supports the idea that DNA hypomethylation is not the trigger for apoptosis, as this cannot be restored by hDNMT1^{C1226Y}. Another feature of AS RNA-injected embryos is the premature activation of transcription prior to the MBT (stage 8.5) (Stancheva and Meehan, 2000). In contrast to xDMO-rescued embryos in which xDNMT1p is depleted by 30-40% (Dunican et al., 2008), co-injection of either the hDNMT1^{C1226Y} or wild-type hDNMT1 (hDNMT1^{WT}) with xDNMT1 AS RNA was unable to restore pre-MBT silencing of xBRA and xID2 to wild-type levels (Fig. 7D). This implies that the premature transcription phenotype before the MBT (stage 8.5) and the activation of apoptosis after MBT (stage 11 and later) are independent processes, and that it is the latter mechanism that is responsible for the phenotypical appearance of xDMO and AS RNA

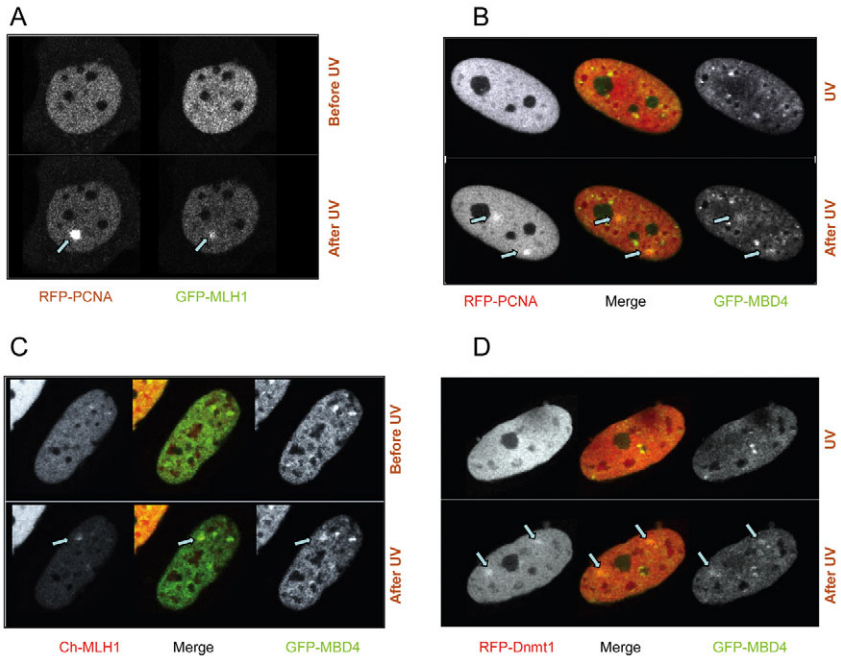

Fig. 6. Recruitment of MLH1, MBD4 and DNMT1 to sites of DNA damage. (**A**) Live cell imaging of HeLa cells transiently transfected with RFP-PCNA and GFP-MLH1 before (top) and after (bottom) microirradiation. RFP-PCNA clearly colocalised with GFP-MLH1 at the microirradiated site (arrows). (**B**) Live cell imaging of HeLa cells transiently transfected with RFP-PCNA (left) and GFP-MBD4 (right) during (top) and after (bottom) microirradiation. RFP-PCNA clearly colocalised with GFP-MBD4 at both microirradiated sites (arrows). (**C**) Live cell imaging of HeLa cells transiently transfected with Ch-MLH1 (left) and GFP-MBD4 (right) before (top) and after (bottom) microirradiation. Ch-MLH1 clearly colocalised with GFP-MBD4 at the microirradiated site (arrows). (**D**) Live cell imaging of HeLa cells transiently transfected with RFP-DNMT1 (left) and GFP-MBD4 (right) during (top) and after (arrows). (**D**) Live cell imaging of HeLa cells transiently transfected with RFP-DNMT1 (left) and GFP-MBD4 (right) during (top) and after (bottom) microirradiation. RFP-DNMT1 clearly colocalised with GFP-MBD4 at both microirradiated sites (arrows). Merged pictures for B-D are shown in the middle. The associated movies are in the supplementary material.

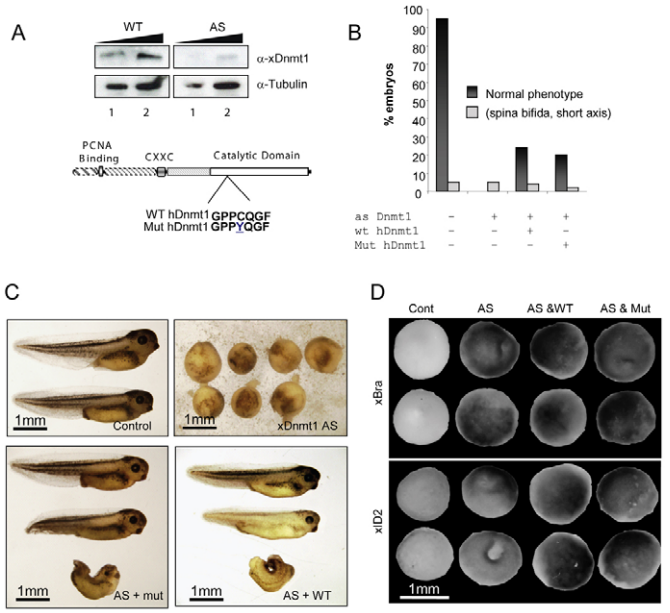

Fig. 7. A catalytically inactive form of hDNMT1 can rescue embryos severely depleted of xDNMT1. (**A**) Western blotting shows that xDNMT1 levels are greatly reduced (>90%) in antisense (AS) *xDnmt1* RNA-depleted stage 8 embryos (pre-MBT). This results in general DNA hypomethylation, premature transcription and activation of apoptosis (Stancheva and Meehan, 2000; Stancheva et al., 2001). In lane 2, four embryo equivalents were loaded in a 1/5 dilution (0.8 embryo) for each sample. The blots were stripped and reprobed with α-tublin antibodies as a loading control. The cartoon indicates functional domains present in human DNMT1 (hDNMT1), including the PCNA-binding domain (PCNA), the Zn finger (CXXC) domain and catalytic domains. An inactive point mutant of hDNMT1 (C1226Y, Mut hDNMT1) has no catalytic activity (Jair et al., 2006). (**B**) Comparison of the percentage of normal and mutant late tadpoles in control, AS RNA, AS and wild-type (WT) hDNMT1 mRNA, and AS and mutant hDNMT1 mRNA-injected embryos. The rescued embryos have a higher percentage of normal embryos than do the AS RNA mutants. The rescuing ability of catalytically active and inactive forms of hDNMT1 was comparable. (**C**) AS *xDnmt1* RNA-depleted rescued embryos are similar in phenotype to their control wild-type siblings. Tadpole (stage 40) embryos are shown. Note the apoptosis in the low number of surviving embryos injected with AS *xDnmt1* RNA only (top right). Co-injection of either wild-type hDNMT1 or mutant hDNMT1 mRNA can rescue AS RNA-injected embryos. Scale bar: 1 mm. (**D**) RNA in situ hybridisation analysis reveals ectopic expression of the indicated genes (xBRA and xID2) throughout the animal pole of AS RNA-injected, but not control stage 8 embryos. Co-injection of either wild-type hDNMT1 or mutant hDNMT1 mRNA cannot reimpose repression in AS RNA-injected embryos. Scale bar: 1 mm.

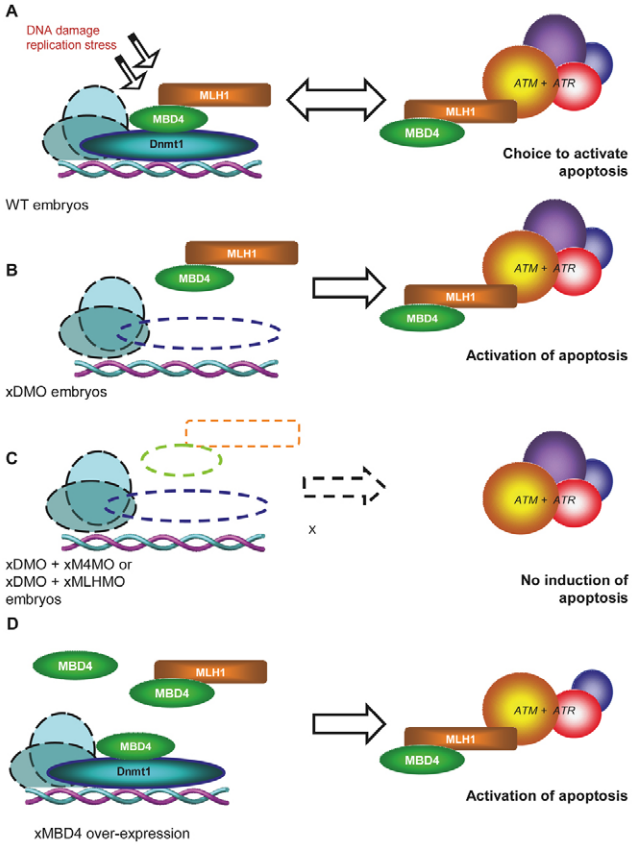

Fig. 8. Model for the activation of xDNMT1-mediated apoptosis in *Xenopus laevis* embryos. (**A**) In normal embryos, we propose that a complex of xDNMT1, xMBD4 and xMLH1 is bound onto chromatin. This complex plays a role in responding to DNA damage or replication stress and is involved in the decision to repair the lesion or to activate an apoptotic response by releasing MBD4/MLH1 from xDNMT1; the MBD4/MLH1 complex signals probably via the DNA damage kinases ATM and ATR. (**B**) In xDMO morphants, a reduction of xDNMT1p levels results in abnormal activation (perhaps caused by release from a chromatin-bound complex) of the p53 apoptotic pathway via unbound MBD4/MLH1. (**C**) In xDMO/xM4MO or xDMO/xMLH1 double morphants, the p53 apoptotic pathway cannot be activated owing to the absence of the xMBD/xMLH1 complex. (**D**) Overexpression of MBD4 induces apoptosis via MLH1.

mutant embryos. The inability to restore silencing in rescued AS RNA embryos is in line with a model in which high levels of DNMT1 are required during pre-MBT stages to maintain gene repression (Dunican et al., 2008).

In Fig. 8A, we present a model for the activation of xDNMT1-mediated apoptosis in *Xenopus laevis* embryos. We propose that a chromatin-associated complex of xDNMT1, xMBD4 and xMLH1 in normal embryos responds to DNA damage or replication stress by deciding either to repair the lesion or to activate an apoptotic response through the activation/release of MBD4/MLH1 from chromatin-bound xDNMT1. The MBD4/MLH1 complex signals, probably via interaction with the DNA-damage kinases ATM and ATR, activate the p53-dependent programmed cell death pathway.

Depletion of xDNMT1 triggers the apoptotic response by activation of the pro-apoptotic MBD4/MLH1 complex (Fig. 8B). Removal of either xMBD4 or xMLH1 prevents the activation of apoptosis in xDMO morphants (Fig. 8C).

DISCUSSION

In previous work, we characterised the molecular pathology of xDNMT1-depleted embryos, which exhibit the typical features of programmed cell death, including DNA fragmentation, the appearance of TUNEL-positive staining, caspase activation and stabilisation of p53 levels (Stancheva et al., 2001). The induction of 14-3-3 proteins in these mutant embryos is consistent with the activation of the G2 DNA damage cell cycle checkpoint. The cell death phenotype of xDNMT1-depleted animal pole explants could be suppressed by overexpression of either the anti-apoptotic protein Bcl2 or HPV-E6, an inhibitor of p53 function. Taken together, this suggests that loss of the DNMT1 protein is linked with the cellular response to DNA damage (checkpoint defects). Exposure of cells to 5-aza also

suggests that DNMT1 is a component of the DNA damage-response system. This results in the robust induction of γ-H2AX, DNA fragmentation, and the activation of repair proteins in both ATM and ATR (DNA damage-response kinases) pathways (Palii et al., 2008). DNMT1 is also strongly colocalised with γ-H2AX in 5-aza-treated cells, which is consistent with its recruitment to sites of DNA damage (Mortusewicz et al., 2005; Palii et al., 2008). A major question that results from these observations is what is the initiating signal for 'damage' in the absence of DNMT1 in cell lines and embryos? An obvious possibility is improper DNA re-methylation; however, our experiments in which partial xDNMT1 depletion by morpholino injection results in an embryonic lethal phenotype and activation of the apoptotic pathway without any global or locus-specific changes in DNA methylation suggests that this model is not applicable (Fig. 1A) (Dunican et al., 2008). Like the effect of AS RNA depletion, the xDMO morphants are TUNEL positive and the cell lethal phenotype (but not the developmental defects) is rescued by inhibiting xp53 function (Fig. 3B; Fig. 2E,I). Similarly, the apoptotic response in (*Dnmt1*$^{-/-}$, *p53*$^{-/-}$) MEFs is suppressed even though up to 10% of genes are ectopically activated, including MBD4, caspase 1 and caspase 4 (Lande-Diner et al., 2007). Our recent observation demonstrating that a catalytically dead version of human DNMT1 can rescue the embryonic (apoptotic) phenotype of xDMO morphants suggested to us that the signal for apoptosis lies not with DNA methylation but with xDNMT1 itself (Dunican et al., 2008).

As DNMT1 has been connected to the MMR and the DNA damage-response pathways (Guo et al., 2004; Palii et al., 2008), we considered what might functionally link xDNMT1 directly to these pathways. We choose to focus on MBD4, as it promotes an apoptotic response to DNA-damaging agents and excises spontaneously deaminated cytosine (i.e. uracil) or methylcytosine (i.e. thymine) from G-T/U mismatches (Cortellino et al., 2003; Hendrich et al., 1999; Sansom et al., 2003). MBD4 also interacts with the MMR/tumor suppressor gene (TSG) MLH1, and inhibition of MLH1 function also causes increased apoptosis resistance with regard to DNA-damaging agents, implying possible checkpoint and apoptotic signalling functions for both proteins (Luo et al., 2004; Zhang et al., 1999; Brown et al., 2003). Our double-depletion experiments (Fig. 2) suggest that both xMBD4 and xMLH1 are required for the embryonic lethal phenotype of xDMO morphants, which we attribute to their ability to activate the apoptotic response (Fig. 1E,F; Fig. 3A-E). This suggests a possible signalling pathway that connects DNMT1 to MBD4/MLH1 and subsequently a DNA damage-sensing response. MLH1 associates with the DNA-damage kinase ATM and MMR complexes formed at sites of DNA damage facilitate the phosphorylation of checkpoint kinases 1 and 2 by ATM (Bartek and Lukas, 2003; Brown et al., 2003). ATM also directly phosphorylates p53 at serine 15, contributing to its stabilisation and increased activity as a transcription factor (Canman et al., 1998). The activation of p53 by these stimuli leads to the transcriptional modulation (both activation and repression) of p53-target genes. In addition, p53 regulates cell cycle progression by controlling the G1-S and G2-M checkpoints. Thus, p53 is an obvious candidate for mediating the DNMT1-dependent apoptotic response (Bellacosa, 2001; Levine et al., 2006). Phosphorylation of ATM is also observed after radiation-induced DNA double-strand breaks that lead to activation of the G2/M checkpoint and the double-strand break-response pathway (Shiloh, 2003). The potential colocalisation of DNMT1, MBD4 and MLH1 implies that they might participate in a cellular checkpoint that monitors potential DNA hypomethylation events by a 'proxy' mechanism that detects the presence or absence of DNMT1, perhaps at or adjacent to the replication fork (see Fig. S5 in the supplementary material). The recruitment of these components in response to localised DNA damage also suggests that they can have a role in the cellular decision whether to repair the lesion or activate apoptosis (Fig. 8). We hypothesise that it is the absence of DNMT1 at this checkpoint that triggers apoptosis by releasing MLH1/MBD4 to activate ATM in mutant embryos and somatic cell lines. Interestingly, complete inactivation of DNMT1 in HCT116 cancer cells also activates G2 arrest; however, most if not all of these *DNMT1* mutant cells eventually escape this arrest and exhibit mitotic defects, including broken chromosomes, chromosome congression or alignment; defects that result in mitotic catastrophe (Chen et al., 2007). It is possible that, in agreement with our model, DNMT1-deficient HCT116 cells could escape G2 arrest without the activation of apoptosis because they lack functional MLH1, and that this contributes to the observed mitotic catastrophe phenotype (Papadopoulos et al., 1994).

What is the potential importance of the mechanism that we have identified? Cancers arise from the sequential acquisition of genetic alterations in specific genes. The high number of mutations in cancer cells led to the hypothesis that an early step in tumor progression is the generation of genetic instability. The potential role of genetic instability in the initiation and progression of colorectal cancers has been well defined in the hereditary nonpolyposis colon cancer syndrome (Bodmer, 2006). Constitutive activation of the DNA-damage response is a feature of the pre-invasive stages of many human tumors, including colorectal cancer (CRC), and this checkpoint response is believed to delay or prevent the malignant transformation of early lesions (Bartkova et al., 2005). MBD4 and other MMR components are frequently mutated in CRCs that exhibit micro-satellite instability (Miquel et al., 2007). These observations suggest that DNMT1 is a crucial part of a signalling cascade that activates an inducible barrier against tumour progression and genetic instability resulting from replicative stress in pre-cancerous lesions or the perturbation of epigenetic regulatory mechanisms during development (Halazonetis et al., 2008). Cellular sensitivity to changes in DNMT1 levels is thus lost when components of the signalling cascade are either absent or mutated. In this context, it is notable that cell cycle regulation of DNMT1 is often abrogated in tumor cells and this has been suggested to be indicative of a poor prognosis (Agoston et al., 2005; De Marzo et al., 1999; Saito et al., 2003).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/13/2277/DC1

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