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Summary

DNA methylation in animals is thought to repress transcription via methyl-CpG specific binding proteins, which recruit enzymatic machinery promoting the formation of inactive chromatin at targeted loci. Loss of DNA methylation can result in the activation of normally silent genes during mouse and amphibian development. Paradoxically, global changes in gene expression have not been observed in mice that are null for the methyl-CpG specific repressors MeCP2, MBD1 or MBD2. Here, we demonstrate that *xKaiso*, a novel methyl-CpG specific repressor protein, is required to maintain transcription silencing during early *Xenopus laevis* development. In the absence of *xKaiso* function, premature zygotic gene expression occurs before the mid-blastula transition (MBT). Subsequent phenotypes (developmental arrest and apoptosis) strongly resemble those observed for hypomethylated embryos. Injection of wild-type human kaiso mRNA can rescue the phenotype and associated gene expression changes of *xKaiso*-depleted embryos. Our results, including gene expression profiling, are consistent with an essential role for *xKaiso* as a global repressor of methylated genes during early vertebrate development.

Key words: DNA methylation, *Kaiso*, Transcriptional repression, *Xenopus*, Differentiation

Introduction

The presence of methylated CpGs in the DNA sequence of a promoter is generally incompatible with gene expression in animal somatic cells (Bird, 2002). Over 70% of CpGs in vertebrate DNA contain the 5-methyl cytosine modification, presenting a potential for global gene silencing. Distinct patterns of DNA methylation exist at specific loci, which can vary between different tissues and developmental stages. In *Xenopus laevis* embryos, global DNA methylation levels are high in early stages, but drop by about 40% in late blastula and subsequently recover during later gastrula stages (Stancheva et al., 2002; Stancheva and Meehan, 2000). This correlates with the period of transcriptional quiescence observed during the first 12 cleavage cycles of *Xenopus* development. Transcription activation begins in a coordinated manner at the midblastula transition (MBT), when DNA methylation levels decrease, accompanied by changes in chromatin composition and increased activity of the basal transcription machinery (Almouzni et al., 1994; Almouzni and Wolffe, 1995; Newport and Kirschner, 1982a; Newport and Kirschner, 1982b; Veenstra et al., 1999).

The general model for indirect repression by DNA methylation predicts that methyl-CpG binding proteins mediate silencing in pre-MBT embryos by recruiting enzymatic complexes that modify local chromatin structure (Jones et al., 1998; Nan et al., 1998; Wade et al., 1999). This results in a closed heterochromatic structure that is refractory to transcription (Jaenisch and Bird, 2003). Conversely, loss of DNA methylation can lead to a relaxation in chromatin-based silencing mechanisms in somatic cells such that the threshold for gene activation and consequent developmental potential is altered (Jaenisch and Bird, 2003; Meehan, 2003). In *Xenopus*, as in mice, inactivation of the maintenance DNA methyltransferase *xDnmt1* results in hypomethylation of genomic DNA, ectopic expression of normally silent genes and embryonic lethality (Jackson-Grusby et al., 2001; Stancheva and Meehan, 2000). *xDnmt1* deficient embryos exhibit premature gene expression at least two cell cycles earlier than normal (prior to the MBT), implying that the correct timing of zygotic activation is regulated in part by the repressive effect of DNA methylation during early blastula stages (Stancheva and Meehan, 2000).

In mammals, three repressor proteins, MeCP2, MBD1 and MBD2, bind methylated CpGs via a conserved motif called the methyl-CpG binding domain (Cross et al., 1997; Hendrich and Bird, 1998). The MBD motif is ~70 amino acids long and is found in a diverse number of nuclear proteins, only a subset of which can bind methylated DNA (Hendrich and Tweedie,

2003). In vitro and transient transfection experiments provide good evidence that MeCP2, MBD1 and MBD2 act as transcriptional repressors of methylated reporter templates (Nan et al., 1997; Ng et al., 1999; Ng et al., 2000). In addition, chromatin immunoprecipitation (ChIP) experiments localise MeCP2, MBD2 and MBD1 to methylated and silenced genes. The chromatin of these genes is also hypoacetylated and contains H3 that is methylated on lysine 9 (H3K9Me), both hallmarks of inactive chromatin (Ballestar et al., 2003; Gregory et al., 2001). A number of histone deacetylases (HDAC) and histone methyltransferases (HMT) are associated with MeCP2, MBD1, MBD3 and DNA methyltransferases. This reinforces the mechanistic link between DNA methylation and specific histone modifications in promoting gene silencing (Jaenisch and Bird, 2003).

Mbd2$^{-/-}$-derived embryonic cells have a reduced ability to repress methylated reporter genes in transient transfection assays, and the activation threshold of specific genes is altered upon differentiation (Hendrich et al., 2001; Hutchins et al., 2002). However, the expectation that MeCP/MBDs might act as global repressors of transcription from methylated templates in vivo was not supported: the absence of MeCP2, MBD1 or MBD2 function does not disrupt specific programs of developmental gene expression in mice (including X-chromosome inactivation), nor does it phenocopy the effects of Dnmt1 inactivation (Chen et al., 2001; Guy et al., 2001; Hendrich et al., 2001; Tudor et al., 2002; Zhao et al., 2003). Only MBD3 is required for early murine development, but as this MBD does not actually bind methylated DNA, MBD3$^{-/-}$ embryo lethality is probably the result of its role as a core component of the NuRD/MeCP1 transcription repressor complex (Hendrich et al., 2001).

A very different methylated DNA-dependent binding domain was identified in the transcriptional repressor kaiso (*ZBTB33* – Human Gene Nomenclature Database), which binds methyl CpGs through a zinc-finger (ZF) motif (Prokhortchouk et al., 2001). Kaiso recognizes DNA sequences that contain at least two methyl-CpGs, and represses transcription from reporter templates in a methyl-CpG-dependent manner (Prokhortchouk et al., 2001; Yoon et al., 2003). It was originally discovered in a two-hybrid screen using the cell adhesion co-factor p120^{ctn} as bait (Daniel and Reynolds, 1999). Like the armadillo-related protein β-catenin, to which it is structurally homologous, p120^{ctn} interacts with the cytoplasmic domain of the transmembrane cell adhesion molecule E-cadherin. Kaiso could possibly mediate p120^{ctn}/E-cadherin signalling to the nucleus to regulate the expression of methylated target genes. In HeLa cells, kaiso mediates silencing of the MTA2 gene by interacting with the N-CoR co-repressor complex via its POZ domain (Yoon et al., 2003). A *Xenopus* homologue, *xKaiso*, has recently been described (Kim et al., 2002). We noted that its expression pattern matched that of *xDnmt1* (A.R. and R.M., unpublished), which hinted that it could have a potential role in regulating transcription silencing in pre-MBT embryos (Stancheva and Meehan, 2000).

We wished to determine the binding specificity of *xKaiso* and its ability to act as a methylation-dependent repressor, and to investigate its role in transcriptional silencing during early amphibian development. We show that *xKaiso* has a similar, if not more pronounced preference for methylated DNA compared with its mammalian counterpart. Depletion of *xKaiso* function in *Xenopus* embryos results in premature activation of pre-MBT zygotic transcription. The depletion phenotype can be rescued by overexpression of wild-type human kaiso, but not by a mutant lacking a functional methyl-CpG binding domain. Our analysis suggests that *xKaiso* is essential for methylation-dependent transcriptional silencing in early amphibian embryos.

Materials and methods

Plasmids and recombinant proteins

GST-*xKaiso* ZF encoded the *xKaiso* zinc fingers (amino acids 470-609; AY044336) cloned in frame into pGEX-4T1. GST-*hKaiso* and GST-C552R*hKaiso* fusions were cloned into pGEX-2T. Point mutation C552R was generated by QuikChange® Site-Directed Mutagenesis (Stratagene) using GGGGAGCGAAGGTATCAG-CGTTTGGCCTGTGGC and reverse complement primer. Proteins were expressed in BL-21 cells and purified on GST Sepharose.

EMSA experiments

Binding reactions were as described using 5% PAGE in 0.3×TBE to resolve DNA-protein complexes with Non-Sm and Me-Sm probes (Prokhortchouk et al., 2001) and the human matrilysin (Hmat) oligo (Daniel et al., 2002).

Methyl CpG-dependent repression test

The ability of *xKaiso* to repress transcription from a methylated reporter gene using *Mbd2*$^{-/-}$ cells was performed as described previously (Hendrich et al., 2001; Prokhortchouk et al., 2001).

Protein extracts and immunoblots

Total protein extracts for western blotting were prepared from staged wild-type eggs, and wild-type and KMO-injected blastulae and gastrulae as described (Stancheva et al., 2001). Extracts were run in 10% SDS-polyacrylamide gels and electro-transferred to PVDF membrane. *xKaiso* was detected by a monoclonal antibody against the conserved N-terminal domain of mouse kaiso (Zymed, clone 6F). Anti-mouse HRP-conjugated IgGs were used as secondary antibodies.

Kaiso – p120 catenin interaction test

p120 mouse cDNA (forms 1A and 3A) was cloned into pFASTBAC1 vector (Invitrogen) from pMS2-p1201A and pMS2-p1203A (provided by Dr Al Reynolds). The proteins (1A and 3A – co-transfected) were expressed in Sf9 insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Cell extracts were added to GST-*Kaiso* Sepharose, incubated for 1 hour at room temperature, washed three times with PBS. Bound proteins were eluted with glutathione and resolved by SDS-PAGE, blotted and visualised with antibodies specific either to p120 catenin (mAb clone 15D2) or to kaiso (ZF6).

Embryos and microinjections

Xenopus embryos were obtained from in vitro fertilized wild-type and albino eggs or by natural mating of sexually mature frogs. They were grown, staged and microinjected according to standard procedures (Stancheva and Meehan, 2000). At the two-cell stage, the embryos were injected into the animal half of the embryos with 0.5-10 ng/cell of *xKaiso* morpholino oligo (KMO), GATCAGCTTTTTTGTCT-CCATGTCT; *xDnmt1* morpholino oligo (DMO), GGACAGGCGT-GAAACAGACTCGGC; or control morpholino, CGCTCAGCT-CCTCCATGTCTGCCGC (Gene-Tools); and/or 200-750 pg of sense capped RNA synthesized in vitro (T3/T7 Cap-Scribe kit, Boehringer). Whole-embryo run-on experiments with [α-^{35}S]UTP incorporation were performed using 5 ng KMO-injected and wild-type embryos, as described (Stancheva and Meehan, 2000). The whole-mount TUNEL staining of embryos was as described previously (Hensey and Gautier, 1998).

RT-PCR analysis

RNA was isolated as described previously (Stancheva and Meehan, 2000). cDNA was synthesised from five embryos using Superscript II reverse transcriptase (RT) (Invitrogen). RT-PCR was performed over a range of cDNA dilutions and PCR cycles to ensure exponential amplification. *xODC* was used as a loading control, and cDNA was synthesised in the absence of RT to control for genomic DNA contamination. Primer sequences used were: xBEF-U, 5′-CCGAAA-CAGCTTCCAGACAA-3′; xBEF-L, 5′-TGAAAGGAAAGCAGA-CGCTC-3′; xDRAK1-U, 5′-ACCGAGAGGAGGAAGTCACT-3′; xDRAK-L, 5′- GGCTTAAAGGAAACAAGTCC-3′; xOct-25-U, 5′-TAATGGAGAGATGCTTGATG-3′; xOct-25-L, 5′-TTCTCTATGT-TCGTCCTCC-3′; xODC-U, 5′-GGAGCTGCAAGTTTGGAGA-3′; xODC-L, 5′-TCAGTTGCCAGTGTGGTC-3′.

Magex array hybridisation and analysis

cDNA filters were obtained from Viagenx (Canada). Arrays were probed as described previously with minor modifications (Helbing et al., 2003). In brief, *Xenopus* total RNA was isolated and cDNA was synthesised using the SuperSMART kit (Clontech, USA) (Barnett et al., 1998). cDNA was amplified in the exponential phase by PCR using 21-23 cycles at an annealing temperature of 63°C. cDNA probes were labelled with [α-^{32}P]dCTP by random priming using the HexaLabel kit (Fermentas, UK). Arrays were hybridised in Church & Gilbert hybridisation solution (0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA, pH 7.2) at 65°C and washed to high stringency in 40 mM Na_2HPO_4, 1% SDS at 65°C. Images were acquired using AIDA software and the FLA2000 phosphorimager. Scanalyze (Eisen et al., 1998) was used to acquire gene spot intensities and non-signal backgrounds. Data across hybridisation experiments were normalised to the 18S rRNA spots intensity.

CpG island analysis

DNA sequences were analysed using the CpG island analysis tool at the Potential Promoter Regulatory Element Database (http://idealab.cs.uiowa.edu/cgi-bin/HPD/cg.cgi). The following parameters were used: minimum CpG island length 200 bp, minimum CpG content 0.5, and observed/expected CpG ratio 0.6. The genes from the MAGEX array were divided into three groups: genes containing (1) a CpG-rich region within first 100 bp of cDNA sequence; (2) CpG-rich region(s) outside the first 100 bp of cDNA sequence; and (3) no CpG-rich regions.

Results

xKaiso is a methyl-CpG dependent transcription repressor

We identified a *Xenopus* EST with homology to mammalian kaiso and subsequent sequencing showed it to be full length (AY044336). Comparison of the evolutionary conserved mammalian and *xKaiso* sequences reveals a domain-specific divergence in the amino acid sequence of the third zinc finger, which has only 61% similarity to human kaiso, compared with 90% for the other zinc fingers (Fig. 1A). Because this could possibly affect the sequence recognition of the ZF domain, we performed electrophoretic mobility shift assays (EMSA) with recombinant GST proteins containing the ZF domain. We found that *xKaiso* ZFs preferentially bind the methylated Sm (Me-Sm) DNA sequence, which contains three methylated CpG pairs (Prokhortchouk et al., 2001). No band shift was observed with the non-methylated Sm (Non-Sm) oligo (Fig. 1B). Despite the amino acid divergence in the third zinc finger, the DNA-binding affinity of the *xKaiso* ZFs for eight other methyl-CpG containing sequences tested previously for the

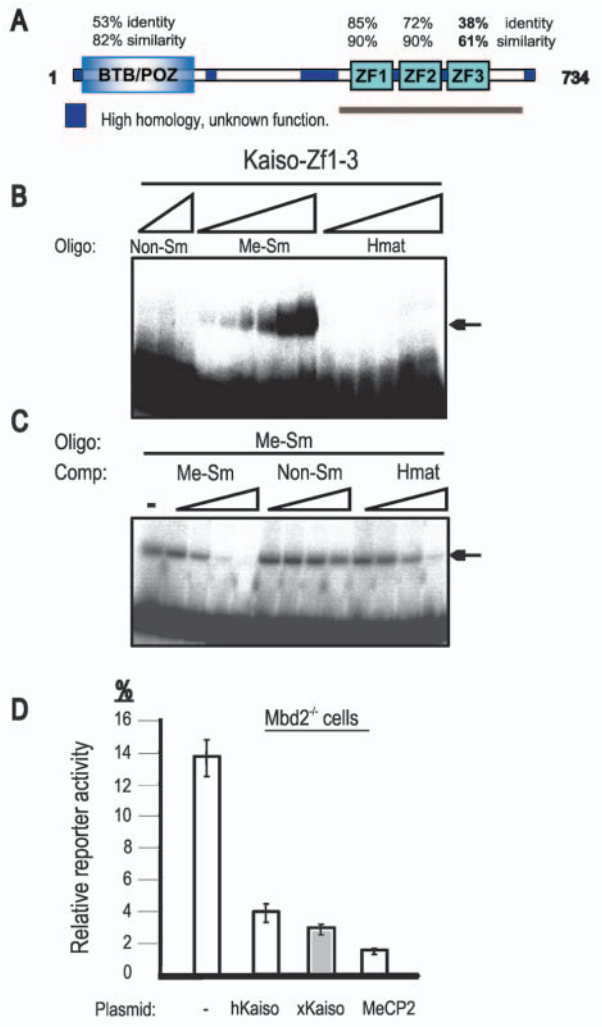

Fig. 1. *xKaiso* is a methyl-CpG dependent transcriptional repressor. (A) Schematic of *xKaiso* illustrating the BTB-POZ and zinc-finger domains and identity and similarity (in %) with human and zebrafish protein sequences. The blue box indicates a region of high homology of unknown function. The bar indicates the region used in EMSA assays. (B) EMSA experiment using recombinant ZF domain of *xKaiso* (*Kaiso*ZFs) (1, 2, 4, 10, 20 and 40 ng of protein) with Sm (methylated and non-methylated) or human matrilysin (Hmat) probes. Arrow indicates the *xKaiso* ZF specific band shift in the reaction with methylated Sm, but not with non-methylated Sm or Hmat probe. (C) EMSA experiment with *xKaiso* ZF, methylated (Sm) probe and non-labelled competitors: either methylated or non-methylated Sm, or Hmat, at 5×, 10×,100×, 1000× molar excess. No competitor is added to the reaction in the first lane. *xKaiso* ZF specific band (arrow) completely disappears at 1000× molar excess of methylated Sm. Non-methylated Sm oligo shows virtually no competition with the methylated Sm probe. The *xKaiso* ZF band in the presence of 1000× molar excess of Hmat competitor is stronger than in the presence of 100× molar excess of methylated non-labelled Sm oligo. (D) Methyl-CpG-dependent repression by *xKaiso* in a transient transfection assay. Kaiso expression constructs were co-transfected with an SV40-luciferase reporter into mouse cells that are compromised in methyl-CpG-dependent transcriptional repression (*Mbd2*$^{-/-}$). Relative percentage (methylated reporter expression/nonmethylated reporter expression) is the average of at least three experiments. Human kaiso (h*Kaiso*) and human MeCP2 (MeCP2) expression constructs were used as positive controls for methyl-CpG dependent transcriptional repression.

human homologue (Prokhortchouk et al., 2001) displayed the same preference for two adjacent methyl-CpGs in the substrate, as the presence of intervening nucleotides reduced binding (data not shown). However, the *xKaiso* ZF domain was more specific for methylated DNA than its human counterpart: it did not copy the affinity of human kaiso for the non-methylated matrilysin (Hmat) sequence in EMSA (Fig. 1B, Fig. 3I). In a competition experiment, the matrilysin oligo was at most one-tenth as efficient a competitor compared with Me-Sm itself (Fig. 1C). Together, these results suggests that the *xKaiso* ZF domain has a preference for methylated DNA, but differs from human kaiso in its ability to bind certain non-methylated sequences.

xKaiso has transcription repression activity when targeted to a GAL4-responsive luciferase reporter in *Xenopus* embryos (Kim et al., 2002). We verified whether *xKaiso*, like human kaiso, can repress transcription in a methyl-CpG dependent manner, using murine *Mbd2*$^{-/-}$ cells that are defective in their ability to repress expression from a methylated reporter gene (Hendrich et al., 2001; Prokhortchouk et al., 2001). In this cell line, a normally silent, fully CpG-methylated reporter gene was de-repressed to ~15% of control non-methylated reporter transcription activity. Expression of either *xKaiso* or human kaiso in this cell line restored repression from the methylated reporter, but not from the non-methylated control (Fig. 1D, and data not shown). Thus, *xKaiso* shows the same ability to repress transcription from a methylated reporter gene in the *Mbd2*$^{-/-}$ cell system as its mammalian homologue (Prokhortchouk et al., 2001).

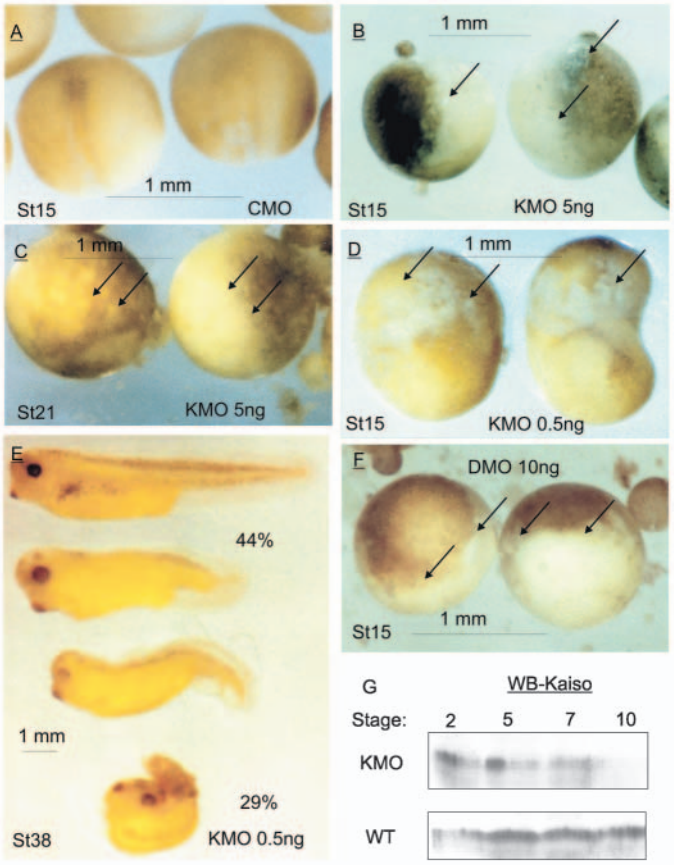

Fig. 2. The phenotypes of *xKaiso*-depleted embryos. (A) Embryos injected at two-cell stage with 10 ng of a control morpholino (CMO) show normal neurulation (stage 15). (B) Injection of 5 ng of the *xKaiso* morpholino (KMO) leads to a failure of blastopore closure and developmental arrest of neurulation at stage 15. Arrows indicate the appearance of white apoptotic cells from the borders of the blastopore. (C) Apoptotic cells (arrowed) cover almost the entire surface of embryos at stage 21 and cell shedding is present. (D) Injection of low dose (0.5 ng) of KMO causes defects of neurulation and delay of blastopore closure. Apoptotic cells are arrowed. (E) The range of phenotypes produced at low dose (0.5 ng) of KMO: 44% of embryos look normal by stage 38 (upper embryo), 29% exhibit failure to develop normal dorsal structures (spina bifida, lower embryo). Other phenotypes are intermediate. (F) Embryos injected with 10 ng of *xDnmt1* morpholino (DMO) show apoptotic phenotype virtually identical to that of 5 ng KMO. (G) Western blot using anti-x*Kaiso* antibody and whole embryonic extracts derived from wild type (WT) and 5 ng KMO-injected embryos (KMO). Stages of development are indicated above the lanes. The kaiso-specific band in KMO-injected embryos disappears by stage 10.

xKaiso is essential for *Xenopus* development

To analyse *xKaiso* function during development, we injected *Xenopus* two-cell stage embryos with a stabilised antisense *xKaiso* morpholino oligonucleotide (KMO) corresponding to the 5′-UTR and first six codons (see Materials and methods). This efficiently blocks *xKaiso* protein translation (Fig. 2G). Injection with 10 ng of a control non-inhibitory morpholino (CMO) resulted in normal development (see Table S1 in the supplementary material; Fig. 2A). By contrast, embryos injected with 0.5-10 ng KMO morpholino failed to develop and exhibited a variety of dose-dependent phenotypes (see Table S1 in the supplementary material; Fig. 2B-E). The characteristic feature of the 5 ng KMO-injected embryos is the appearance of white apoptopic cells near the edge of the blastopore in neurulating embryos (Fig. 2B-D; Fig. 4). By stages 21-22, the KMO embryos are developmentally arrested and the embryo surface is covered with apoptotic cells that are associated with cell shedding (Fig. 2C). Injection of a low dose (0.5 ng) of KMO into two-cell stage embryos led to a less severe phenotype with a delay in blastopore closure at stage 15 (Fig. 2D); many of these embryos exhibited short axis or reduced dorsal structures (spina bifida) by tadpole stage (Fig. 2E). Phenotypically, the effect of the KMO injection was similar to that of *xDnmt1* depletion (Fig. 2F) (Stancheva et al., 2001; Stancheva and Meehan, 2000). This overlap with the effects of loss of DNA methylation suggests that *xKaiso* may be responsible for mediating silencing of methylated genes in pre-MBT embryos, and is equally essential to their survival.

A functional methyl CpG binding domain is required to rescue KMO injected embryos

To ascertain the specificity of the KMO injection phenotype, we tried to rescue them by co-injection with human kaiso RNA, which will not bind the KMO because of sequence differences. Embryos injected at the two-cell stage with 750 pg of wild type human kaiso RNA developed normally (see Table S1 in the supplementary material, compare Fig. 3A,B). Co-injection of 5 ng KMO with 750 pg of wild type human kaiso enabled over half of the embryos (56%) to successfully complete neurulation compared with none for the KMO alone (see Table S1 in the supplementary material). By tadpole stage, 26% of KMO/750 pg wild type human kaiso injected embryos

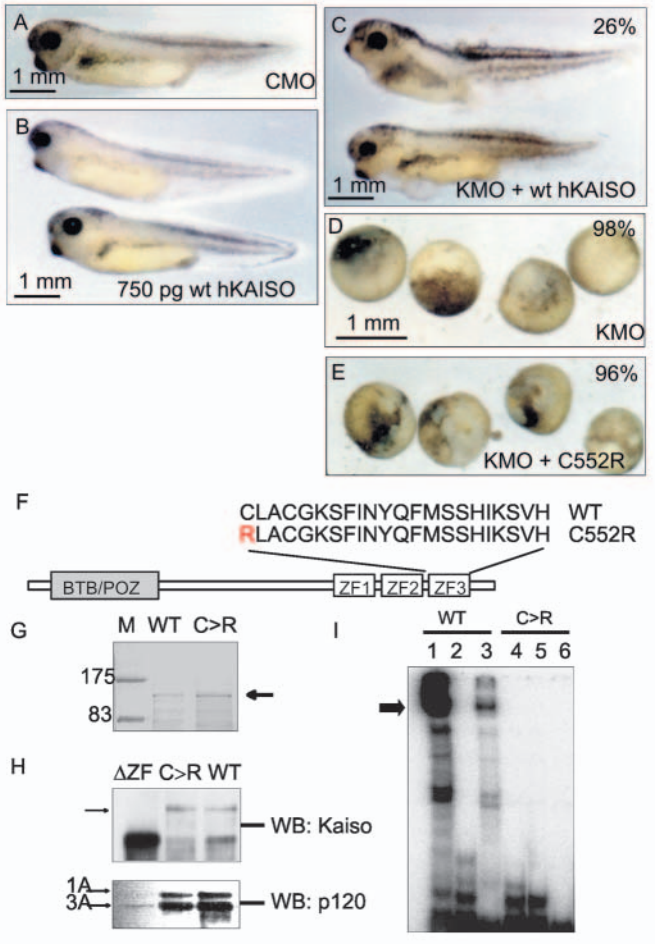

Fig. 3. The methyl-CpG binding function of kaiso is required to rescue the *xKaiso* knockdown phenotype. The percentages of presented phenotypes are indicated. (A) Embryos injected with 10 ng of CMO develop normally by tadpole stage. (B) Over-expression of 750 pg wild-type human kaiso RNA does not affect normal development of *Xenopus* embryos. (C) Injection of 750 pg of wild-type human kaiso RNA together with 5 ng KMO leads to complete rescue of 26% of the embryos. (D) Apoptotic phenotype produced by injection of 5 ng KMO. (E) Co-injection of 750 pg of C522R human kaiso mutant RNA with 5 ng KMO cannot rescue the phenotype of *xKaiso* depletion. (F) Location of the kaiso mutant C522R amino acid substitution (red) in the third zinc finger, leading to loss of the ability to bind methylated DNA. (G) Protein gel of recombinant wild-type human kaiso and C522R (C>R) mutant proteins (arrow). (H) Pull-down experiment showing $p120^{ctn}$ (arrow) binds both wild-type human kaiso and C522R (C>R) mutant proteins in vitro, but not human kaiso protein lacking the ZF domain (ΔZF). (I) EMSA experiment using recombinant C522R (C>R) mutant and wild-type human kaiso proteins with methylated (lanes 1, 4), non-methylated Sm oligos (lanes 2, 5) and human matrilysin (Hmat) oligo (lanes 3, 6) as probes. The kaiso-specific band shift is arrowed. The C522R mutant shows no DNA-binding activity.

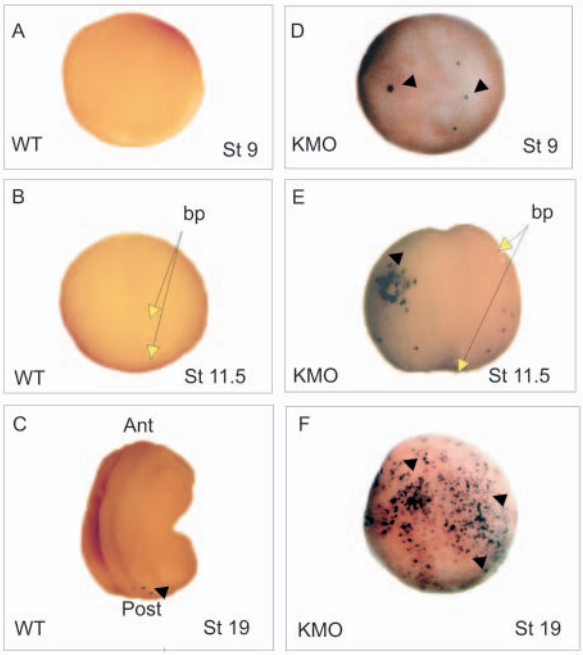

Fig. 4. The loss of *xKaiso* induces an apoptotic response in *Xenopus* embryos. Wild-type (A-C) and 5 ng KMO-injected (D-F) *Xenopus* embryos were assayed by TUNEL for the appearance of apoptotic cells. TUNEL-positive cells were not detectable in normal late blastula (A) and gastrula (B) embryos. Small numbers of apoptotic cells (arrowhead) appeared in 1-2% of wild-type embryos at late neurula stage (C). TUNEL-positive cells were detected in 11% and 15% of KMO-injected embryos at late blastula (D) and gastrula stages (E), respectively. More than 90% of KMO-injected embryos exhibited a general pattern of apoptosis at a stage corresponding to late neurula of normally developing embryos (F). Abbreviations: bp, blastopore; ant, anterior; post, posterior.

had developed normally, while 15% exhibited axial defects (see Table S1 in the supplementary material, Fig. 3C). We also attempted to rescue the KMO-induced phenotype with a mutant human kaiso RNA carrying a cysteine to arginine substitution at position 552 (C552R) in the third zinc finger (Fig. 3F). This disrupts the human kaiso DNA and methyl-CpG-dependent binding activity but not the ability to bind its interacting partner, $p120^{ctn}$ (Fig. 3H,I) (Daniel and Reynolds, 1999). Embryos injected with 5 ng of KMO and 750 pg of C522R RNA showed exactly the same phenotype as those injected with 5 ng KMO only, without any (even partial) rescue (see Table S1 in the supplementary material, Fig. 3D,E). These experiments strongly suggest that the DNA binding function of human kaiso is required to rescue KMO-injected embryos.

Kaiso-depleted embryos are apoptotic

x*Dnmt1* depletion in *Xenopus* embryos results in induction of apoptosis (Stancheva et al., 2001). We therefore checked whether KMO-injected embryos were apoptotic, using the whole-mount TUNEL assay, a highly sensitive indicator of DNA fragmentation in situ (Hensey and Gautier, 1998; Stancheva et al., 2001). A small number (1-2%) of non-injected control embryos have low levels of apoptotic cells that are restricted mainly to the developing nervous system at later stages of neurulation (Fig. 4A-C) (Hensey and Gautier, 1998). By contrast, in 11% of embryos injected at the two-cell stage with 5 ng of KMO, a small number of TUNEL-positive cells already appeared at late blastula stage (Fig. 4D). Fifteen percent of KMO injected embryos showed TUNEL-positive cells at mid-gastrula stage 11, mainly restricted to the ectodermal layer (Fig. 4E). By neurulation, a general pattern

of apoptosis was detected in more than 90% of KMO-injected embryos (Fig. 4F), consistent with their visible appearance (Fig. 2C), and development was arrested. Overall, the cellular apoptotic patterns caused by *xKaiso* depletion are similar to those observed for x*Dnmt1*-deficient embryos, further emphasizing the functional equivalence of loss of this CpG-dependent repressor, or loss of CpG methylation itself.

Loss of *xKaiso* activity results in premature activation of gene expression in blastula stage embryos

Reduction in genomic levels of ^{5}mC in *Xenopus* embryos results in transcription activation approximately two cycles earlier than normal (Stancheva et al., 2002; Stancheva and Meehan, 2000). We tested for premature activation of zygotic transcription in *xKaiso*-depleted embryos by measuring the incorporation of α-^{35}S labelled UTP in KMO-injected and control embryos at blastula and gastrula stages (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b; Stancheva and Meehan, 2000). As expected, control embryos begin to incorporate label above background after MBT. By contrast, ^{35}S-UTP incorporation in *xKaiso*-depleted embryos was detected two cell cycles earlier (Fig. 5A), as had been previously described for *xDnmt1*-depleted embryos (Stancheva and Meehan, 2000). In diploid embryos, premature global activation has only been observed upon *xDnmt1* depletion. This strongly suggests that x*Dnmt1* and x*Kaiso* may participate in the same pathway to silence genes in pre-MBT embryos.

In a cDNA screen, we have identified transcripts that are upregulated in pre-MBT embryos hypomethylated as a result of *xDnmt1* depletion (D.D. and R.M., unpublished). Three examples (xOct-25, xBef and xDrak) are shown in Fig. 5B; derepression also occurred in KMO-injected embryos but was less marked than that observed for *xDnmt1* depletion. All transcripts that were upregulated in KMO-injected blastula embryos were also upregulated in DMO embryos (D.D. and R.M., unpublished).

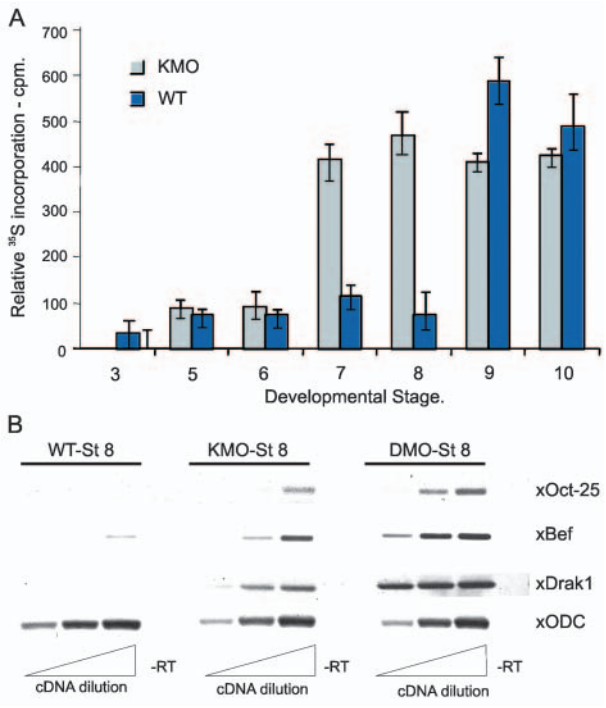

Fig. 5. *xKaiso* depletion leads to premature activation of transcription of a subset of genes regulated by *xDnmt1*. (A) [α-^{35}S]UTP incorporation detecting the activation of gene expression in wild-type (WT) and 0.5 ng KMO-injected embryos. WT and KMO-injected two-cell embryos were treated with 50 nCi [α-^{35}S]UTP, cultured in parallel and collected at the same developmental stage. (B) RNA derived from wild type, 5 ng KMO and 5 ng DMO (*xDnmt1* morpholino) injected embryos at stage 8 were assayed by RT-PCR for the presence of transcripts of genes (xOct-25, xBef, xDrak1) regulated by *xKaiso* and *xDnmt1*, respectively, with xODC as a loading control.

xKaiso is a genome wide repressor of transcription in the developing embryo

Our results suggest that the methyl-CpG-dependent repression function of *xKaiso* is required to maintain transcriptional silence in pre-MBT *Xenopus* embryos. To explore the capacity of *xKaiso* as a general transcription repressor, we screened a *Xenopus* cDNA mini-array with cDNA probes synthesized from pre-MBT stage 8 wild-type embryos (WT8), and *xKaiso* morpholino-injected (KMO8) and *xKaiso* morpholino/human kaiso mRNA co-injected (KMO8^{rescue}) embryos. The fold expression changes between KMO8:WT8 and KMO8^{rescue}:WT8 were computed using ScanAlyze and compared using a threshold of 1.5-fold expression change (Fig. 6A; Table S2 in the supplementary material). Fifty-seven genes (13%) were misregulated in the KMO:WT8 experiments, of which 55 (96%) were upregulated 1.5-fold or more in KMO8 embryos, and two genes [Mad2 and p33 ringo (ls26)] exhibited a decrease in expression relative to WT8 embryos (Fig. 6A). Many (25/55) of the genes upregulated in KMO8 were in the range of a 5- to 10-fold increase. We observed a similar change in expression pattern for this gene set in *xDnmt1*-deficient embryos (D.D. and R.M., unpublished). By sharp contrast, the overexpression of the 55 genes analysed in the KMO8:WT8 comparison was largely neutralised in KMO8^{rescue} embryos. Only 18 (31%) of the genes were greater than 1.5 upregulated (e.g. GemH1, xMEK2, CycA1 and CycB2 in Fig. 6A,B). Six genes (11%) were essentially expressed at similar levels, while expression of the remainder (58%) was reduced in KMO8^{rescue} embryos. This suggests that the rescued embryos have reduced levels of ectopic gene activation compared with *xKaiso*-depleted embryos.

The 55 genes that were up-regulated in the KMO:WT experiments did not represent 13% of genes across the board. Some functional categories, such as 'cell growth control' (27% of genes affected, P<0.0003) and 'chromatin/DNA structure' (15%, P<0.02), were enriched over twofold relative to the input array, whereas others, in particular 'transcription' (6%, P<0.0003), were significantly underrepresented (Fig. 7A,B). This is in marked contrast to the genes showing increased levels of expression at normal MBT (Altmann et al., 2001), where approximately half of the genes are involved in transcription (24%), regulatory functions (17%) and signal transduction (10%). After MBT, a greater number of the downregulated genes were involved with energy metabolism (16%) and significantly fewer genes serve regulatory functions (2%) (Altmann et al., 2001), whereas downregulated genes were an exception in the KMO:WT set (Fig. 7B).

In previous work we demonstrated that genes that are

regulated by DNA methylation at MBT contain clusters of CpGs at their promoters, so called 'CpG islands', which are methylated in pre-MBT embryos (Stancheva et al., 2002). At present, the *Xenopus laevis* genome has not been sequenced. However, it is possible to identify potential CpG island regions in the 5′ region of the MAGEX cDNAs based on the assumption that the initial 5′sequence (first exon) overlaps with the promoter region (Bird, 1986; Tykocinski and Max, 1984). On this basis, 30% of the genes on the MAGEX array contain potential CpG islands and 38% percent do not (Fig. 7C). The remaining 32% contains CpG rich sequences outside the 5′ region. In the KMO:WT set of cDNAs, the proportion of potential CpG island containing genes is enriched (52% compared with 30%, $P<0.00004$; Fig. 7D). If these regions are methylated in pre-MBT embryos, then they may represent direct targets for *xKaiso*-dependent silencing during early development.

Discussion

In the developing embryo, the midblastula transition (MBT) marks the time point when dependence on maternal transcripts gives way to the start of zygotic transcription (maternal-zygotic transcription, MZT). This key event in the development of all metazoans takes place early after the first few cell divisions in mammalian embryos, but occurs at later stages in embryo species sustained on large supplies of maternal RNA. In amphibians, where the MBT was first noted (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b), the transition is preceded by a period of transcriptional silence lasting 12 cell divisions. DNA methylation has a clearly identified role in the regulation of developmental gene activation at the MBT (Stancheva et al., 2001; Stancheva et al., 2002; Stancheva and Meehan, 2000). Loss of methylation at individual *Xenopus* gene promoters coincides with their zygotic activation at MBT. No changes in methylation patterns are observed for repeated sequences, genes that are inactive at MBT, or in coding regions (Stancheva et al., 2002). Experimentally induced depletion of DNA methylation in the developing embryo causes premature activation of zygotic gene transcription at least two cell cycles earlier than normal. This incorrect timing of the MBT leads to developmental arrest and cell death (Stancheva et al., 2001). Loss of DNA methylation is also deleterious to mammalian development (Jackson-Grusby et al., 2001; Lei et al., 1996).

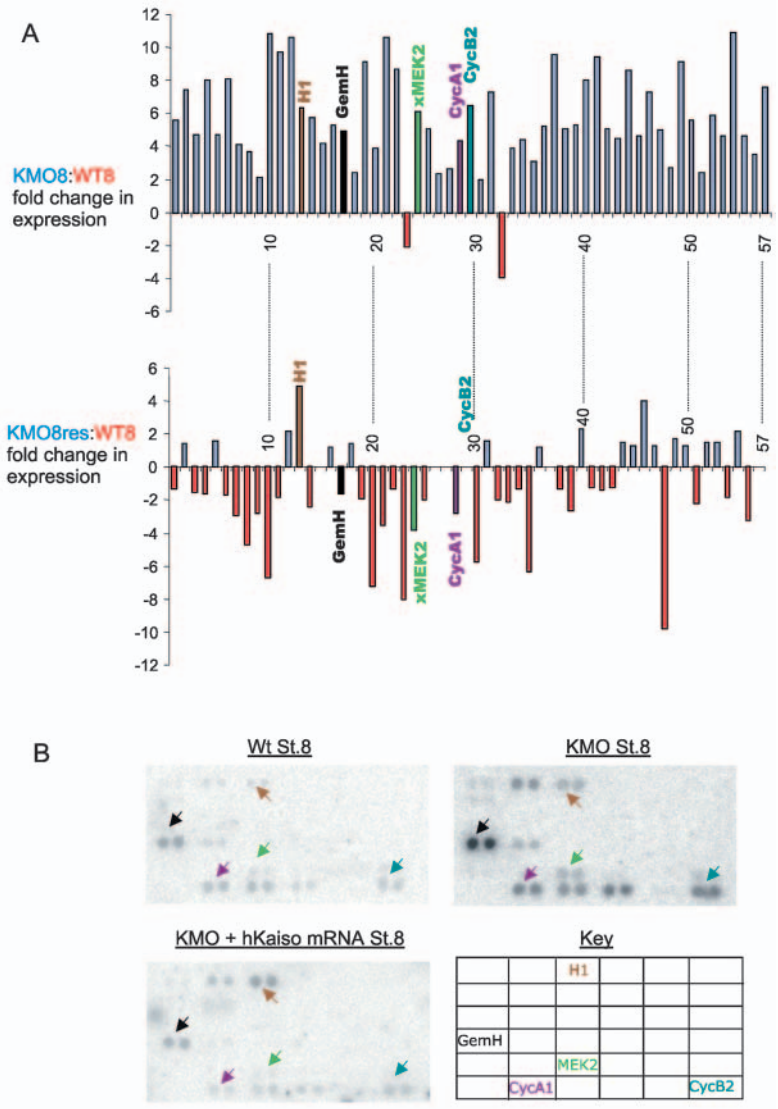

Fig. 6. Depletion of x*Kaiso* relieves transcriptional silence but can be rescued by overexpression of human kaiso mRNA. (A) Two array experiment comparisons were performed: WT8 versus KMO8 and WT8 versus KMO8^{rescue}. The *x*-axis shows 57 genes that were differentially expressed over a minimum 1.5-fold threshold, and the *y*-axis shows the fold expression changes. Genes that are elevated in KMO8 or KMO8^{rescue} relative to wild-type expression are shown in blue, and downregulated genes in red. (B) A representative array region showing the expression changes of five genes [Histone H1 (H1), Geminin H (GemH), cyclin A1 (CycA1), cyclin B2 (CycB2) and MEK1 (MEK1)], which are also indicated on the graph of genes shown in A. H1 expression was not rescued in KMO8^{rescue} embryos, whereas CycB2 is, and GemH, MEK1 and CycA1 have reduced expression levels.

The mechanism by which transcriptional silencing is maintained in early development is not clear. CpG methylation can directly inhibit transcription factor binding or affect nucleosome positioning (Davey et al., 2004; Meehan, 2003), but it is also a target for methyl-CpG binding domain (MBD)-containing proteins such as MeCP2, MBD1 and MBD2. However, the absence of these proteins in MeCP2-, MBD1- and MBD2-null mice does not have the same deleterious consequences as *Dnmt1* depletion (Chen et al., 2001; Guy et al., 2001; Hendrich et al., 2001; Li et al., 1992; Tudor et al., 2002; Zhao et al., 2003). This could be due to functional redundancy between MBD proteins. However, this does not seem to be the case for MBD2 and MeCP2 (Guy et al., 2001). Possibly, the exceptionally early MZT before the first cell division in mice is functionally different from the period of transcriptional quiescence typical for other organisms (Beaujean et al., 2004a; Beaujean et al., 2004b). A further difference exists in the DNA binding ability of MBD3:

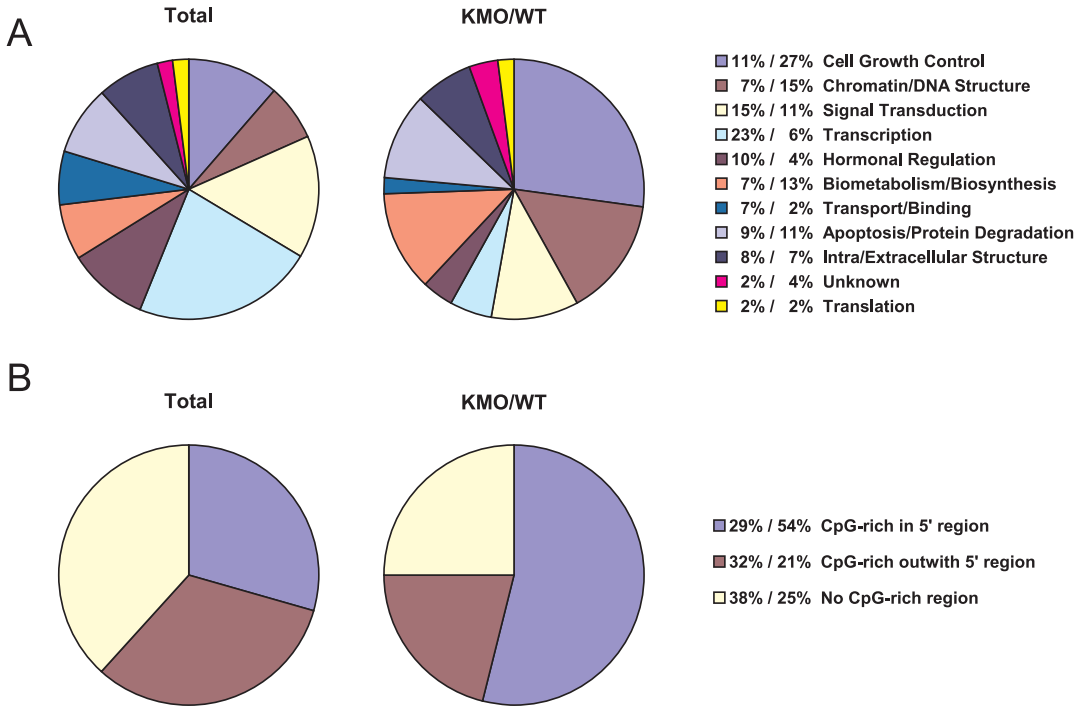

Fig. 7. xKaiso-repressed genes: functional categories. (A) The 429 input (Total) genes and 55 genes upregulated relative to WT in Kaiso-depleted embryos (KMO/WT) were categorized as described (http://www.viagenx.ca/) and their distributions shown in separate pie charts. The numbers refer to the percentages of genes in each functional category in the left/right chart respectively. (B) CpG island analysis of the input (Total) and KMO/WT upregulated genes is shown in separate pie charts. Colour coding for the different groupings and their percentages of the total in each chart are shown on the right.

Xenopus contains an MBD3 isoform that, unlike its mammalian counterpart, binds to methylated DNA (Wade et al., 1999). This is due to a tyrosine to phenylalanine substitution at position 29 of the MBD that is crucial for contacts with the phosphate backbone (Saito and Ishikawa, 2002). Alternatively, additional methyl-CpG activities could exist that mediate global silencing of methylated genes in animals.

In this work, we identify *xKaiso* as the likely candidate for mediating CpG methylation-dependent gene repression in the developing embryo. Kaiso is a transcription repressor with a distinct methyl-CpG binding ability contained in its zinc-finger domain. We demonstrate that despite considerable amino acid divergence in the third zinc-finger motif, *xKaiso* binds methylated DNA in vitro with a similar binding specificity to its mammalian counterpart, but with less non-methylated DNA sequence specificity than observed for mouse kaiso (Daniel et al., 2002). In addition, we show that ectopic expression of *xKaiso* in *MBD2*$^{-/-}$ deficient fibroblasts restores repression of a methylated reporter construct, establishing its credentials as a CpG methylation-dependent repressor, rather than a general transcription repressor (Kim et al., 2002). In stark contrast with the minor consequences of depletion/knockouts of MBD proteins in mouse, x*Kaiso* is essential for normal *Xenopus* development. An *xKaiso* knockdown mimics *xDnmt1* depletion in every aspect studied: embryos show precocious activation of gene expression, apoptosis and developmental arrest. The specificity of the depletion phenotype and functional conservation was demonstrated by expressing human kaiso in *Xenopus* KMO embryos. This rescued a high proportion of these embryos, while enabling a majority to complete neurulation. Importantly, a mutation in the third zinc finger that abolished the ability of kaiso to bind methylated DNA could not rescue the KMO phenotype. Our results strongly suggest that the main developmental changes observed after loss of CpG methylation are normally under control of the *xKaiso* transcriptional repressor and dependent on its methyl CpG binding domain.

Our gene array data reveal that *xKaiso* may control more than 10% of genes in early development. This is consistent with an important role for *xKaiso* in these processes, its absence leading to developmental arrest. The reported interaction with p120^{ctn} is interesting with regard to the possible role of kaiso in the MBT. Apart from the onset of zygotic transcription, the MBT is also associated with the slowing of cell division and the onset of cell migration (Masui and Wang, 1998; Veenstra, 2002). The p120^{ctn} interaction links E-cadherin cell adhesion with transcription in the nucleus, and loss of this connection may be responsible for the dose-dependent effects of *xKaiso* depletion on gastrulation movements. Loss of cell adhesion is observed with *xDnmt1* as well as kaiso knockdown embryonic tissues (I.S., A.R. and R.M., unpublished). Over-expression experiments nevertheless do not support a mutual inhibition mechanism for *p120*^{ctn} versus kaiso function in vivo. Milder phenotypes result from over-expression of murine p120^{ctn} or reduction of *xp120*^{ctn} in *Xenopus* compared with kaiso depletion (Fang et al., 2004; Geis et al., 1998; Paulson et al., 1999). Conversely, an excess of kaiso does not appear to disturb essential developmental processes, as our embryo controls developed normally when injected with 750 pg of wild-type human kaiso RNA. The point mutant C552R-kaiso is able to bind p120^{ctn}, but could not rescue the depletion phenotype through a p120^{ctn}-mediated pathway.

xKaiso depletion, like *xDnmt1*, causes the premature activation of gene transcription at least two cycles prior to MBT. Our results indicate that it is unlikely that the MBT is simply brought forward; rather, gene silencing is relieved outside the normal context of the MBT. This unscheduled entry into transcription is not accompanied by the increase in transcription-related gene products normally observed at the MBT (Altmann

et al., 2001). Levels of maternal mRNA and/or cytoplasmic volume may trigger this production of transcription machinery independently (Masui and Wang, 1998; Veenstra, 2002). The premature transcription we observe could therefore be rate limiting with regards to these components.

However, it is clear that *xKaiso* normally represses an abundance of genes involved in cell growth and control. Only a small set of this class of genes is normally activated at MBT (Altmann et al., 2001). This specialisation of a methylation-dependent transcription repressor is perhaps not surprising in view of the role of DNA methylation in cancer. It is also consistent with the observation of tumours in *xDnmt1*-depleted, apoptosis-inactivated *Xenopus* embryos (Stancheva et al., 2001). We demonstrate that apoptosis is triggered by the depletion of *xKaiso,* as observed with *Dnmt1* depletion. Elevated expression in both *xKaiso*- and *xDnmt1*-deficient pre-MBT embryos suggest a common pathway of repression of a set of candidate genes previously implicated in MBT (Stancheva and Meehan, 2000). Their scheduled release from repression implies a regulatory control over the methylation-dependent repression pathway subject to developmental checkpoints by apoptosis. Receptor signalling pathways could be involved based on the recruitment of N-CoR in association with HDAC3 observed for human kaiso at the human *MTA2* gene, when abnormally methylated in HeLa cells (Yoon et al., 2003). N-CoR is expressed at all stages of *Xenopus* development and may also be required to mediate transcriptional silencing in pre-MBT embryos (Koide et al., 2001). In addition, methylation mapping data suggest that *Dnmt1* may contribute specificity by selectively maintaining CpG methylation at certain loci while total levels decrease prior to the MBT (Stancheva et al., 2002). It is also possible that xMBD3, which has been shown to be part of the NuRD repressor complex has a general role in developmental gene activation at MBT and preliminary analysis suggests that it is also essential for *Xenopus* development (D.D. and R.M., unpublished) (Iwano et al., 2004).

The point at which embryonic transcription initiates in mammals varies between species with MZT occurring at the end of the one-cell stage in mice, the four- to eight-cell stage in humans, and at the eight- to 16-cell stage in rabbits, sheep and cattle (Kanka, 2003). Amphibians, and also zebrafish (Martin et al., 1999), exemplify the situation where high initial levels of CpG methylation correlate with transcriptional quiescence in the presence of large supplies of maternal transcripts. In species where the MZT occurs after more than one cleavage, transcriptional silence is likely to be maintained because of a non-permissive chromatin state that may involve DNA methylation and methyl-CpG repressor proteins, such as *xKaiso*. Our results raise the issue of whether the global role of *xKaiso* in CpG-dependent transcriptional silence is specific to early development. The indications are that MBD1, MBD2 and MeCP2 are not required to regulate MZT; instead they are used at later stages of development (Jaenisch and Bird, 2003; Stancheva et al., 2003).

A key point is that CpG methylation in differentiated somatic cells is implicated in stable gene silencing that may involve the sequestering of genes in the heterochromatic (late replicating) compartment of the nucleus. This is associated with the restriction of developmental potential and maintenance of differentiation. A very different situation applies to the pre-MBT embryo, where the genome is quiescent but in fact poised to start transcription at MZT to provide a pluripotent cell type. We propose that this poised repression, which is different from stable silencing, requires *xKaiso*, a transcriptional repressor (rather than silencing mediator) with connections to signalling pathways that permit the scheduled release from transcriptional quiescence at MZT.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/24/6185/DC1

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