Development 136, 729-738 (2009) doi:10.1242/dev.025569

The non-methylated DNA-binding function of Kaiso is not required in early *Xenopus laevis* development

Alexey Ruzov[1,*], Ekaterina Savitskaya[2,*], Jamie A. Hackett[1], James P. Reddington[1], Anna Prokhortchouk[2], Monika J. Madej[1], Nikolai Chekanov[2], Minghui Li[3], Donncha S. Dunican[1], Egor Prokhortchouk[2], Sari Pennings[4] and Richard R. Meehan[1,†]

Mammalian forms of the transcription repressor, Kaiso, can reportedly bind methylated DNA and non-methylated CTGCNA motifs. Here we compare the DNA-binding properties of Kaiso from frog, fish and chicken and demonstrate that only the methyl-CpG-binding function of Kaiso is evolutionarily conserved. We present several independent experimental lines of evidence that the phenotypic abnormalities associated with xKaiso-depleted *Xenopus laevis* embryos are independent of the putative CTGCNA-dependent DNA-binding function of xKaiso. Our analysis suggests that xKaiso does not play a role in the regulation of either xWnt11 or Siamois, key signalling molecules in the Wnt pathway during *X. laevis* gastrulation. The major phenotypic defects associated with xKaiso depletion are premature transcription activation before the mid-blastula transition and concomitant activation of a p53-dependent cell-death pathway.

KEY WORDS: Evolution, Kaiso, MBT, Siamois, Methyl-CpG binding, *Xenopus laevis*

INTRODUCTION

Reduction in xKaiso protein levels by morpholino (xKMO) injection into *Xenopus laevis* embryos results in developmental delay during gastrulation relative to control morpholino (CMO)-injected embryos (Kim et al., 2004; Ruzov et al., 2004). xKMO morphants subsequently die during neurulation with all the hallmarks of apoptosis (Ruzov et al., 2004). Differing interpretations exist as to the molecular basis of the mutant phenotype. Our work suggests that xKaiso regulates general gene silencing before the mid-blastula transition (MBT) through its ability to bind methylated DNA via its zinc-finger domains (ZF1-3) (Ruzov et al., 2004). In this respect, it is notable that the ectopic gene expression profile in pre-MBT xKMO embryos corresponds to a subset of genes that are prematurely activated when levels of the maintenance methyltransferase, xDnmt1, are decreased (Ruzov et al., 2004). A different study (using the same xKaiso morpholino) was restricted to the analysis of potential gastrulation defects (stages 10-12), in which the same gastrula phenotype, corresponding to developmental delay and an open blastopore, was observed (Kim et al., 2004). Here it was suggested that xKaiso could also directly repress canonical and non-canonical Wnt gene targets (Siamois, Fos, Cyclin-D1, Myc and xWnt11) based on its ability to bind non-methylated CTGCNA sites that are present in target promoters (Kim et al., 2004; Park et al., 2005). These distinct reports suggested that xKaiso has bimodal gene regulatory roles during animal development; as a participant in an embryonic general transcription repression pathway and as a regulator of canonical and non-canonical Wnt-signalling pathways during gastrulation. These data also raise questions as to the underlying molecular pathology of the observed phenotypes. Do they result from Kaiso's role as a component of the xDnmt1/DNA methylation repression pathway in pre-MBT embryos and subsequent activation of apoptosis, or are the phenotypes due to its ability to specifically regulate the expression of genes such as Siamois and xWnt11 via defined non-methylated DNA binding sequences? One way to discriminate between these potentially differing roles in *X. laevis* development is to try to rescue the mutant phenotype with a Kaiso variant that can only bind methylated DNA and not CTGCNA-binding sites.

The original DNA-binding site selection experiments with mouse Kaiso under low stringency conditions identified a non-methylated DNA-binding motif, Hmat, with a conserved 6 bp core sequence CTGCNA that was first identified in the promoter of the human matrilysin gene (Daniel et al., 2002). Previously we had noted that xKaiso was not as robust as its mammalian counterparts in binding Hmat (Ruzov et al., 2004). We therefore undertook a characterisation of the DNA-binding properties of Kaiso in three species (zebrafish, frog and chicken) to determine if their methylated and non-methylated DNA-binding functions are conserved. In this study we demonstrate that the ZF1-ZF2 region of all three Kaiso homologues is sufficient for binding methylated DNA but that the ability to bind Hmat is not conserved. Zebrafish Kaiso is unable to bind Hmat or CTGCNA sequences present in the Siamois and xWnt11 promoters. Despite its reduced DNA-binding repertoire (compared with frog and human Kaiso), co-injection of dKaiso mRNA rescues developmental defects associated with xKMO morphants. This observation suggests that the reported CTGCNA-binding function of xKaiso does not have a key role during early *Xenopus laevis* development. In agreement with this observation, we did not observe ectopic activation of Siamois or xWnt11 expression in xKMO morphants. A global analysis of Kaiso occupancy in chromatin derived from human 293 cells also did not find any evidence for enrichment of CTGCNA-containing sequences. We propose that the main role of Kaiso in early *X. laevis* development is more restricted than previously suggested and intimately linked with the maintenance of transcriptional silencing before the onset of zygotic transcription at the MBT (Park et al., 2005; Ruzov et al., 2004).

[1]Human Genetics Unit, MRC, Western General Hospital, Edinburgh EH4 2XU, UK. [2]Center 'Bioengineering', 60-let Oktyabrya 7-1, Moscow, 117312, Russian Federation. [3]Shanghai Biochip Company, No.151 Libing Road, Zhangjiang Hi-Tech Park, Pudong, Shanghai, 201203, China. [4]Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, UK.

*These authors contributed equally to this work
†Author for correspondence (e-mail: r.meehan@hgu.mrc.ac.uk)

Accepted 15 December 2008

MATERIALS AND METHODS

Plasmids, recombinant proteins and reporter assays

The coding regions of *Danio rerio* and *Gallus gallus* Kaiso were amplified from genomic DNAs and cloned into pGEM-T-easy vector (Promega). A Kozak sequence and stop codon were introduced to the dKaiso plasmid used for in vitro transcription in rescue experiments. xKaiso ZF123 (GST-*x*ZF, aa 470-609) was described previously (Ruzov et al., 2004). The same region was cloned into a modified pet25A-6xHis-tag expression vector (Allen et al., 2006). The xBTB/POZ region (aa 2-120) of xKaiso was cloned into pGEX-4T1. dKaisoZF123 (aa 371-550) and gKaisoZF123 (aa 419-611) were cloned into both pGEX-6P-1 and pet25A-6xHis-tag. Full-length xKaiso was cloned into pGEX-6P-2. xKaisoZF12 (aa 470-558) and xKaisoZF23 (aa 523-608) were cloned into pGEX-6P-1 and pGEX-6P-3, respectively. gKaisoZF12 (aa 419-558) and gKaisoZF23 (aa 524-611) were cloned into the pGEX-6P1 vector. GST-fusion proteins were expressed in Rosetta-gami cells (Novagen) and purified on GST Sepharose. 6xHis-tag fusions were expressed in BL21-Codon Plus (DE3)-RIPL (Stratagene) and affinity purified on Ni-NTA Superflow (Qiagen) according to the manufacturer's instructions. The mKaiso construct was made by cloning the full-length mKaiso cDNA into the *Eco*RI site of the LZRS vector. The HA-tagged dKaiso construct was made by cloning into the C-terminal polylinker of CMV2-FLAG together with an HA epitope. The T7-tagged dKaiso and xKaiso expression constructs were made in pCGT7 by cloning into *Xba*I-*Bam*HI sites (Cazalla et al., 2005). These were expressed in 293T cells and T7-tagged proteins affinity purified (Cazalla et al., 2005). To obtain VP16 fusions zinc-finger domains of xKaiso (aa 447-635) and the HMG domain of xTcf3 were cloned into the *Eco*RI and *Xba*I sites downstream of the VP16 activation domain in pVP16 (Clontech). The –304 to +198 bp region of the mouse Tex19 promoter was cloned into the *Mlu*I and *Bgl*II sites of pGL3-basic (Promega) using primers with restriction site overhangs. S01234 and S constructs were previously described (Brannon et al., 1997). The constructs were in vitro methylated according to standard techniques. Luciferase reporters were transfected (Lipofectamine 2000) into 293T cells and analysed according to Dunican et al. (Dunican et al., 2008).

Electrophoretic mobility shift assay (EMSA) experiments

Binding reactions were as described, using 5% PAGE in 0.5 × TBE to resolve DNA-protein complexes with S (non-methylated), Sm (methylated-Sm) probes (Prokhortchouk et al., 2001), the human matrilysin (Hmat) oligo (Daniel and Reynolds, 1999), wild-type KCS-*Siamois* oligo (Park et al., 2005), wild-type *Xenopus Wnt11* oligo (Kim et al., 2004) and TCFbs oligo derived from *Xenopus Siamois* promoter: F catcagaatcATCAAAGgacctccc, R gggaggtccTTTGATgattctgatg. Purified GST proteins, 6xHis-tag proteins, T7-tagged proteins, *Escherichia coli* extracts containing GST-fusion proteins or in vitro translated myc-xTcf3 were used in EMSAs. After gel scanning on an FLA2000, signal quantification was performed using AIDA software.

Embryos and microinjections

Xenopus embryos were obtained from in vitro fertilised eggs. 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 with 10-40 ng/cell of the xKMO or control morpholino (Gene-Tools), and/or 200-750 pg of sense capped RNA (dKaiso or myc-xKaiso mRNA) synthesized in vitro (T3/T7 Cap-Scribe kit, Boehringer) (Ruzov et al., 2004). Zebrafish Wik embryos, obtained from in house breeding, were maintained at 28°C as described previously (Detrich et al., 1999). Between 5 and 15 ng/embryo of dKaiso morpholino (ATATCAGCTTCAGTTTCGACATGCC) was injected according to Nasevicius and Ekker (Nasevicius and Ekker, 2000). A second MO, TGCAGAGCGACCCGTACAAATCCAC, was also used and gave similar phenotypes. For the rescue experiment equal volumes of dKMO and dKMO plus xKaiso mRNA (1 ng/nl) were injected. The phenotypes of surviving embryos (48) were scored after 24 hours. In experiments on apoptosis inhibition embryos were placed in 0.1XMMR containing 20 μM caspase-3 inhibitor Z-DEVD-FMK (R&D Systems) immediately after microinjection. xp53 morpholino was described in Cordenonsi et al. (Cordenonsi et al., 2003). All in situ hybridisations were performed according to published procedures (Hauptmann and Gerster, 1994). xWnt11 full-length cDNA was cloned into pGEMT-easy and used as a probe. xID2 probe was provided by Richard Harland (Liu and Harland, 2003).

Semi-quantitative and real-time RT-PCR

Semi-quantitative RT-PCR was performed as reported before (Ruzov et al., 2004) using published primers for *xWnt11* (Kim et al., 2004), *Siamois* (Park et al., 2005) and H4 (Ruzov et al., 2004). Quantitative real-time RT-PCR of *xWnt11*, *Siamois*, *Caspase7* and *Caspase9* was evaluated as follows (Houston et al., 2002). Primer sequences for Caspase7 and Caspase9 are available upon request. Total RNA was extracted with TriReagent (Sigma). Samples were reverse transcribed using random primers (Promega) and Superscript II RT (Invitrogen). Products were detected using SYBR Green PCR Mastermix (Applied Biosystems) and a PTC-200 cycler with a Chromo-4 detection system (MJ Research). Data were normalised relative to both GAPDH and H4 RNA, with comparable results. Error is expressed as s.e.m.

Chromatin immunoprecipitation (ChIP) assay and bisulfite sequencing

The chromatin immunoprecipitation (ChIP) assay was performed in an A6 *Xenopus* cell line according to (Dunican et al., 2008) using HA-tagged xKaiso or T7 tagged dKaiso with published ChIP primers for the *Siamois* promoter region (Park et al., 2005) or the Oct91 distal promoter region (Dunican et al., 2008). Bisulfite sequencing was performed according to standard procedures (Dunican et al., 2008).

HEK cells were used for genome-wide ChIP. Anti-HA tag polyclonal antibody (Bethyl Laboratories), ZFH6 rabbit polyclonal mKaiso antibody (Prokhortchouk et al., 2001), IgG or anti-T7 tag antibody (Novagene) were used. The transfection levels were checked by western blot hybridisation. ChIP DNA was amplified using anWGA4 kit (Sigma) and sequenced using the Genome Sequencer FLX System (Roche).

Genome-wide ChIP/sequencing data analysis

The ChIP DNA sequences were analysed using Perl, Blast and GS FLX Mapper software.

Initially PCR primer sequences were excluded from the analysis. Sequences obtained in the same experiment that were 97% or more homologous to each other and had the same 5′ end (with not more than 5 bp difference) were regarded as the same sequence that had become amplified during whole-genome amplification (WGA) PCR. All the remaining sequences were mapped on to the human genome (version 36.1) with a homology threshold of at least 95% throughout the whole length of the sequence. Only the unique genomic sequences were selected for further analysis. For all selected sequences the central positions were used as reference points for mapping onto the genome. If less than three such central positions were mapped onto a 1 kb segment of the genome the corresponding sequences were excluded from further analysis. In the case where central sequences positions from four different experiments (using different antibodies) were scored onto the same 1 kb region of the genome, the corresponding sequences were also excluded from the analysis. After this filtration protocol, the numbers of sequences for mKaiso ChIP with ZFH6 antibody, mKaiso ChIP with preimmune serum, dKaiso-HA ChIP with HA antibody and mock transfection with HA antibody experiments were 27,000, 72,000, 55,000 and 28,000, respectively. The 1 kb genomic regions anchored by the central sequences after filtration were analysed for the presence of CpG-rich regions or CTGCNA sites. Raw genome-wide ChIP/sequencing data are available upon request to Egor Prokhortchouk (Prokhortchouk@biengi.ac.ru).

CpG island array design

To design the CpG island array, CpG islands were selected from the human genome sequence (version 36.1) according to the following parameters: CpG island length >250 bp, expected/observed ratio >0.6, percentage of CpGs >50. There were 46,957 CpG islands chosen in total. These CpG islands were used by NimbleGene for the synthesis of 36,7802 isotermic oligonucleotides (38-70 bp each).

Genome-wide methylation status analysis

The MBD domain of human MBD2 was cloned into *Eco*RI, *Sal*I sites of pGEX 4T-1 vector to make MBD2 GST fusion construct. To prepare MBD2B-GST sepharose the MBD2B-GST fusion was purified on glutathione sepharose 4b (Amersham Biosciences, Piscataway, NJ) without elution. Fifty microlitres of sepharose saturated with GST-tagged MBD2B were incubated with 200 μl binding buffer (25 mM Hepes KOH, pH 7.5, 300 mM KCl, 12.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT). ChIP HEK293 genomic DNA, 500 ng fragmented with an average size of 200-300 bp and ligated with adaptors (5′-GCGGTGACCCGGGAGATC-TGAATTC-3′ and 5′-GAATTCAGATC-3′), was used for binding with MBD2-GST-sepharose using a procedure adapted from Rauch and Pfeifer (Rauch and Pfeifer, 2005). Fifty nanograms of DNA was used as an input control. Briefly, DNA was incubated with MBD2 resin for 2 hours, washed three times with washing buffer (25 mM Hepes KOH, pH 7.5, 600 mM KCl, 12.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT), eluted with elution buffer (25 mM Hepes KOH, pH 7.5, 1.5 M KCl, 12.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT), purified using Qiaquick PCR purification kits (Qiagen, Valencia, CA), amplified and Cy3(Cy5) labelled. Equal amounts of MBD2B-GST bound and input labelled DNA were hybridised with our NimbleGene CpG island array for 40 hours according to the manufacturer's instructions. After hybridization, the array was processed with NimbleGene buffers and washes. The arrays were scanned on a GenePix scanner to measure Cy3 and Cy5 intensity. To identify signal peaks SignalMap software (NimbleGene) was used with a threshold 2. The CpG rich sequences obtained in the genome-wide ChIP/sequencing experiment were analysed for their correspondence (overlap) with the methylated CpG islands using a ChIP sequence/CpG island distance thresholds from 500 to 2000 bp.

RESULTS

The methyl-CpG-binding function of Kaiso is conserved

We identified full-length zebrafish (*Danio rerio*), *Fugu rubripes* and chicken (*Gallus gallus*) Kaiso homologues by database screening with human and *Xenopus* Kaiso sequences. In each case, the structural organisation was similar, with an N-terminal BTB/POZ domain and a C-terminal region containing three zinc fingers (ZF1-3). Alignment of these Kaiso protein sequences with their mammalian counterparts showed that BTB/POZ domains are very similar (53% identity, 82% similarity), as are ZF1 (85% identity, 90% similarity) and ZF2 (72% identity, 90% similarity). The ZF3 regions are much more variable, exhibiting 38% identity and 61% similarity overall (Fig. 1A). By contrast, the intervening region between the BTB and ZF domains exhibits low levels of overall similarity. Phylogenetic analysis reflected the evolutionary relationship between vertebrate species and suggested that fish Kaiso proteins form a group that is most distantly related from their tetrapod counterparts (see Fig. S1A,B in the supplementary material).

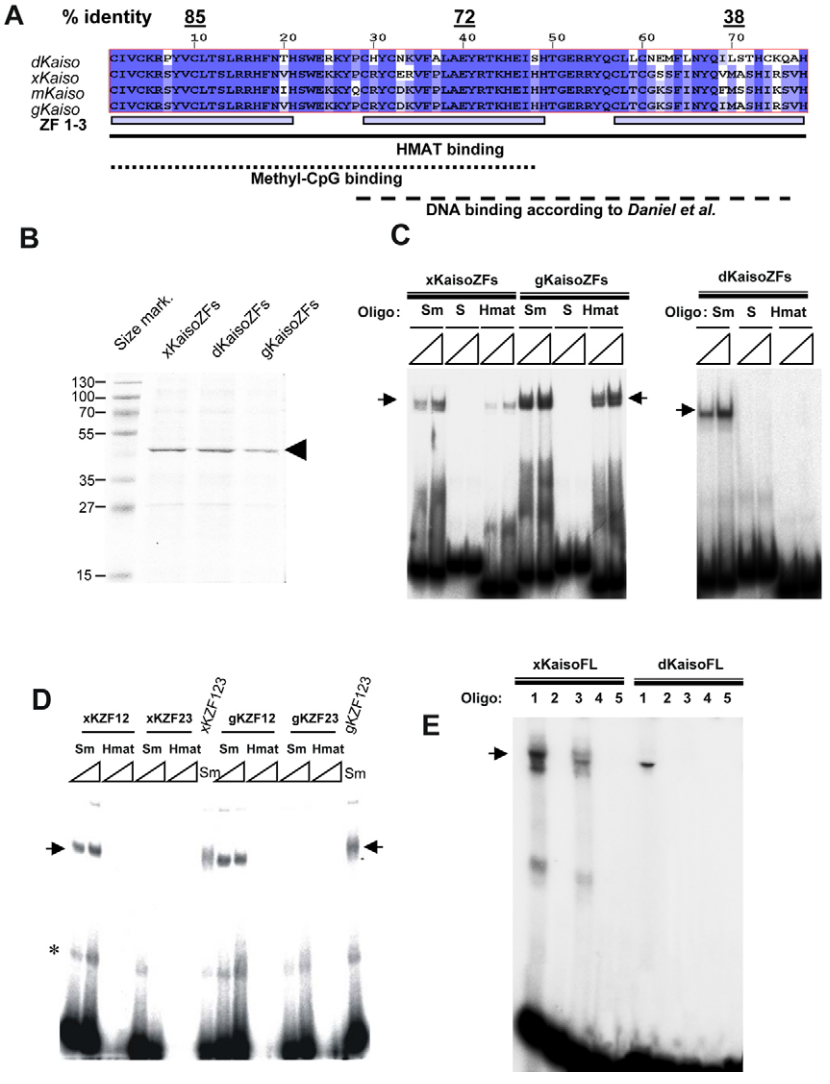

Fig. 1. The methyl-CpG binding activity of Kaiso is confined to ZF1 and 2. (**A**) Alignment of the zinc-finger domains of *Gallus*, *Xenopus*, mouse and *Danio* Kaiso proteins. The regions required for Hmat-dependent binding (solid line) and methyl-CpG-specific binding (dotted line) according to our results are compared with the previously published DNA-binding motif (dashed line) (Daniel et al., 2002). (**B**) SDS-PAGE showing the indicated purified 6xHis-tag ZF1-3 fusion proteins (arrow) used in the EMSAs. Size markers are on the left. (**C**) EMSA experiment with the indicated purified ZF1-3 proteins of xKaiso, dKaiso and gKaiso with methylated Sm, non-methylated S or human matrilysin (Hmat) probes in the presence of 2 μg pdIC competitor. Arrow indicates the Kaiso ZF-specific band shift. (**D**) EMSA using GST-ZF12 (xKZF12, gKZF12) and GST-ZF23 (xKZF23, gKZF23) deletion constructs from xKaiso and gKaiso, respectively, with Sm and Hmat oligos. xKaiso and gKaiso ZF domains (KZF123) were used with Sm probe as positive controls. (**E**) EMSA using eukaryotically expressed and affinity-purified full-length *Xenopus* (xKaisoFL) and *Danio* (dKaisoFL) Kaiso proteins with Sm (1), S (2), Hmat (3) and CTGCNA-containing probes from the promoter regions of Siamois (4) and xWnt11 (5).

The sequence variability in ZF3 is intriguing, as the ZF2-3 modules were reported to be necessary and sufficient for DNA binding (Daniel et al., 2002). We therefore determined the DNA-binding specificity of recombinant Kaiso-ZF1-3 modules in band shift assays. All the Kaiso proteins tested (*Xenopus*, zebrafish and chicken) were able to specifically bind the Sm oligo (3 MeCpGs) but not the S oligo, its non-methylated counterpart (Fig. 1C). By contrast, gKaiso had a similar affinity for the non-methylated Hmat sequence as for Sm, whereas xKaiso had a lower affinity for the Hmat oligo. Remarkably, dKaiso had no detectable binding to Hmat. This is probably due to sequence differences in ZF3. The same results were obtained using GST-ZF1-3 fusions (see Fig. S2A in the supplementary material). For a summary of the binding results see Fig. S2B in the supplementary material. It is notable that ZF3 in Kaiso proteins that can bind Hmat contains a core SHIR/KS sequence. This is replaced by THCKQ or THCKS in zebrafish and fugu, respectively (Fig. 1A), which may account for the lack of Hmat binding by dKaiso.

ZF1-2 is sufficient for binding to methylated DNA

Our comparative analysis demonstrates that the methyl-CpG-binding function is highly conserved in the three species tested, despite obvious sequence differences in ZF3. We investigated the ZF requirement for DNA binding using recombinant GST fusion proteins. We found that ZF1-2 from *Xenopus* and chicken is sufficient for binding to the Sm oligo (Fig. 1D). By contrast, an intact *Xenopus* and chicken ZF1-3 module is required to bind Hmat (Fig. 1C,D). This suggests that there is a correlation between the evolutionary conservation of ZF1-2 and the ability to bind methyl-CpGs. Full-length *Xenopus* and *Danio* Kaiso expressed and purified from 293 cells had the same DNA-binding specificity as their ZF1-3 counterparts (Fig. 1E); xKaiso could bind Sm (lane 1) but had a reduced affinity for Hmat (lane 3), whereas dKaiso could bind only Sm (lane 6). Our observations contrast with published work suggesting that ZF2-3 in mKaiso is sufficient for Hmat and Me-Sm binding (Daniel et al., 2002). The mKaiso analysis was based on EMSAs done under reduced stringency conditions with low amounts of competitor DNA and high amounts of fusion protein (Daniel et al., 2002). This may account for our differing conclusions; in our experiments, such EMSA conditions led to non-specific DNA binding (see Fig. 3E).

dKaiso is a methylation-specific repressor and is essential for zebrafish development

Apart from expression in *X. laevis* being limited to the animal pole, Kaiso mRNAs do not exhibit any regionally restricted expression pattern that would suggest Kaiso has a specific role in dorsoanterior specification (see Fig. S3A-C in the supplementary material). Given the unique DNA-binding properties of dKaiso, we tested whether it can repress transcription in a methyl-CpG dependent manner. We used a murine *Kaiso/Mecp2/Mbd2*$^{-/-}$ recipient cell line, which is defective in its ability to inhibit expression from methylated reporter plasmids (Filion et al., 2006). In these cells the methylated reporter plasmid was de-repressed to approximately 23% of a control non-methylated plasmid. Co-transfection of *Xenopus*, zebrafish or human Kaiso (*hKaiso*) expression plasmids resulted in enhanced repression of the methylated reporter, but not from the non-methylated control (Fig. 2A). Thus, dKaiso is a methyl-CpG repressor protein like its amphibian and mammalian counterparts. Since dKaiso cannot bind the non-methylated Hmat sequence, we determined whether it was essential for early zebrafish development using used a fluorescein-labelled morpholino (dKMO), which has the same specificity of action as its unlabelled counterpart. Embryos microinjected with dKMO at the 1- to 4-cell stage (Nasevicius and Ekker, 2000) were scored for survival and morphology after 24 and 48 hours of development (Fig. 2B,C). The dKMO morphants had significantly higher rates of embryo mortality at 48 hours: 89% compared with 17% for a non-inhibitory control morpholino, including any associated microinjection damage. Surviving dKMO morphants exhibited gross phenotypic defects, including microcephaly, that were coincident with the presence and dose of the morpholino. All the surviving control embryos (83%) went on to develop normally compared with only 2.5% of the dKMO morphants. Most of the remaining dKMO survivors at 48 hours

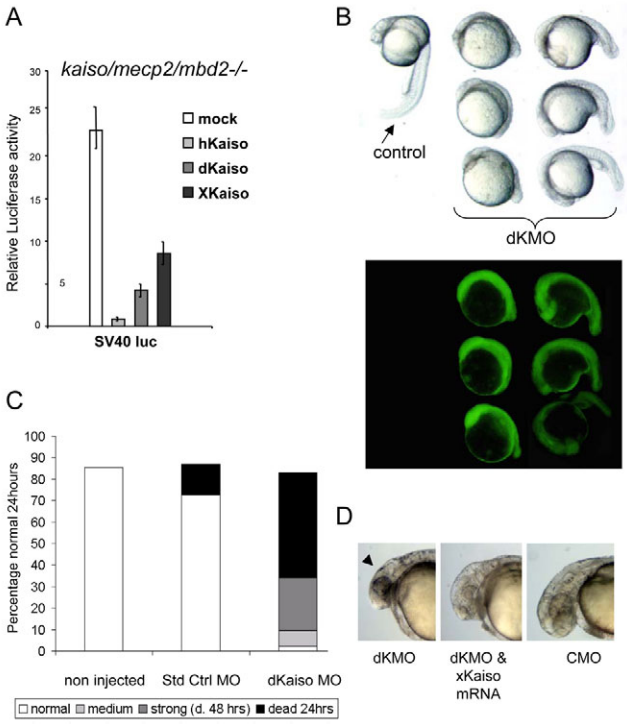

Fig. 2. *Danio rerio* Kaiso is a methyl-CpG-dependent repressor that is necessary for zebrafish development. (**A**) Methyl-CpG-dependent repression by dKaiso in a transient transfection assay. Kaiso expression constructs were co-transfected with a methylated SV40-luciferase reporter into mouse cells that are compromised in methyl-CpG-dependent transcriptional repression (*Kaiso/Mecp2/Mbd2*$^{-/-}$). The methylated SV40-luciferase reporter is repressed in the presence of dKaiso. The relative percentage (methylated reporter expression/nonmethylated reporter expression) is the average of at least three experiments. Human Kaiso (hKaiso) and xKaiso expression constructs were used as positive controls for methyl-CpG-dependent transcriptional repression. (**B**) The phenoptypes of KMO-injected zebrafish embryos compared to control embryos 24 hours after fertilisation. Lower panel is an FITC image of the upper panel and shows that the severity of phenotypical defects correlates with the amount of injected fluorescein-labelled morpholino. (**C**) The percentages of normal embryos, embryos dead at 24 hours of development and embryos with strong (non-viable) and medium developmental abnormalities at 48 hours after fertilisation are shown for non-injected (*n*=259), standard control MO (Std Ctrl MO, *n*=62) and dKMO (*n*=182) embryos. (**D**) The microcephaly phenotype of dKMO morphants at 24 hours post-fertilisation (left panel) can be rescued by xKaiso mRNA (middle panel). A control (CMO-injected) embryo (right panel) at the same stage is shown for comparison.

(8.5%) exhibited developmental delay, axial defects and incomplete head formation. Although 2.5% were normal in appearance, these embryos displayed abnormal neural responses compared with controls (not shown) and represented a low-dose phenotype. These features are similar to the phenotypes associated with xKaiso or xDnmt1 depletion in *Xenopus* embryos and dDnmt1 depletion in zebrafish (Rai et al., 2006; Ruzov et al., 2004; Stancheva and Meehan, 2000). The range of dose-dependent phenotypes could be ameliorated by co-injection of xKaiso mRNA (Fig. 2D; see Fig. S4A,B in the supplementary material) and we observed similar phenotypes with a second non-overlapping dKaiso morpholino (not shown). We conclude that dKaiso is essential for zebrafish development.

Neither dKaiso or xKaiso can bind to CTGCNA sequences in the Siamois or xWnt11 promoters

Although it was unable to bind Hmat, it was possible that dKaiso could bind the CTGCNA-containing sequences associated with the Siamois and xWnt11 promoters (Park et al., 2005), and this feature underlies its essential function in zebrafish development. However, we could only detect binding by ZF1-3 (*Xenopus*, zebrafish and chicken) recombinant proteins to the Sm sequence and not the Siamois- and xWnt11-derived sequences (Fig. 3A,B; see Fig. S2C,D in the supplementary material), irrespective of using poly dI-dC or *E. coli* DNA as a non-specific competitor (data not shown). No binding to the promoter CTGCNA sequences was observed even with either a full-length GST-xKaiso fusion (see Fig. S2D in the supplementary material) or T7-tagged xKaiso or dKaiso (Fig. 1E). In competition experiments, unlabelled Sm oligo could efficiently compete with binding to itself, whereas Hmat was less efficient as a competitor of Sm binding to xZF1-3 (Fig. 3C,D). The competition experiments suggest that Hmat is 10-fold less efficient in binding xKaiso compared with Sm, whereas a 200-fold excess of either the Siamois- or xWnt11-derived sequences did not interfere with Sm binding (Fig. 3D). The specificity of the Kaiso/DNA complex is demonstrated by a supershift with an anti-His tag antibody (Fig. 3C). As a further test of stringency, we used a fixed amount of purified xZF1-3 protein in the presence of decreasing amounts of competitor DNA (2-10 ng) with labelled Hmat, Siamois, xWnt11 probes or a control duplex oligo with xTcf3- (non-CTGCNA) binding site. As expected, lowering the amount of competitor DNA led to increased formation of the xZF1-3/Hmat complex (Fig. 3E). However, we did not observe an interaction between Siamois- or xWnt11-derived sequences and xZF1-3 until the competitor was reduced to 10 ng; under these same conditions the unrelated xTcf3 target oligo also bound (Fig. 3E). On this basis we conclude that the Siamois- or xWnt11-derived sequences do not represent specific xKaiso-binding sites in vitro.

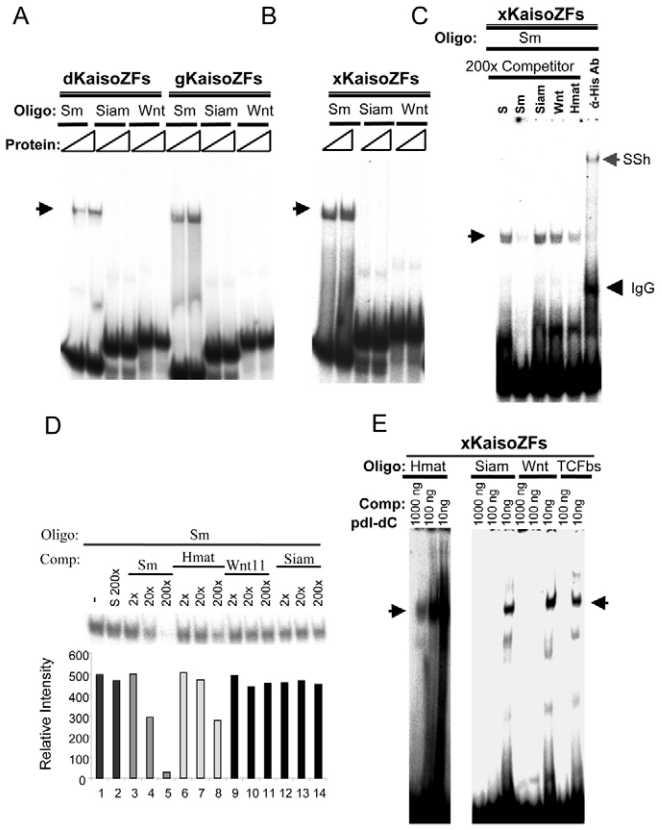

Fig. 3. xKaiso has no specific affinity for CTGCNA-binding sites in the *Siamois* and *xWnt 11* promoters. (**A**) EMSA experiment with the indicated purified 6xHis-ZF1-3 proteins dKaiso and gKaiso with methylated Sm probe and CTGCNA-containing probes derived from the promoter regions of Siamois (Siam) and xWnt11 (Wnt) in the presence of 2 μg pdIC competitor (**B**) Same experiment as in A but with xKaiso. (**C**) Competition experiment with 6xHis-ZF1-3 xKaiso protein under standard EMSA conditions with the Sm probe but with 200-fold excess of the following cold competitors: S, Sm, Siamois, xWnt11 and Hmat. Notice only the Sm and Hmat probes compete effectively. The last lane is a super-shift experiment in which an anti-His-tag antibody is included that shifts the 6xHis-Kaiso-specific complex. (**D**) Competition experiment as in C, with increasing amounts of cold competitors (2×, 20× and 200×). The signal quantification using AIDA software is shown below. Note that the Hmat oligo competes at least 10 times less efficiently than Sm. (**E**) EMSA with purified GST fusions of ZF1-3 domains of xKaiso with labelled Hmat, Siamois, xWnt11 and (non-CTGCNA) TCF3-binding site (TCFbs) probes. A fixed amount of protein and the indicated decreasing amount of pdIC competitor was used. Arrows indicate the shifted complex for each probe.

Kaiso preferentially associates with methylated CpGs rich sequences but not with CTGCNA sequences in vivo

To validate our in vitro EMSA results we performed a global analysis of Kaiso-binding sites in human HEK293 cells that were transiently transfected with either murine or *Danio* Kaiso expression plasmids. We sequenced and mapped chromatin-derived DNA fragments that were bound by these proteins and found a significant enrichment of CpG-rich sequences in both cases (Fig. 4A,C). A genome-wide analysis of DNA methylation in HEK293 cells showed that these CpG sequences bound by *Danio* and murine Kaiso are also enriched in methylated CpGs (see Fig. S5A in the supplementary material; data not shown). By contrast, our analysis did not detect an enrichment of CTGCNA sites in the chromatin fraction associated with mKaiso and dKaiso (Fig. 4B,D). These data do not exclude the possibility that mKaiso binds to a small number of CTGCNA-containing sequences in vivo but it does suggest that CTGCNA does not represent a general consensus sequence for mKaiso binding in vivo. The results are in remarkable agreement with our EMSA analysis and a ChIP experiment in which we could not detect any binding of transiently transfected dKaiso or xKaiso at the Siamois promoter in *Xenopus* A6 cells (Fig. 4F). By contrast, we could detect promoter occupancy by both dKaiso and xKaiso at the methylated distal promoter region of the Oct91 gene in the A6 cell line (Fig. 4E,F). An additional proof of the inability of xKaiso to

bind the Siamois promoter in vivo comes from luciferase reporter assays in which the xKaiso zinc fingers are fused to the VP16 activator domain (xKaisoZFVP16). xKaisoZFVP16 cannot activate transcription from a Siamois reporter (Fig. 4G, upper) but can activate transcription from a methylated Tex19 reporter 2.7 times (Fig. 4G, lower). It is important to note that a VP16 fusion with the xTcf3 DNA-binding HMG domain activated the Siamois-driven luciferase reporter 5-fold in the same set of experiments (Fig. 4G). These experiments support the view that xKaiso has a preference towards methylated DNA and not for the CTGCNA sequence present in the Siamois promoter.

dKaiso can rescue Kaiso-depleted *Xenopus laevis* embryos

The unique DNA-binding and transcriptional repressor specificity of dKaiso provides us with a perfect tool to test if the ability to bind methylated DNA is sufficient to rescue xKMO morphants. Although we previously found that 10 ng morpholino is sufficient to elicit a phenotype, we injected a high dose (40 ng), as used by Kim and colleagues, into *Xenopus* two-cell-stage embryos (Kim et al., 2004; Ruzov et al., 2004). In xKMO morphants the dorsal lip was less prominent at stage 10 and by stage 12 there was an extended open blastopore (see also Fig. 7A). This resulted in gastrulation delay followed by the characteristic appearance of white apoptopic cells near the edge of the open blastopore by stage 15 (Fig. 5A, xKMO and Fig. 7A). This gastrulation phenotype is identical to that reported by Kim and colleagues (however, they did not report on later stage phenotypes) with the same morpholino sequence and dose (Kim et al., 2004) and can be rescued by co-injection of either xKaiso, dKaiso or hKaiso mRNAs (Fig. 5B) (A.R. and R.R.M., unpublished). We found at stage 14 (neurulation) that a high rate of mortality (80%) occurred in the xKMO morphants compared with controls (Fig. 5B), and the embryos were developmentally arrested with, as noted before, a phenotype resembling that of xDnmt1-depleted embryos (Ruzov et al., 2004; Stancheva and Meehan, 2000). Embryos injected at the two-cell stage with dKaiso RNA and xKMO increased the proportion of normally gastrulating embryos (stage 12), from just over 10% to more than 45%. By stage 14 (neurulation) the mortality rate was reduced to 18% compared with more than 80% for xKMO-only injected embryos (Fig. 5A,B, xKMO+dKaiso). The presence of dKaiso mRNA enabled nearly half of the morphants (46%) to successfully complete gastrulation/neurulation compared with none for the xKMO alone (Fig. 5B). By tadpole stage, the rate of survival for dKaiso/xKMO-injected embryos was reduced, but it is notable that a few phenotypically normal embryos could develop even though the morpholino was still present (Fig. 5A,B). These rescued embryos differed in appearance from the few arrested xKMO morphants that survived to late stages (Fig. 5A). Importantly, the rescuing capacity of dKaiso mRNA was similar to that observed with wild-type (but not a DNA-binding mutant) hKaiso mRNA, which binds the Sm and Hmat sequences (Fig. 5B) (Ruzov et al., 2004). These experiments strongly suggest that the capacity to bind CTGCNA-containing sequences is not obligatory for rescuing xKMO-injected embryos; instead, the ability to bind methylated DNA correlates with the capacity to rescue early developmental deficits. This observation is in line with the proposed role of Kaiso as a transcriptional silencer in pre-MBT embryos (Ruzov et al., 2004). The ability of dKaiso to rescue the xKMO morphants is incompatible with a model in which a CTGCNA-binding activity is necessary to target the repression function of Kaiso to Wnt-signalling target genes (Kim et al., 2004; Park et al., 2005).

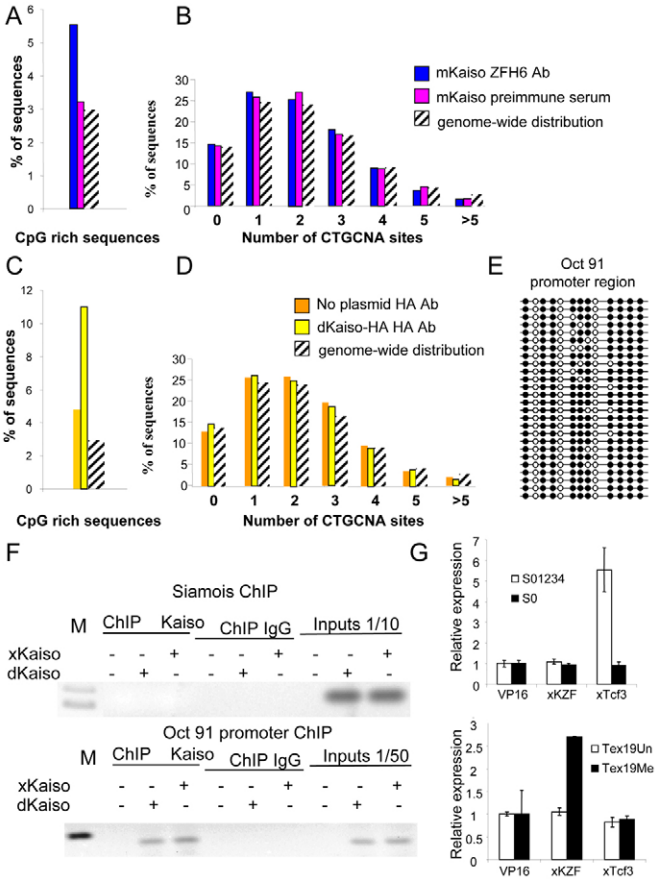

Fig. 4. Kaiso preferentially interacts with methylated CpGs but not with CTGCNA sequences in vivo. (**A-D**) The results of genome-wide ChIP/sequencing experiments in HEK293 transiently transfected with mouse Kaiso (mKaiso) and HA-tagged *Danio* Kaiso (dKaiso-HA). The ChIPs were performed using anti-mKaisoZF or anti-HA-tag antibodies with additional controls using preimmune serum (control for mKaiso ChIP) or anti-HA-tag antibody on non-transfected cells (control for dKaiso-HA experiment). The DNA obtained in the ChIP was amplified and 454 sequenced. After initial data filtering all the remaining sequences were mapped on to the human genome, susbsequently 1 kb regions in the vicinity of the ChIP sequences were analysed for the presence of either CpG-rich regions or CTGCNA sites. The ChIP sequences for both the mKaiso and dKaiso-HA experiments were enriched in CpG-rich regions in comparison to either to preimmune serum or anti-HA-tag antibody on non-transfected cells, respectively (A,C), but not in CTGCNA sites (B,D). The data were normalised with respect to the genome-wide distribution of CpG rich regions and CTGCNA sites as shown. (**E**) Diagram indicating the DNA methylation status of the Oct 91 distal promoter fragment used for ChIP in (F) in A6 cells. Filled circles represent methylated, and empty circles non-methylated, CpGs. (**F**) ChIP experiment using transiently transfected *Xenopus* HA-tagged Kaiso (xKaiso) and T7 tagged *Danio* Kaiso (dKaiso) on A6 cells. Both xKaiso and dKaiso bind to the heavily methylated distal region of the Oct91 gene, but do not show any detectable binding to the Siamois promoter under the same experimental conditions. IgG was used as an antibody control. 1/10 and 1/50 of inputs are loaded for the Siamois and Oct91 experiments, respectively. (**G**) The xKaiso ZF domain VP16 fusion (xKZF) does not activate transcription of a Siamois-driven luciferase reporter (S01234) but does activate transcription from a methylated Tex19 promoter (Tex19Me). The xTcf3 HMG domain fusion (xTcf3) activates transcription from the Siamois reporter 5.5 times. A Siamois luciferase reporter containing mutated xTcf3-binding sites (S0) and an unmethylated Tex19 promoter reporter (Tex19Un) were used as controls.

Inhibition of apoptosis in KMO embryos results in their successful gastrulation

xKMO morphants exhibit a developmental delay in closing the blastopore relative to control morpholino injected or wild-type embryos and subsequently die during neurulation with all the hallmarks of apoptosis (Fig. 7A) (Ruzov et al., 2004). We reasoned that inhibitors of the apoptotic pathway would reduce the high rates of lethality associated with the high-dose xKMO morphants. This would allow us to determine whether the resulting embryos exhibit a phenotype that would be indicative of ectopic Wnt signalling function. Incubation of wild-type embryos with the caspase-3 inhibitor, Z-DEVD-FMK, did not interfere with normal development (Fig. 6A). In high-dose xKMO morphants, the presence of Z-DEVD-FMK inhibited apoptosis and allowed up to one-third of embryos to progress through to the equivalent of tadpole stage (St. 38) (Fig. 6A; see Fig. S6 in the supplementary material). However, even though these embryos still underwent developmental delay, they gastrulated successfully but exhibited a short axis phenotype at later stages with obvious eye defects (Fig. 6A). We also suppressed the apoptotic effect of xKaiso depletion by directly inhibiting xp53 function by co-injecting a well-characterised xp53 morpholino (xp53MO) (Cordenonsi et al., 2003). The double xKMO/xp53MO morphants also underwent developmental delay but eventually underwent blastopore closure (Fig. 6B,C). The survival rate of these double morphants was high (90%) but none developed normally (Fig. 6C; see Fig. S6 in the supplementary material). These morphants did not exhibit an axis duplication phenotype that might be indicative of hyperactivation of downstream Wnt-signalling target genes (Tao et al., 2005). More importantly, these experiments imply that much of the phenotypic defects associated with xKaiso depletion are associated with a general failure to complete proper gastrulation/neurulation along with a concomitant activation of a p53-mediated cell-death pathway.

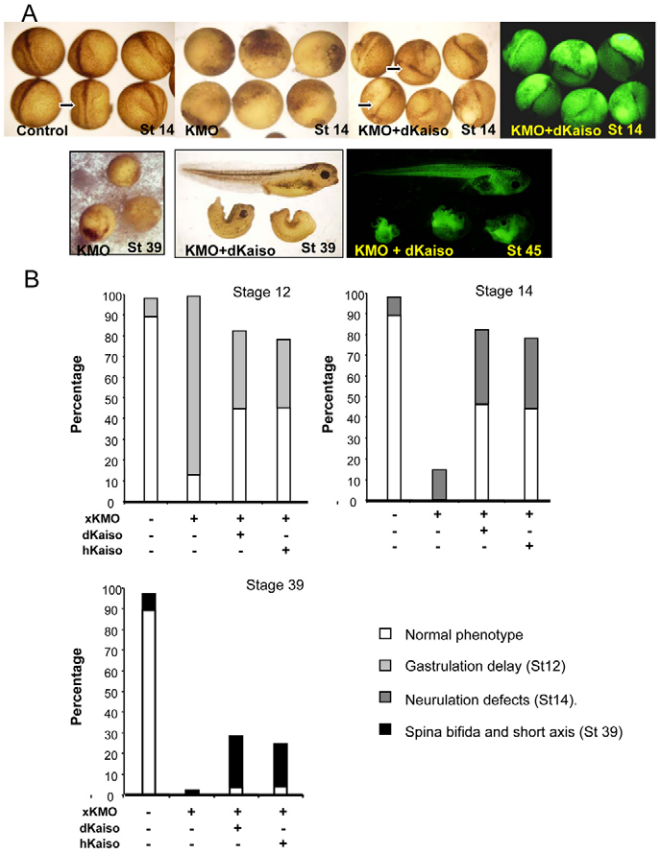

Fig. 5. dKaiso can rescue Kaiso-depleted *Xenopus laevis* embryos to the same extent as its human counterpart. (**A**) The phenotypes of uninjected control (*n*=150), KMO (*n*=53) and xKMO co-injected with dKaiso RNA (*n*=84) embryos (KMO+dKaiso). Development stages are indicated. FITC image of two pictures are presented as well as an injection control; arrow indicates neural fold. Notice that even at the later stage (St. 39), when there are reduced numbers of survivors, the xKMO morphants are arrested whereas the rescued embryos can form complete tadpoles or attenuated tadpoles that differ in appearance from the xKMO morphant. (**B**) Bar graphs showing the percentages of normal embryos and embryos with developmental defects in the rescue experiments using xKMO co-injected with dKaiso or human Kaiso (hKaiso) RNA. Dead embryos are not included. The stages of development are indicated.

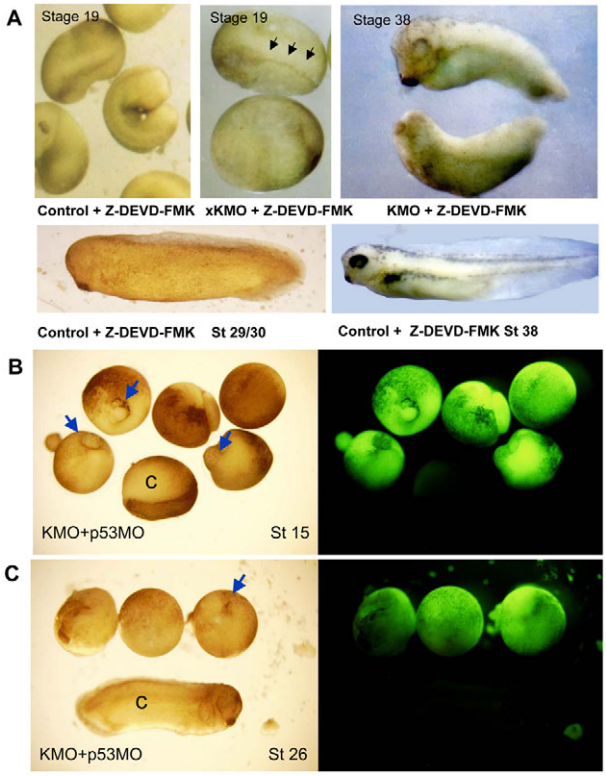

Fig. 6. Inhibition of apoptosis in KMO embryos results in their successful gastrulation. (**A**) The presence of a caspase inhibitor, Z-DEVD-FMK, prevents apoptosis in xKMO morphants and allows development to proceed. The rescued embryos can complete gastrulation but neurulation is impaired and they exhibit development delay compared to control embryos. Arrows indicate a poorly developed neural fold in the rescued xKMO morphant at stage 19. The rescued xKMO morphants do not show evidence of axis duplication (indicating no hyper β-catenin activation during gastrulation) at stage 38. In addition they exhibit developmental delay and axis defects that result from poor neurulation. Control embryos incubated with Z-DEVD-FMK are phenotypically normal. (**B**,**C**) Phenotypes of embryos co-injected with xKMO together with an xp53 morpholino (p53MO) are presented at stages 15 and 26. Uninjected control embryos are also shown (C in figures). Note the completion of delayed gastrulation in KMO/p53MO embryos (blue arrow points to the blastopores).

xWnt11 and Siamois are not mis-expressed in xKMO morphants

As result of our phenotypic analysis and the inability of xZF1-3 to bind the Siamois and xWnt11 probes, we decided to verify if these genes were aberrantly expressed in xKMO morphants, as reported previously (Kim et al., 2004; Park et al., 2005). Although xKMO embryos are delayed in gastrulation compared to wild-type embryos (Fig. 7A), we did not observe upregulation of Siamois and xWnt11 levels in xKMO morphants by semi-quantitative RT-PCR (Fig. 7B). Real-time quantitative RT-PCR suggests that Siamois levels are actually reduced by 30% in xKMO stage 10 morphants, which may reflect the developmental lag (Fig. 7A,C). By contrast, there were no significant differences in xWnt11 levels at the equivalent of stage 12 (Fig. 7C). In the same sets of embryos Caspase-7 and Caspase-9 RNA levels were increased 2.5-3.5 times in stage 10 and 12 xKMO embryos, respectively, compared with controls, which is in line with activation of a programmed cell-death pathway (Fig. 7C). We also used whole-mount RNA in situ analysis to compare the expression of xWnt11 in wild-type and xKMO pre-MBT embryos. Unlike previous reports (Kim et al., 2004), we could not find differences in expression: the maternal xWnt11 transcript was present at similar levels in both types of embryo (Fig. 7D; see Fig. S7A in the supplementary material). A control in situ with xID2, identified in a screen for genes that are mis-expressed in KMO embryos (Ruzov et al., 2004), confirms that it is upregulated in xKMO morphants (Fig. 7D; see Fig. S7B in the supplementary material). XWnt11 expression can be induced by the mesoderm-specific transcriptional activator Xbra (Tada and Smith, 2000), but we have not observed ectopic induction of Xbra in xKMO embryos, suggesting that xKaiso depletion does not result in the direct or indirect activation of xWnt11 (D.S.D., unpublished). We conclude that it is unlikely that xKaiso strongly influences the expression of xWnt11 and Siamois either before the MBT or during subsequent gastrulation.

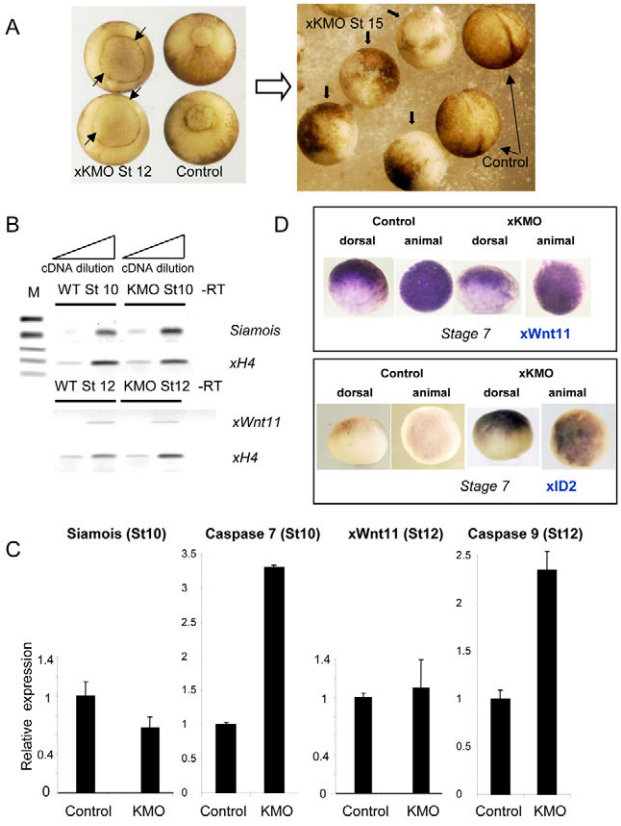

Fig. 7. The Wnt signalling pathway is not activated in xKMO morphants. (**A**) The xKMO morphants exhibit a delay in closing the blastopore (short arrows) compared with wild-type embryos. This phenotype appears in 85-90% of the embryos and is identical to that presented by McCrea and colleagues (Kim et al., 2004; Park et al., 2005). By stage 15 (neurulation), the xKMO morphants (downward arrow) cannot form a neural fold; they are apoptotic and are shedding cells through the open blastopore. Control embryos are shown with a proper neural fold (long arrows). (**B**,**C**) Neither *Siamois* nor *xWnt11* are ectopically activated in xKMO morphants when assayed by semi-quantitative RT-PCR according to Park et al. (Park et al., 2005) or real-time PCR relative to a histone H4 control at stage 10 (Siamois) or 12 (xWnt11). Caspase7 and Caspase9 expression is activated in 2.5-3.5 times compared to control in the same sets of KMO embryos at stages 10 and 12, respectively. (**D**) Whole-mount RNA in situ analysis demonstrates that xWnt11 is not prematurely activated in pre-MBT xKMO morphants in comparison to a control transcript, xID2, that is activated prematurely (Ruzov et al., 2004).

DISCUSSION

We find that the methyl-CpG-binding function of the Kaiso transcription repressor is highly conserved in three species; by contrast, the non-methylated Hmat-binding affinity varied from zero activity and weak binding for dKaiso and xKaiso, respectively, to levels comparable with methyl-CpG binding for gKaiso. In addition, our localisation of the Kaiso methyl-CpG-binding domain to ZF1-2 conflicts with previously published work that defined ZF2-3 as sufficient for both methyl-CpG-dependent and CTGCNA-based binding (Daniel et al., 2002). This previous observation may be attributable to differences in the stringency of the DNA-binding assays employed. However, the evolutionary conservation of the first two Kaiso ZF domains is in step with the experimental outcome of our DNA-binding analysis, which indicated that the methyl-CpG binding function resides in ZF1-2. Two other Kaiso-like proteins (ZBTB4 and ZBTB38) in mice also have a strong methyl-CpG-binding function, but only ZBTB4 has a weak affinity for the Hmat sequence, whereas ZBTB38 does not (Filion et al., 2006). Based on this, we come to a broad conclusion that the primary DNA-binding activity of all members of the Kaiso-like family (Kaiso, ZBTB4 and ZBTB38) is for methyl-CpGs, which can act as ligands in chromatin for the transcription repression function associated with these proteins (Filion et al., 2006; Prokhortchouk et al., 2001; Ruzov et al., 2004). Our global analysis of Kaiso-binding sites in HEK293 cells also supports the view that they are associated with methylated CpGs and not CTGCNA sites. In addition it has recently been shown that Kaiso represses methylated tumour suppressor genes and can bind in a methylation-dependent manner to the CDKN2A gene in human colon cancer cell lines (Lopes et al., 2008).

The DNA-binding ability of Kaiso is primarily dependent on its three zinc fingers (ZF1-3), which belong to the C2H2 class. It has been proposed that each ZF can be regarded as an independent DNA-binding module, and an additional ZF in an array specifies three base pairs of adjacent, but discrete, subsites (Choo and Klug, 1997). This would predict a 9 bp binding site for Kaiso if each ZF bound equally to its respective subsite. An inference of the requirement for all three ZFs to bind Hmat is that its true recognition sequence may correspond to a 9 bp sequence and not 6 bp as previously reported (Daniel et al., 2002). The requirement for ZF1-2 for binding to the Sm oligo is consistent with a minimum recognition sequence of 6 bp that contains two methyl-CpGs (Prokhortchouk et al., 2001). Comparison of the three CTGCNA substrates used (Hmat, Siamois and xWnt11) shows that they are

flanked by distinct sequences that probably account for their differential binding to gKaiso and xKaiso. Notably, like xKaiso, gKaiso cannot bind the CTGCNA-containing sequences in the xWnt11 and Siamois promoter regions (Fig. 3) despite its high affinity for Hmat. This suggests that the non-methylated binding ability of gKaiso is restricted to one or a few unique sequences that have yet to be identified. Our analysis suggests that CTGCNA sequences in the promoters of Siamois and xWnt11 are not high-affinity Kaiso-binding sites, which would be incompatible with the proposed role of xKaiso as a direct repressor of these genes (Park et al., 2005).

We also show, that like its amphibian counterpart, dKaiso is essential for early zebrafish development. The phenotypes of dKMO-injected embryos, the percentages of abnormal embryos and the developmental stages in which these embryonic defects are detected resemble those of xKMO-injected *Xenopus* embryos (Ruzov et al., 2004). In our previous study we showed that xKaiso is required for genome-wide transcription silencing in embryos before the MBT. In zebrafish the MBT begins at cycle 10 and, as in amphibians, is characterised by cell-cycle lengthening, loss of cell synchrony, appearance of cell motility and activation of transcription (Kane and Kimmel, 1993). Noting the similarity of the *Danio rerio* and *Xenopus* Kaiso loss-of-function phenotypes, we can hypothesise that in both cases mis-regulation of similar mechanisms leads to close phenotypic abnormalities. Another conclusion from the comparison of zebrafish and *Xenopus* KMO embryos and from the dKaiso ability to rescue the xKMO phenotype, is that the main phenotypical features of xKMO-injected embryos are not dependent on CTGCNA-binding ability, which is not conserved between zebrafish and *Xenopus*. The observation that mice do not have an extended period of transcriptional silencing during early development may partly explain the absence of an embryonic lethal phenotype in Kaiso null mice (Prokhortchouk et al., 2006). These mutant mice also do not exhibit mis-regulation of candidate target genes such as Wnt11.

In parallel with the mouse study, we did not observe the reported mis-regulation of the Wnt signalling pathway in xKMO stage 8 or stage 10 morphants. Indeed, inhibition of the cell death pathway in xKMO morphants by incubation with apoptotic inhibitors does not uncover any gross axis duplication phenotypes, which is also indicative that β-catenin signalling is not ectopically activated under these conditions. A potential role for xKaiso in Wnt signalling was initially suggested by non-physiological experiments in which xKaiso and Wnt-signalling components are overexpressed. This is probably mediated by Kaiso/TCF3 interactions and not via CTGCNA-binding sites (Kim et al., 2004; Park et al., 2005; Ruzov et al., 2009). In a companion paper, we demonstrate that xKaiso interacts directly with TCF3/4 and thereby masks their HMG DNA-binding domains (Ruzov et al., 2009). As a result, overexpression of xKaiso inhibits the ability of β-catenin to mediate transcription activation through xTcf3 by displacing it from a target promoter. This mode of action can account for the inhibition of dorsal axis formation when Kaiso mRNA is co-injected with β-catenin mRNA into the ventral marginal region of four-cell-stage embryos (Park et al., 2005).

We thank Josh Brickman, Patricia Yeyati and Nick Hastie for helpful comments and corrections during manuscript preparation and Nick Hastie for general support. Work in the R.R.M. lab (R.R.M., D.S.D., A.R. and J.A.H.) is supported by the MRC. We thank Richard Harland, Vladimir Korinek, Pierre McCrea, Randall Moon and Ralph Rupp for reagents, Mike McGrew and Helen Sang for chick embryos, Nicola Grey and Bill Richardson for *Xenopus* embryos and members of the Chromosome and Gene expression group for technical advice. E.P. and A.P. were supported by Russian Academy of Sciences program grant "Molecular and Cellular Biology", RFBR 06-04-49216-a and CRDF RB-2808. M.L. was supported by CR10-A-04 and 08410708700. Deposited in PMC for release after 6 months.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/5/729/DC1

References

Allen, M. D., Grummitt, C. G., Hilcenko, C., Min, S. Y., Tonkin, L. M., Johnson, C. M., Freund, S. M., Bycroft, M. and Warren, A. J. (2006). Solution structure of the nonmethyl-CpG-binding CXXC domain of the leukaemia-associated MLL histone methyltransferase. *EMBO J.* **25**, 4503-4512.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T. and Kimelman, D. (1997). A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Genes Dev.* **11**, 2359-2370.

Cazalla, D., Sanford, J. R. and Caceres, J. F. (2005). A rapid and efficient protocol to purify biologically active recombinant proteins from mammalian cells. *Protein Expr. Purif.* **42**, 54-58.

Choo, Y. and Klug, A. (1997). Physical basis of a protein-DNA recognition code. *Curr. Opin. Struct. Biol.* **7**, 117-125.

Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C. and Piccolo, S. (2003). Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* **113**, 301-314.

Daniel, J. M. and Reynolds, A. B. (1999). The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol. Cell. Biol.* **19**, 3614-3623.

Daniel, J. M., Spring, C. M., Crawford, H. C., Reynolds, A. B. and Baig, A. (2002). The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. *Nucleic Acids Res.* **30**, 2911-2919.

Detrich, H. W., 3rd, Westerfield, M. and Zon, L. I. (1999). Overview of the Zebrafish system. *Methods Cell Biol.* **59**, 3-10.

Dunican, D. S., Ruzov, A., Hackett, J. A. and Meehan, R. R. (2008). xDnmt1 regulates transcriptional silencing in pre-MBT Xenopus embryos independently of its catalytic function. *Development* **135**, 1295-1302.

Filion, G. J., Zhenilo, S., Salozhin, S., Yamada, D., Prokhortchouk, E. and Defossez, P. A. (2006). A family of human zinc finger proteins that bind methylated DNA and repress transcription. *Mol. Cell. Biol.* **26**, 169-181.

Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. *Trends Genet.* **10**, 266.

Houston, D. W., Kofron, M., Resnik, E., Langland, R., Destree, O., Wylie, C. and Heasman, J. (2002). Repression of organizer genes in dorsal and ventral Xenopus cells mediated by maternal XTcf3. *Development* **129**, 4015-4025.

Kane, D. A. and Kimmel, C. B. (1993). The zebrafish midblastula transition. *Development* **119**, 447-456.

Kim, S. W., Park, J. I., Spring, C. M., Sater, A. K., Ji, H., Otchere, A. A., Daniel, J. M. and McCrea, P. D. (2004). Non-canonical Wnt signals are modulated by the Kaiso transcriptional repressor and p120-catenin. *Nat. Cell Biol.* **6**, 1212-1220.

Liu, K. J. and Harland, R. M. (2003). Cloning and characterization of Xenopus Id4 reveals differing roles for Id genes. *Dev. Biol.* **264**, 339-351.

Lopes, E. C., Valls, E., Figueroa, M. E., Mazur, A., Meng, F. G., Chiosis, G., Laird, P. W., Schreiber-Agus, N., Greally, J. M., Prokhortchouk, E. et al. (2008). Kaiso contributes to DNA methylation-dependent silencing of tumor suppressor genes in colon cancer cell lines. *Cancer Res.* **68**, 7258-7263.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Park, J. I., Kim, S. W., Lyons, J. P., Ji, H., Nguyen, T. T., Cho, K., Barton, M. C., Deroo, T., Vleminckx, K., Moon, R. T. et al. (2005). Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev. Cell* **8**, 843-854.

Prokhortchouk, A., Hendrich, B., Jorgensen, H., Ruzov, A., Wilm, M., Georgiev, G., Bird, A. and Prokhortchouk, E. (2001). The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. *Genes Dev.* **15**, 1613-1618.

Prokhortchouk, A., Sansom, O., Selfridge, J., Caballero, I. M., Salozhin, S., Aithozhina, D., Cerchietti, L., Meng, F. G., Augenlicht, L. H., Mariadason, J. M. et al. (2006). Kaiso-deficient mice show resistance to intestinal cancer. *Mol. Cell. Biol.* **26**, 199-208.

Rai, K., Nadauld, L. D., Chidester, S., Manos, E. J., James, S. R., Karpf, A. R., Cairns, B. R. and Jones, D. A. (2006). Zebra fish Dnmt1 and Suv39h1 regulate organ-specific terminal differentiation during development. *Mol. Cell. Biol.* **26**, 7077-7085.

Rauch, T. and Pfeifer, G. P. (2005). Methylated-CpG island recovery assay: a new technique for the rapid detection of methylated-CpG islands in cancer. *Lab. Invest.* **9**, 1172-1180.

Ruzov, A., Dunican, D. S., Prokhortchouk, A., Pennings, S., Stancheva, I., Prokhortchouk, E. and Meehan, R. R. (2004). Kaiso is a genome-wide repressor of transcription that is essential for amphibian development. *Development* **131**, 6185-6194.

Ruzov, A., Hackett, J. A., Prokhortchouk, A., Reddington, J. P., Madej, M. J., Dunican, D. S., Prokhortchouk, E., Pennings, S. and Meehan, R. R. (2009). The interaction of xKaiso with xTcf3: a revised model for integration of epigenetic and Wnt signalling pathways. *Development* **136**, 723-727.

Stancheva, I. and Meehan, R. R. (2000). Transient depletion of xDnmt1 leads to premature gene activation in Xenopus embryos. *Genes Dev.* **14**, 313-327.

Tada, M. and Smith, J. C. (2000). Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X. and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell* **120**, 857-871.