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Kaiso-Deficient Mice Show Resistance to Intestinal Cancer†

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Kaiso is a BTB domain protein that associates with the signaling molecule p120-catenin and binds to the methylated sequence mCGmCG or the nonmethylated sequence CTGCNA to modulate transcription. In Xenopus laevis, Kaiso deficiency leads to embryonic death accompanied by premature gene activation in blastulae and upregulation of the xWnt11 gene. Kaiso has also been proposed to play an essential role in mammalian synapse-specific transcription. We disrupted the Kaiso gene in mice to assess its role in mammalian development. Kaiso-null mice were viable and fertile, with no detectable abnormalities of development or gene expression. However, when crossed with tumor-susceptible ApcMin+ mice, Kaiso-null mice showed a delayed onset of intestinal tumorigenesis. Kaiso was found to be upregulated in murine intestinal tumors and is expressed in human colon cancers. Our data suggest that Kaiso plays a role in intestinal cancer and may therefore represent a potential target for therapeutic intervention.

The methyl-CpG binding proteins act as intermediates between the transcriptional machinery and methylated DNA, specifically recognizing 5-methylcytosine in the context of a CpG dinucleotide and imposing a chromatin structure that is unfavorable to transcription. Two types of methylated DNA binding motifs have been identified: methyl-CpG binding domains (MBD) and C2H2 zinc fingers. Vertebrate proteins in mammals that contain an MBD domain are MBD1 to -4 and MeCP2 (10). MBD1, MBD2, and MeCP2 interact with different corepressor complexes, but each depends for its transcriptional repression activity on a different chromatin-modifying complex (18, 29, 30, 41, 49). Kaiso, with three C-terminal zinc fingers, is a distinct member of the class that recognizes a consecutive pair of methyl-CpG sequences (33, 34) but also has binding specificity for the nonmethylated sequence CTGCNA (6). Like the MBD proteins, Kaiso can behave as a DNA methylation-dependent transcriptional repressor (6, 33) and recruit a histone deacetylase-containing corepressor complex (N-CoR) to methylated sites in the genome (55).

Kaiso was first isolated through its ability to interact with the Armadillo-repeat catenin p120 (5). The interaction was surprising as p120-catenin associates with cadherins at the cell membrane, whereas Kaiso behaves as a DNA-binding protein. This raised the possibility that the p120-catenin:Kaiso pair may functionally resemble the β-catenin:LEF/TCF system by participating in the transmission of extracellular signals from the cell membrane to the nucleus, where Kaiso could act as a regulator of target genes (1). Immunostaining experiments have shown that Kaiso can be either nuclear or cytoplasmic, its intracellular localization and levels of expression being determined by unidentified factors that respond to the cellular microenvironment (44). Support for Kaiso’s role in responding to signals from the cell surface has come from studies in Xenopus which showed that the xWnt11 gene, a target of noncanonical Wnt signaling, is regulated by Kaiso (22). Kaiso-mediated repression of xWnt11 and other targets of canonical Wnt signaling are antagonized by p120-catenin (22), which is consistent with the finding that p120-catenin competes with DNA for access to the Kaiso zinc finger domain (6). Repression of the Xenopus genes xWnt11 and Siamois appears to be DNA methylation independent (32), but Kaiso has also been shown to repress transcription of methylated genes (33, 53). In addition, Kaiso has been detected in HeLa cells as part of a multiprotein histone deacetylase complex, where it directly interacts with N-CoR. Likely Kaiso target genes in mammalian cells include S100A4, MTA2, Matrilysin, and the synapse-specific gene Rapsyn (22, 37, 45). Interestingly, Kaiso is reported to be a transcriptional activator at the Rapsyn promoter (37).

Methyl-CpG binding proteins have been implicated in a variety of cellular processes using the technique of gene disruption in mice. For example, Mbd4 deficiency causes an increase in mutation at methyl-CpG sites and reduces the apoptotic response to DNA damage (28, 40, 51). Mbd2 deficiency
FIG. 1. Generation of Kaiso-null mice. (A) Schematic representation of the genomic Kaiso locus, targeting vector, targeted locus, and deleted locus. Boxes represent Kaiso exons that are either translated (black) or untranslated (open). LoxP sites are shown as triangles. The position and direction of primers that were used to validate the targeting are depicted with arrows.
causes premature activation of the interleukin-4 and gamma interferon genes in T cells (14), and Mbd1 deficiency causes defects in neurogenesis (54). A lethal phenotype is demonstrated by Mecp2-null mice, which acquire neurological defects at 6 weeks of age and show misregulation of several genes in brain tissue (3, 12, 26, 31). Depletion of Kaiso in Xenopus embryos leads to premature gene activation at the blastula stage (38), abnormal gastrulation, and early embryonic lethality. It was therefore proposed that Kaiso is an essential component of a developmental gene regulatory pathway that controls vertebrate morphogenesis (22). Here, we show that deletion of the mouse Kaiso gene does not result in any obvious phenotype. Nor does absence of Kaiso detectably alter expression of the putative target genes Wnt11, S100A4, MTA2, or Rapsyn. Kaiso is therefore dispensable for mouse morphogenesis. Kaiso-deficient mice do, however, show resistance to intestinal tumorigenesis when bred onto an ApcMin/+ genetic background, indicating a role in tumor development. This effect is reminiscent of the tumor resistance seen in Mbd2-deficient mice (39). Consistent with a contribution of Kaiso expression to tumorigenesis, we also observe elevated Kaiso expression in mouse intestinal tumors and expression in a series of human colorectal tumors. Together, our data indicate that Kaiso augments tumorigenesis in the colon.

MATERIALS AND METHODS

Northern blots. Total RNA was isolated from mouse tissues by using RNAzol according to the manufacturers’ protocol (Biorosics, Ltd.), and 30 μg was loaded per lane for Northern blotting. RNA was transferred to Hybond-N+ (Amersham Pharmacia Biotech), and all blots were hybridized in Modified Church and Gilbert buffer (7% sodium dodecyl sulfate [SDS], 0.5 M phosphate buffer [pH 7.2], 10 mM EDTA) with denatured herring sperm DNA at 65°C. After overnight hybridization blots were washed in 0.3 M NaCl–0.03 M sodium citrate–1% SDS at 65°C. Signal was detected by using a Storm PhosphorImager (Molecular Dynamics), and analysis was performed using ImageQuant software (version 3.3). Gene specific probes were prepared by PCR amplification of coding sequences from either wt genomic DNA or cDNA. A 334-bp Rapsyn exon 2 probe was amplified with the following primers: 5’-CCGGTGTCCAGATTTG ATACT and 5’-TGGACCTGGGCGTAGAAACT. A 572-bp MTA2 exon 1-2 cDNA probe was amplified with the following primers: 5’-CGGGTGGGAGATGTTCT and 5’-CCACCACGAGAAACTGATC. A cDNA of mouse S100A4 gene was excised from p271 plasmid as described previously (9).

Chromatin immunoprecipitation. Chromatin was prepared from Kaiso-FLAG animals (livers and lungs) as described by the manufacturer (http://www.upstate.com/misc/protocols.q.prot.e.chips/Chromatin

Immunoprecipitation#ChIP). Chromatin was immunoprecipitated with 20 μg of anti-Flag antibody (M2; Sigma) overnight at 4°C on a rotating platform. Subsequent steps for recovery of the immunoprecipitated DNA were performed as described in the Upstate protocol cited above. The PCR conditions consisted of 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s. The primers for IAP

were 5’-TGAGGTTTCCCGTGTTGAGTCA. The primers for Wnt11 were 5’-AGGGGGCCTGTGTTCTGC and 5’-AGGGGGCCTGTGTTCTGC.
Real-time PCR was performed with an ABI Prism 7000 using SYBR Green I. Mean values of $C_T$ (cycle threshold) and standard deviations were calculated for duplicate samples. Analysis was performed with independent RNA samples from two mice, with equivalent results. Depicted data represent analysis of one animal.

**Clinical samples.** Informed consent was obtained from patients to obtain normal and malignant tissue prior to surgical resection of their colon carcinomas in accordance with and under the supervision of the Institutional Review Board of the Montefiore Medical Center.

**Kaiso gene disruption.** A mouse genomic DNA fragment containing the Kaiso locus was identified through screening of the RPCI-21 genomic PAC library with $^{32P}$-labeled Kaiso cDNA. Clone 382-D23 was subcloned to generate the targeting vector. We first cloned two fragments (all coordinates assume the Kaiso transcriptional start ATG codon as 0): a Smal fragment (~2041 to ~116) and a ScaI fragment (~2391 to ~3679) were subcloned into the pBS/SK− plasmid. A neo/tk selectable marker cassette was created by excising the tk gene from plasmid pBS/Sp6tk(XbaI) using XbaI and cloning it into pBT/MTneo(RI) version17 at the EcoRV site. An XbaI-HindIII fragment containing the neo and tk genes was then cloned into pBS246 (Invitrogen). A Nhel-Scal fragment from the resulting plasmid was subcloned into pBS246 to generate pBS246-neo/tk, which contained three loxP sites flanking a BamHI site and the neo/tk cassette. A C-terminal Flag tag was added to the Kaiso cDNA (~115 to ~2394) by introducing a synthetic double-stranded Flag oligonucleotide at an artificially introduced EcoRI site at position +88. The resulting tagged Kaiso cDNA was subcloned into pBS246-neo/tk via BamHI. Finally, Kaiso cDNA and neo/tk were excised by NotI and cloned into the NotI site of plasmid pBS/SK−. The vector was linearized prior to transfection.

We carried out gene targeting in the embryonic stem (ES) cell line E14 Tg2a from the Jackson Laboratories. Cells were grown on gelatinized dishes without feeder cells in the presence of recombinant human LIF (a gift from A. Smith) in from mouse substrain 129/Ola. Cells were grown on gelatinized dishes without M phenylmethylsulfonyl fluoride, and 0.1 μg of pepstatin/ml. Equal protein amounts were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with anti-Kaiso monoclonal antibody 6F (Upstate, Lake Placid, NY). Colo 201, Colo 205, Colo 320, DLD-1, HCT116, and SW48 cells were grown in Dulbecco modified Eagle medium with 10% fetal bovine serum (Gemini Biosciences). Cells were lysed in 50 mM Tris-HCl (pH 7.4)–NP-40 1%–sodium deoxycholate 0.2%–NaCl 150 mM buffer with protease inhibitors and assayed by Western blotting with the Kaiso 6F monoclonal antibody.

**Gel shift analysis of cell line extracts.** Kaiso-deficient mouse tail fibroblasts were prepared and immortalized with simian virus 40 virus as described previously (11). The CG11 (nonmethylated) and McCG11 (methylated) probes were prepared and labeled as described previously (27). The gel was performed in agarose gels as described previously (11).

**Immunohistochemistry.** Colorectal tumors and matched normal mucosa from Muc2−/− mice were fixed in 4% neutral buffered formalin, processed, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. For Kaiso expression, tissue sections were deparaffinized and rehydrated through a xylene and graded ethanol series. For antigen retrieval, slides were immersed in citrate buffer (pH 6) and brought to boil in a steamer for 20 min. Slides were cooled to room temperature in a running water bath for 15 min and then incubated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity for 15 min. After three washes with phosphate-buffered saline (PBS), slides were incubated with peroxidase buffer (0.5% Triton X-100, 20 mM HEPES [pH 7.4], 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose) for 20 min at room temperature. After one wash with PBS, slides were incubated with universal blocking solution (CAS Block; Zymec Laboratories, California) for 10 min at room temperature. The solution was drained, and primary immunoglobulin G1 (IgG1) mouse monoclonal anti-Kaiso antibody (1:200 dilution in PBS; Upstate) or mouse IgG1 was added as a negative control (1:200 dilution in PBS; Jackson ImmunoResearch). Slides were then incubated at 4°C overnight. After three washes with PBS, the slides were incubated for 1 h with biotinylated goat anti-mouse antibody (1:250 dilution in PBS; Zymed Laboratories) at room temperature. Slides were washed three more times with PBS and then incubated for 1 h with preformed avidin-biotinylated peroxidase complex (Vectastain ABC; Vector Laboratories) at room temperature. Color was developed by the addition of diaminobenzochromate peroxidase substrate (Vector Laboratories). Slides were then counterstained with 10% Harris hematoxylin (Lerner Laboratories), dehydrated through a graded ethanol series and xylene, mounted (VectorMount; Vector Laboratories), and visualized by using a light microscope (Zeiss Axioskop).

**Neutral stem cells assay.** Monolayer differentiation to neuroectoderm and isolation of neural stem cell lines was performed as described previously (4, 52). Briefly, ascites were harvested from animals obtained from the Developmental Studies Hybridoma Bank, the anti-β-tubulin III was obtained from Covance, and the anti-GFAP antibody was obtained from Sigma.

**Confocal immunofluorescence.** Kaiso-deficient cells were plated at 105 cells per coverslip and grown for 12 h. A plasmid expressing a green fluorescent protein (GFP)-Kaiso fusion was generated by inserting the human Kaiso coding region in frame with a FLAG coding sequence into pFLAG-CMV2 vector (Sigma) with subsequent cloning of the GFP gene in frame at 3′ end of Kaiso to give an N-terminal GFP tag. The cells were transfected with the GFP-Kaiso construct by using Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Two days after transfection the cells were fixed in 3% paraformaldehyde for 30 min, followed by two washes with PBS-glycine, permeabilized in 0.2% Triton X-100 for 5 min, and blocked with 5% milk solution. Monoclonal antibody 6H11 against p120-catenin (kindly provided by A. B. Reynolds) was used at 2 μg/ml. Secondary goat anti-mouse IgG labeled with Alexa 594 (Molecular Probes) was diluted 1:600. The slides were mounted with Vectashield reagent (Vector Laboratories) and examined with Leica DM IRE 2 confocal microscope with a ×100 oil immersion objective lens.

**RESULTS**

**Deletion of the Kaiso gene causes no overt phenotype in mice.** The X-linked Kaiso locus was targeted in male ES cells to generate a cell line with a “floxed” allele of Kaiso that could be conditionally deleted (Fig. 1A; see Materials and Methods).
The genomic structure at the targeted Kaiso allele was confirmed by Southern blot (not shown) and PCR analysis with a set of locus-specific primers (Fig. 1B). Chimeric mice derived from the Kaiso-null cells were bred, and germ line progeny were identified. A null Kaiso allele was then generated by intercrossing with Cre-expressing mice, leading to deletion of the single loxP flanked coding exon of the Kaiso gene. Subsequent progeny were intercrossed to generate Kaiso-null animals that lacked Kaiso mRNA as determined by a Northern blot assay (Fig. 1C). Absence of the ∼100-kDa Kaiso protein was confirmed in liver nuclear extracts derived from the mutant mice by using antisera raised against amino acids 124 to 492 of Kaiso (Fig. 1D). We further showed that the protein-DNA complex seen in wild-type (wt) mice between Kaiso and the methylated probe MeCG11 (KGB) (33) was absent in extracts from mutant mice (Fig. 1E). An anti-Kaiso antibody supershifted the wt KGB complex but had no effect on complexes formed in mutant extracts (Fig. 1E). Kaiso-null mice showed no overt phenotype and could be maintained as a robust line for >10 generations. The mice were of normal weight and gave birth to litters of normal size.

**Analysis of Kaiso-null mice at the cellular level.** The corepressor N-CoR has been biochemically purified in association with Kaiso and shown to mediate Kaiso repression (53). Since N-CoR has been implicated in development of the central nervous system, erythrocytes, and thymocytes (16), we sought to determine whether Kaiso deficiency affected these tissues. Analysis of blood cells in Kaiso-null mice showed no significant difference from wt mice with respect to the composition of the leukocyte and erythrocyte fractions or erythrocyte morphology (see Fig. S1 in the supplemental material).

We next tested the developmental potential of Kaiso-null stem cells, since N-CoR is implicated in development of the nervous system (16). Also, mice deficient for the methyl-CpG binding protein Mbd1 are viable and fertile but show a defect in adult neurogenesis and hippocampal function, and Mbd1-null neural stem cells show reduced neuronal differentiation compared to wt cells (54). To test for a comparable phenotype in Kaiso-null cells, ES cells in which GFP is expressed from the Sox1 locus (52) were targeted with the floxed Kaiso construct. Properly targeted cells were transfected with a Cre-expression plasmid to induce deletion of the Kaiso gene. The genotype of targeted cells was confirmed by PCR and Southern blotting.

**FIG. 2.** Kaiso-null cells show no defects in neural differentiation. (A) Wild-type or Kaiso-deficient ES cells expressing GFP from the Sox1 locus were induced toward neural differentiation for 3, 4, 5, 6, 7, or 8 days (D3, D4, etc.) and fluorescence-activated cell sorted for GFP expression. The experiment was performed in triplicate, with average values (± the standard error of the mean) plotted. (B) Kaiso gene expression was analyzed by RT-PCR in wt and null (clone A and B) sorted Sox1-positive cells. (C to F) Wild-type (C and E) and Kaiso-null (D and F) cultures were stained for β-tubulin (C and D) or Gfap (red) and DAPI (4',6'-diamidino-2-phenylindole; blue) (E and F) after 12 days of monolayer differentiation. (G to N) Wild-type (G, I, K, and M) and Kaiso-null (H, J, L, and N) neural stem cells were stained for nestin (G and H) and RC2 (I and J) or were induced to differentiate and stained for β-tubulin III (K and L) or Gfap (M and N). Cells were counterstained with DAPI (blue). Scale bars: 100 μm (C, D, E, F, M, and N) and 50 μm (G, H, I, J, K, and L).
FIG. 3. No evidence for abnormal gene expression in Kaiso-null animals. (A) Total RNA from four different animals born in two independent families was isolated from wild-type (WT) and Kaiso-null (KO) strains. Sources of RNA were brain, liver, spleen, and muscle. The same blot was hybridized with S100A4 and Mta2 probes. Separate blots were prepared with muscle RNA and hybridized with a Rapsyn probe. Normalization of the amount of RNA loaded was performed by reprobing with β-actin (S100A4; Mta2) or S26 ribosomal protein (Rapsyn) probes. (B) In the left panel, chromatin immunoprecipitation was performed with M2 anti-FLAG monoclonal antibodies (M2 lanes) and chromatin was prepared from kidney of wild-type (WT) and Kaiso-null (KO) animals. PCR products amplified with IAP-specific primers from chromatin immunoprecipitated with or without (“no Ab”) the addition of antibodies are designated. Amplification without DNA (−) and with kidney genomic DNA (+) were used as negative and positive controls, respectively. PCR products were fractionated on 1% agarose gels. In the right panel is shown IAP expression analysis. RNA from liver of wild-type (WT) and Kaiso-null (KO) animals was either transcribed (+) or not transcribed (−) by reverse transcriptase (RT). Subsequent PCR amplification of IAP cDNA and control 18S cDNA produced DNA fragments that were resolved on agarose gels. Different dilutions of cDNA were used for PCR amplification as depicted. (C) Semiquantitative RT-PCR with serial dilutions of cDNA from WT or KO heart were amplified by using Wnt11-specific primers and compared to 18S rRNA-specific primers used to amplify the same samples. (D) Quantitative “real-time” PCR analysis of Wnt11 mRNA abundance in the hearts, livers, and testes of WT and KO mice. “Delta Ct” expresses the difference in cycle thresholds between Wnt11 and 18S amplification rates.
(as in Fig. 1B), and loss of Kaiso expression was verified by reverse transcription-PCR (RT-PCR) (Fig. 2B). Both Kaiso<sup>+/y</sup> Sox1<sup>GFP</sup> and Kaiso<sup>−/y</sup> Sox1<sup>GFP</sup> ES cell lines were then induced to differentiate into neural ectoderm as described previously (52). Since Sox1 is a specific marker of neural specification, cells that become GFP positive have differentiated into neural precursor cells and can be quantified by fluorescence-activated cell sorting analysis. We found that both Kaiso<sup>+/y</sup> Sox1<sup>GFP</sup> and Kaiso<sup>−/y</sup> Sox1<sup>GFP</sup> ES cells differentiated into Sox1<sup>GFP</sup>-positive neural precursors at similar frequencies (Fig. 2A). After 12 days, cultures were fixed and stained for markers of postmitotic neurons (β-tubulin III; Fig. 2C and D) and astrocytes (GFAP; Fig. 2E and F). Both astrocytes and neurons were produced efficiently in Kaiso-null cultures, indicating that Kaiso is not important for cell fate decisions by this assay.

In order to study whether Kaiso plays a role in maintenance of stem cell state and self-renewal, we made pure neural stem cell lines from Kaiso<sup>−/y</sup> and Kaiso<sup>−/−</sup> ES cells (4). Kaiso<sup>−/−</sup> neural stem cells were efficiently maintained through multiple passages (>20) mirroring the wild-type neural stem cells in morphology and proliferation (data not shown). Cells of both genotypes expressed neural stem cell markers Nestin (Fig. 2K and L) and astrocytes (Fig. 2M and N) that were indistinguishable from wild-type cultures. We conclude that Kaiso function is not important for neural specification, neural stem cell viability, or neuronal and astroglial cell differentiation ex vivo.

We also addressed the effects of Kaiso on localization of p120-catenin in fibroblasts derived from Kaiso-null mice. Transient transfection of a construct expressing a GFP-Kaiso fusion protein had no detectable effect on the cytoplasmic localization of p120-catenin, since transfected and neighboring untransfected cells were indistinguishable in this respect (see Fig. S2 in the supplemental material). Kaiso localization in this assay was predominately nuclear. Our findings are compatible with a previous report that p120-catenin and Kaiso do not colocalize (44).

**Kaiso deficiency does not affect expression of candidate target genes.** Kaiso has been implicated in the regulation of several genes using mammalian cultured cell systems. We initially sought to determine whether Kaiso deletion influenced transcription of the putative targets S100A4, Mta2, and Rapsyn (35, 37, 53) in tissues from Kaiso-null mice. Northern blot analysis revealed no change in expression of Mta2 in RNA from brain, liver, or spleen (Fig. 3A). Rapsyn mRNA, which is normally expressed in muscle, was not affected by the absence of Kaiso and S100A4 expression, which is high spleen (9), was also indistinguishable between wt and Kaiso-null animals (Fig. 3A). To look for global effects of Kaiso deficiency, we examined expression of IAP transposable elements, which is normally suppressed by DNA methylation (50). Chromatin immunoprecipitation established that FLAG-tagged Kaiso expressed from the floxed allele was associated with IAP element sequences in chromatin from liver (Fig. 3B). Semiquantitative PCR analysis, however, failed to detect any difference in IAP expression when wt and Kaiso-null liver RNA preparations were compared (Fig. 3B). A particularly well-characterized Kaiso target gene is xWnt11, which binds Kaiso and is upregulated by its absence in Xenopus embryos (22). Moreover, the human Wnt11 gene has been immunoprecipitated from cross-linked HeLa cell chromatin by anti-Kaiso antibodies (22). Examination of Wnt11 expression in Kaiso-null tissues by RT-PCR showed no obvious effect of Kaiso deficiency by either semiquantitative RT-PCR using heart cDNA (Fig. 3C) or quantitative real-time PCR using cDNA.

![Image](https://example.com/image.png)

**FIG. 4.** Kaiso deficiency decreases tumor size and increases life span of Apc<sup>Min+/−</sup> mice. (A) Kaplan-Meier survival plot of Apc<sup>Min+/−</sup> Kaiso<sup>−/y</sup> (solid line) and Apc<sup>Min+/−</sup> Kaiso<sup>−/−</sup> (hashed line) mice. Apc<sup>Min+/−</sup> Kaiso<sup>−/−</sup> mice live significantly longer (median, 317 days; gray vertical line) than both Apc<sup>Min+/−</sup> Kaiso<sup>−/y</sup> (median, 217; black vertical line, P = 0.006 [log rank]), (B) Tumor number is not altered by Kaiso deficiency. Boxplots show numbers of adenomas per mouse at 180 days and at death. The horizontal boxed line represents the median. Gray boxes, Apc<sup>Min+/−</sup> Kaiso<sup>−/y</sup>; open boxes, Apc<sup>Min+/−</sup> Kaiso<sup>−/−</sup>. No significant differences were observed between Apc<sup>Min+/−</sup> Kaiso<sup>−/y</sup> and Apc<sup>Min+/−</sup> Kaiso<sup>−/−</sup> mice at either 180 days (P = 0.10 [Mann-Whitney], n = 20) or at death (P = 0.62 [Mann-Whitney], n = 20). (C) Tumor size, measured by area, is reduced in Kaiso-deficient mice at 180 days (P = 0.001 [Mann-Whitney], n = 114) but not death (P = 0.55 [Mann-Whitney], n = 239). Gray bars, Apc<sup>Min+/−</sup> Kaiso<sup>−/y</sup>; open bars, Apc<sup>Min+/−</sup> Kaiso<sup>−/−</sup>. Bars represent the standard error of the mean.
from heart, liver, and testis (Fig. 2D). We conclude that in the mouse Kaiso does not play a dominant role in the regulation of these candidate target genes.

**Kaiso-deficient Apc\(^{Min/+}\) mice exhibit delayed intestinal tumorigenesis.** Three lines of evidence led us to examine a possible role for Kaiso in intestinal cancer. First, accumulating data indicate a critical role for DNA methylation-dependent gene silencing in the pathogenesis of colorectal cancer (23). Second, Kaiso DNA binding and transcriptional activity is attenuated by interaction with the p120 catenin protein (22), which is lost in ca. 25% of colorectal tumors, and is abnormally expressed or localized in another 40% of tumors (20, 47). Third, Kaiso is implicated in Wnt signaling (8, 19, 22, 32, 45), which is often disrupted in intestinal tumors. To test for the involvement of Kaiso in colorectal cancer, we used the Apc\(^{Min/+}\) mouse, which is a model for human familial adenomatous polyposis (46). Apc\(^{Min/+}\) mice acquire multiple intestinal polyps within the first 6 months of life. DNA methylation has been previously implicated in this model since deficiencies of either the DNA methyltransferase Dnmt1 (7, 24) or the methyl-CpG binding protein Mbd2 (39) confer striking resistance to tumorigenesis. We therefore crossed Kaiso-null mice with Apc\(^{Min/+}\) mice and assessed survival and tumor burden in the resulting male Kaiso\(^{-/-}\) Apc\(^{Min/+}\) offspring. A significant increase in the survival of the Kaiso\(^{-/-}\) Apc\(^{+/Min}\) mice compared to Kaiso\(^{-/-}\) Apc\(^{+/Min}\) mice was recorded (Fig. 4A). Tumor burden at 180 days and at death was comparable between the two genotypes (Fig. 3B), but the size of polyps was significantly less at 180 days in mice lacking Kaiso (Fig. 4C). Examination of adenomatous polyps from wt and Kaiso-null Min mice showed no significant difference in the mitotic indices or in levels of apoptosis in either normal epithelium or adenomas (see Fig. S3A and B in the supplemental material), arguing that the reduced growth rate of Kaiso-null intestinal polyps is not caused by a lower rate of cell division or increased cell death.

As a further test for a relationship between Kaiso and intestinal tumorigenesis in mice, we examined Kaiso expression levels in colorectal tumors from the Muc2\(^{-/-}\) mouse model, which develops invasive colorectal tumors akin to those of patients with inflammatory bowel disease. The latter have been linked to silencing by CpG island hypermethylation (13, 15, 43, 48). We performed immunohistochemistry and Western blots on Muc2\(^{-/-}\) tumors and matched normal mucosa controls from the same mice. By both assays, Kaiso expression was significantly increased in tumors compared to controls (Fig. 5). Moreover, Kaiso was predominantly nuclear in tumor samples (Fig. 5A), unlike the predominantly cytoplasmic staining reported in human tumors and normal tissues (44). We went on to ask whether human colon carcinomas also express Kaiso. Kaiso protein levels were examined in 14 human primary colorectal tumors and their matched normal colonic mucosa. Kaiso was detectable in all tumors with variable expression ratios between polyps and normal mucosa (see Fig. S4 in the supplemental material).
DISCUSSION

The mild phenotype of Kaiso-null mice is surprising given the severe effects of its absence in Xenopus (22, 38) and its proposed role as an essential component in a regulatory pathway that controls vertebrate morphogenesis (22). This may be due to differences in the roles of DNA methylation in controlling the zygotic gene program in mice and frogs. In frogs, methylation is critical for the silencing of genes through the first eight zygotic cell divisions. In contrast, the paternal genome of mice is actively demethylated before the first zygotic cell division, whereas the maternal genome becomes passively demethylated through cleavage divisions. Zygotic transcription is delayed at the two-cell stage in mice, but is not activated until the ~5,000 cell mid-blastula transition in frogs. Thus, the role of Kaiso in ensuring delayed activation of genes in frogs may have no counterpart in mice.

Regarding the role of Kaiso in transcriptional repression, we were surprised to find no difference in expression of four genes that were previously identified as Kaiso targets (S100A4, Mta2, Rapsyn, and Wnt11). This suggests either that these genes are not targets of Kaiso-mediated repression in the mouse or that there is a level of redundancy in their control. It is not possible to predict which proteins might substitute for the absence of mouse Kaiso, but the related protein CIBZ/Zenon may be a potential candidate (21, 42). It is clear that deficiency of N-CoR is not equivalent to loss of Kaiso, as Kaiso-null mice show none of the abnormalities observed in N-CoR-null embryos, which die before birth (16). N-CoR is also implicated in the regulation of neurogenesis and in blood differentiation (16), both of which appear normal in Kaiso-null mice.

Our data implicate Kaiso in intestinal tumorigenesis, since its absence inhibits the formation of adenomatous polyps in a mouse model of familial adenomatous polyposis, and both mouse and human colorectal tumors express Kaiso. Given the ability of Kaiso to mediate DNA methylation-dependent transcriptional repression (33, 53) and the known dependence of mouse intestinal tumorigenesis on Dnmt1 (7, 24) and Mbd2 (39), it is tempting to speculate that Kaiso plays a part in the gene silencing that contributes to the cancer phenotype. Studies of repression of the MTA2 gene in HeLa cells are compatible with this view (53). MTA2 is a component of the ubiquitously expressed Mi-2/NuRD complex, and its DNA methylation-dependent repression in HeLa cells is therefore likely to be an abnormal gene silencing event of the kind that is common in permanent cell lines (2) and cancer cells (17). Therefore, Kaiso may mediate abnormal gene silencing that occurs in cancer cells. The finding that Kaiso-null Min tumors exhibit the same mitotic and apoptotic indices as wild-type Min tumors suggests that Kaiso does not delay tumor growth. It is therefore possible that Kaiso augments the early stages of tumorigenesis. Kaiso has been implicated in Wnt signaling (8, 19, 22, 32, 45), which is important for the normal differentiation program of intestinal epithelium (36). Since polyps in Min mice are invariably Apc null and therefore hyperactive in Wnt signaling (25), the absence of Kaiso may reduce the impact of this defect and therefore constrain tumor development. Future work will seek to identify the range of Kaiso target genes in the intestine that may contribute to such an effect.

The observation that deficiency of Kaiso attenuates tumorigenesis suggests Kaiso as a possible target for anticancer therapy, as has been suggested for both Dnmt1 and Mbd2. Although the delay of tumorigenesis caused by absence of Kaiso is less pronounced than that due to Dnmt1 or Mbd2, Kaiso has the attraction that its absence does not lead to any deleterious phenotype in the mouse. This contrasts with the embryonic lethality caused by absence of Dnmt1 (24) and abnormal gene expression caused by absence of Mbd2 (14) and may reduce the likelihood that Kaiso antagonists will be toxic.

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