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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*, xKaiso 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 $Apc^{Min/+}$ 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 (53).

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 deacetylation 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

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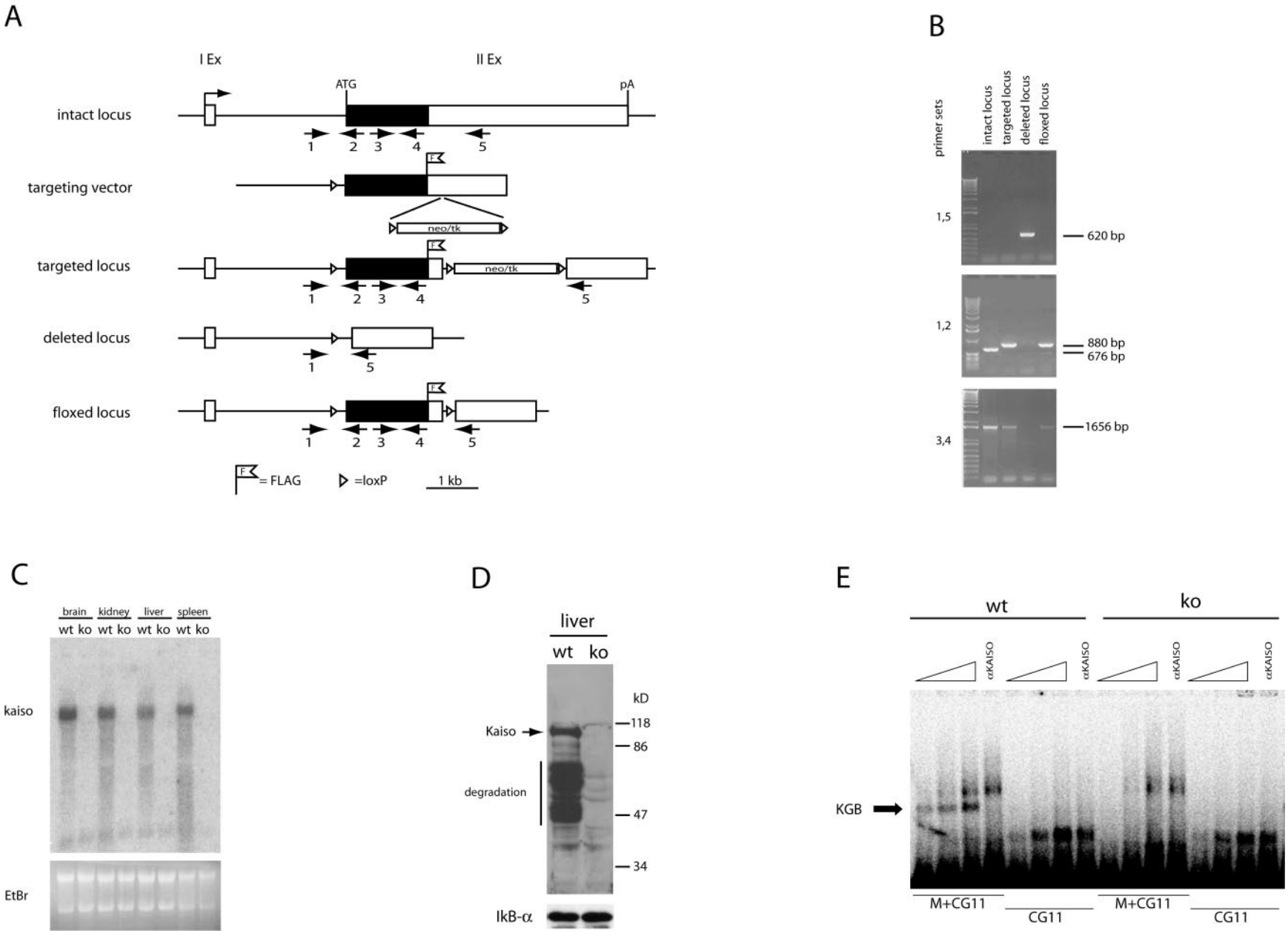


FIG. 1. Generation of Kaiso-null mice. (A) Schematic representation of the genomic *Kaiso* locus, targeting vector, targeted locus, and deleted locus. Boxes represent *Kaiso* exons that are either translated (black) or untranslated (open). LoxP sites are shown as triangles. The position and direction of primers that were used to validate the targeting are depicted with arrows.

(B) Validation of correct targeting by PCR. Primer pairs are indicated on the left. Genomic DNA from a correctly targeted ES clone was used as a template. PCR products were fractionated on 1% agarose gels. DNA fragment sizes are indicated on the right. (C) Northern blot hybridization with *Kaiso* cDNA. RNA was isolated from brain, kidney, liver, and spleen of wild-type (wt) and *Kaiso*-null (ko) animals. *Kaiso* mRNA corresponds to ca. 7 kb, and the hybridization signal is indicated by "kaiso." Prior to the blotting, the gel was stained with ethidium bromide (EtBr) and photographed (bottom panel). (D) Western blot hybridization of liver nuclear extracts from wild-type (wt) and *Kaiso*-null (ko) animals. The Kaiso band (~100 kDa) and products of Kaiso degradation (from 60 to 45 kDa) are indicated on the left. The bottom panel shows a Western blot of inhibitory κB-alpha protein as an internal control. The protein size markers are on the right. (E) Band-shift assays with nuclear extracts from *wt* or mutant (ko) liver. The labeled probe was either M+CG11 (methylated) or CG11 (unmethylated). The lower complex in *wt* M+CG11 lanes is the DNA methylation-specific Kaiso-DNA complex (KGB). This complex is absent in ko lanes. Lanes αKaiso contained anti-Kaiso antibody ZFH6 (33) that specifically supershifts the KGB complex.

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 $Apc^{Min/+}$ 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 RNABee according to the manufacturers' protocol (Biogenesis, 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 (V3.3). 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′-CCGTGGTCCAGATTG ATACT and 5′-TGGACCTGGGCGTAGAAACT. A 572-bp MTA2 exon 1-2 cDNA probe was amplified with the following primers: 5′-CCGGGTGGGAGA TTACGTC 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++ChIPs++ Assay+Kit). 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 IAP chromatin immunoprecipitation primers were 5′-AGCCGCCCCCACATTCGCCGT and 5′-TCACTCCCTGATTGGCTGCAGC.

Reverse transcriptase PCR. Total RNA was isolated from mouse liver by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For first-strand synthesis, the RevertAid First-Strand cDNA synthesis kit (Fermentas) was used. Total RNA (1 to 1.5 μg) plus 0.2 μg of random hexamers were incubated for 5 min at 70°C, chilled, and mixed with 4 μl of 5× reaction buffer, 2 μl of 5 mM deoxynucleoside triphosphates, and 200 U of RevertAid M-MuLV reverse transcriptase. The reaction mix was incubated at 25°C for 5 min at 42°C for 60 min and then at 70°C for 10 min. Freshly synthesized cDNA was used as a template for PCR. The PCR conditions consisted of 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The primers for IAP Q-PCR were 5′-TGTACCCCGAGCACCAAGAGT and 5′-ATAGGATCC GGGCCATACCAT. The primers for 18S rRNA Q-PCR were 5′-AGACGA TCAGATACCGTCGTA and 5′-TGAGGTTTCCCGTGTTGAGTCA. The primers for Wnt11 were 5′-AGCTGGAGGGCCTGGTGTCTGC and 5′-AGG CCCGGGCGATGGTGTG.

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 ^{32}P-labeled *Kaiso* cDNA. Clone 382-D23 was subcloned to generate the targeting vector. We first cloned two fragments (all coordinates assume the Kaiso translational start ATG codon as 0): a SmaI fragment (−2041 to −116) and a SacII fragment (+2391 to + 3673) were subcloned into the pBS/SK− plasmid. A *neo*/*tk* selectable marker cassette was created by excising the *tk* gene from plasmid pBT/SPtk(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 NheI-ScaI 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;+2394) by introducing a synthetic double-stranded Flag oligonucleotide at an artificially introduced EcoRI site at position +2013. The resulting tagged Kaiso cDNA was subcloned into pBS246-neo/tk through 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 mouse substrain 129/Ola. Cells were grown on gelatinized dishes without feeder cells in the presence of recombinant human LIF (a gift from A. Smith) in standard ES cell conditions. ES cells (10e7cells) were transfected with the linearized targeting vector (250 μg of DNA in 0.8 ml of HEPES buffered saline) by electroporation (800 V, 3 μF; Bio-Rad Gene Pulser) and plated in 10-cm dishes at 5×10^6 cells per dish. Correctly targeted clones were identified by PCR with the following primers: 1, TCAAAGGAAGGCGACCAAGGAGAT; 2, AGC AGTACCATCCTGTTCTG; 3, CTGTCACAGGTTAAAAGC; 4, GTAAGA TTCTGGTATTAT; and 5, ATAGTTTAAAGGCATATAGTGGCC. The position of the primers is shown in Fig. 1A. Three primer sets were used for the amplification: 1-5, 1-2, and 3-4. The extension time was calibrated so that only a short (620-bp) DNA fragment was amplified when the 1-5 set was used without amplification of *Kaiso* coding sequences or the *neo*/*tk* cassette. The LoxP flanked allele was identified as an 880-bp band in the 1-2 set, while the intact locus gave rise to a smaller (676-bp) band.

Correctly targeted ES cell clones were passaged the day before injection and injected into blastocysts from naturally mated C57BL/6 females at 3.5 days postcoitum. Injections were performed in M2 medium (Sigma) with 10 to 15 ES cells being injected into each blastocyst before transfer to pseudopregnant recipient females (6 to 12 blastocysts per recipient). Chimeric pups were identified by their agouti coat color and, on maturity, were mated with C57BL/6 mice. Since *Kaiso* is X linked, all agouti F_1 females were heterozygous for the floxed allele. This was confirmed by PCR and Southern blot. We crossed heterozygous females with "deleter" mice that expressed cre recombinase under the cytomegalovirus promoter. The DNA fragment between two loxP sites was deleted in the offspring. After inbreeding, the line was maintained in the homozygous state on a C57BL/6 genetic background.

Assay for intestinal tumorigenesis. *Kaiso*-null mice segregating equally for C57BL/6 and Ola129 genomes were mated to *Apc*$^{Min/+}$ mice on an inbred C57BL/6J background. Progeny from this cross were then interbred to generate cohorts of *Apc*$^{Min/+}$, *Kaiso*$^{+/y}$ (20 mice plus 23 for the 180-day experiment), and *Apc*$^{Min/+}$ *Kaiso*$^{-/y}$ (21 mice plus 18 for the 180-day experiment) mice. These cohorts were therefore segregating for C57BL/6 (75%) and 129/Ola (25%) genomes. All mice were confirmed as congenic for the C57BL/6 *Mom-1* allele via PCR analysis. Two experiments were performed: (i) mice were sacrificed when they displayed overt signs of illness and (ii) mice were culled at 180 days. Intestinal tumor burden was determined by removing the entire intestine and mounting en face. Preparations were fixed in methacarn (methanol-chloroform-glacial acetic acid [4:2:1]), and the lesion number and size were scored macroscopically.

Intestines fixed in methacarn were wound into a "gut roll" and paraffin-embedded for histological analysis and immunohistochemistry. To determine crypt size and the levels of apoptosis and mitosis, gut rolls were stained with hematoxylin and eosin, and then the numbers of apoptotic bodies and mitotic figures were determined. Crypt size, apoptotic bodies, and mitotic figures in normal crypts were scored per 25 full crypts. For each adenoma, the number of apoptotic bodies per 500 cells was scored, and for each mouse at least three adenomas were scored, producing an average value per mouse. At least three mice were used for each time point.

Western blots. Frozen aliquots of human colorectal tissue were thawed, and total cellular protein was isolated in immunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 1 mM EDTA, 5 μg of leupeptin/ml, 1 μg of aprotinin/ml, 1 μM phenylmethylsulfonyl fluoride, and 0.7 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, HCT15, 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 0.25%–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 MeCG11 (methylated) probes were prepared and labeled as described previously (27). The gel shift was performed in agarose gels as described previously (11).

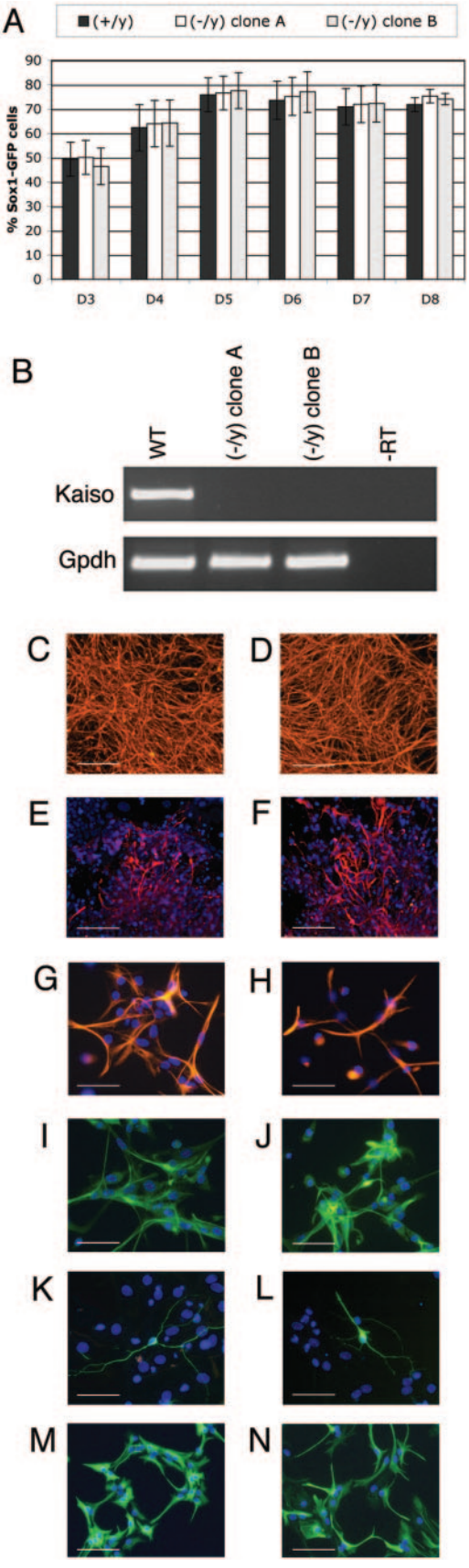
Immunohistochemistry. Colorectal tumors and matched normal mucosa from Muc2$^{-/-}$ mice were fixed in 4% neutral buffered formalin, processed, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. For Kaiso expression, tissue sections were deparaffinized and rehydrated through 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 permeabilization buffer (0.5% Triton X-100, 20 mM HEPES [pH 7.4], 50 mM NaCl, 3 mM $MgCl_2$, 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 IgG 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 diaminobenzoate chromogen peroxidase substrate (Vector Laboratories). Slides were then counterstained with 10% Harris hematoxylin (Lerner Laboratories), dehydrated through a graded ethanol series and xylene, mounted (VectaMount; Vector Laboratories), and visualized by using a light microscope (Zeiss Axioskop).

Neural stem cells assay. Monolayer differentiation to neuroectoderm and isolation of neural stem cell lines was performed as described previously (4, 52). Antibodies against RC2 and Nestin were 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 10^3 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 3% 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).

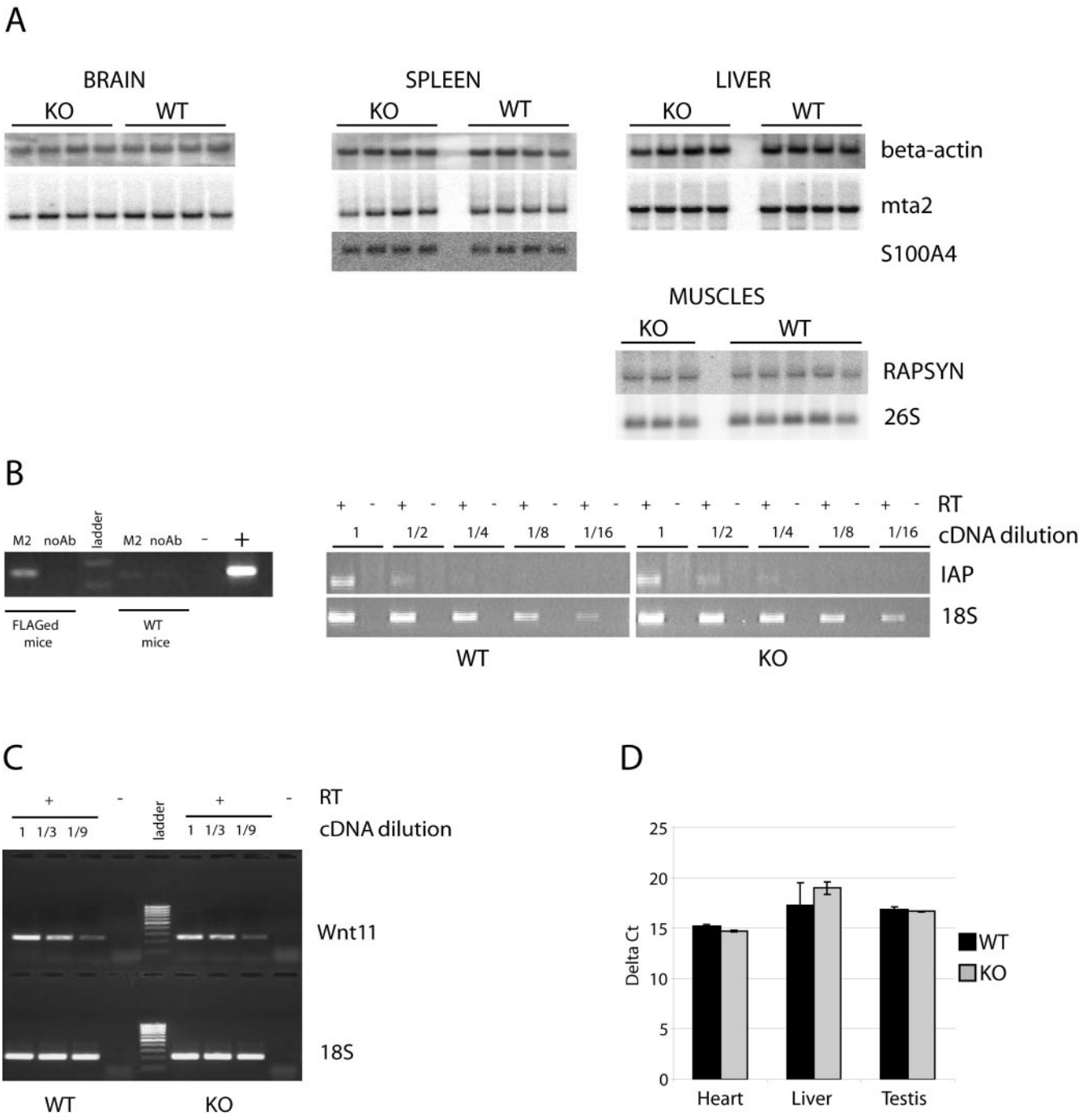


FIG. 3. No evidence for abnormal gene expression in *Kaiso*-null animals. (A) Total RNA from four different animals born in two independent families was isolated from wild-type (WT) and *Kaiso*-null (KO) strains. Sources of RNA were brain, liver, spleen, and muscle. The same blot was hybridized with *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*$^{+/y}$ *Sox1*^{GFP} and *Kaiso*$^{-/y}$ *Sox1*^{GFP} 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*$^{+/y}$ *Sox1*^{GFP} and *Kaiso*$^{-/y}$ *Sox1*^{GFP} ES cells differentiated into Sox1^{GFP}-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*$^{+/y}$ and *Kaiso*$^{-/y}$ ES cells (4). *Kaiso*$^{-/y}$ 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. 2G and H) and RC2 (Fig. 2I and J). Multipotency was verified by efficient differentiation into postmitotic neurons (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 predominantly 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

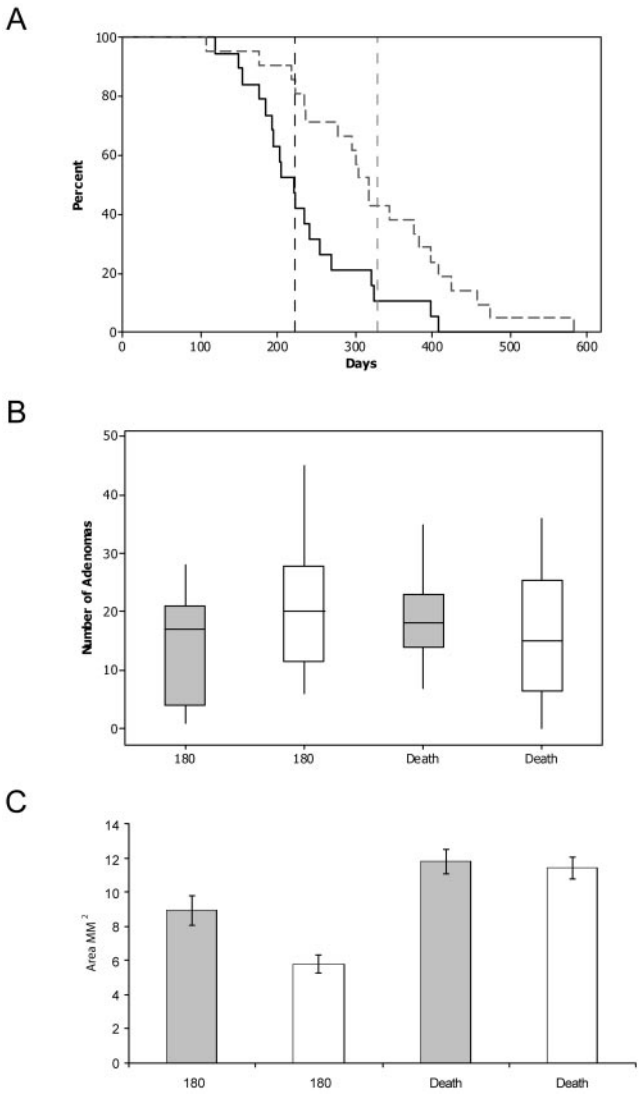


FIG. 4. Kaiso deficiency decreases tumor size and increases life span of *Apc*$^{Min/+}$ mice. (A) Kaplan-Meier survival plot of *Apc*$^{Min/+}$ *Kaiso*$^{+/y}$ (solid line) and *Apc*$^{Min/+}$ *Kaiso*$^{-/y}$ (hashed line) mice. *Apc*$^{Min/+}$ *Kaiso*$^{-/y}$ mice live significantly longer (median, 317 days; gray vertical line) than both *Apc*$^{Min/+}$ *Kaiso*$^{+/y}$ (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*$^{Min/+}$ *Kaiso*$^{+/y}$; open boxes, *Apc*$^{Min/+}$ *Kaiso*$^{-/y}$. No significant differences were observed between *Apc*$^{Min/+}$ *Kaiso*$^{+/y}$ and *Apc*$^{Min/+}$ *Kaiso*$^{-/y}$ mice at either 180 days ($P = 0.10$ [Mann-Whitney], $n \geq 20$) or at death ($P = 0.62$ [Mann-Whitney], $n \geq 20$). (C) Tumor size, measured by area, is reduced in Kaiso-deficient mice at 180 days ($P = 0.001$ [Mann-Whitney], $n \geq 114$) but not death ($P = 0.55$ [Mann-Whitney], $n \geq 239$). Gray bars, *Apc*$^{Min/+}$ *Kaiso*$^{+/y}$; open bars, *Apc*$^{Min/+}$ *Kaiso*$^{-/y}$. Bars represent the standard error of the mean.

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

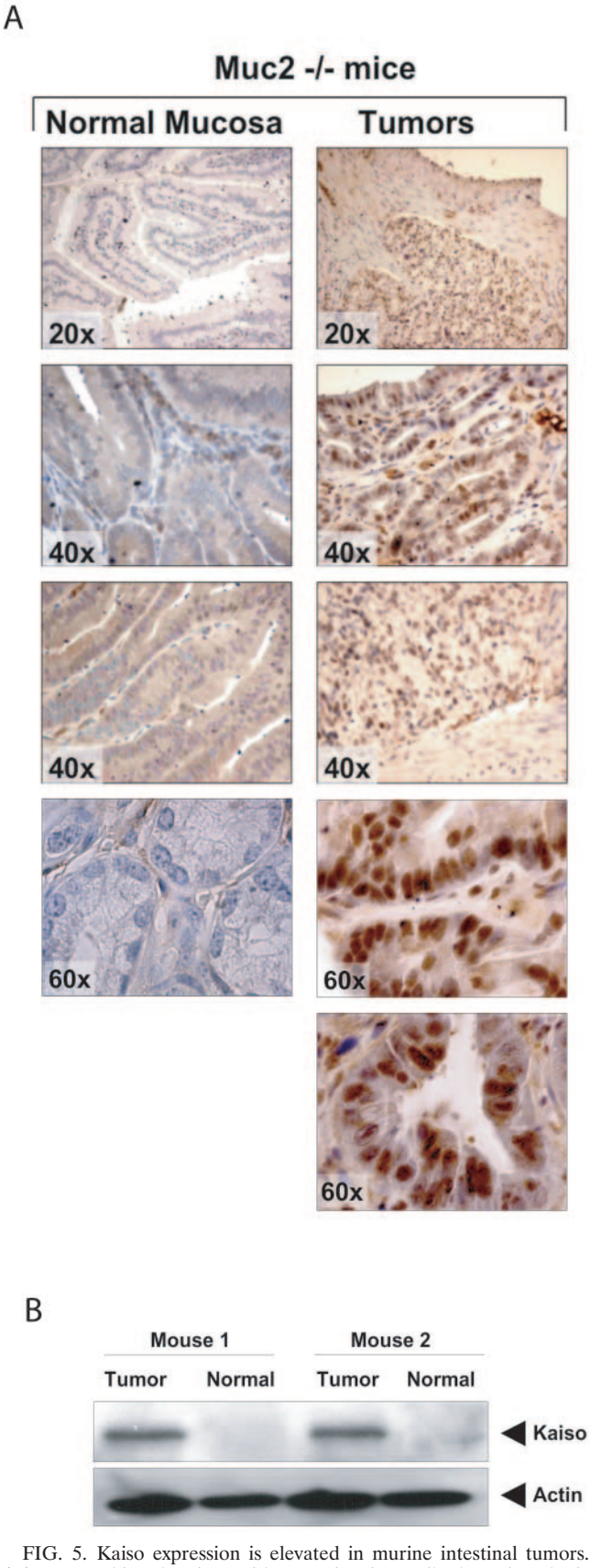


FIG. 5. Kaiso expression is elevated in murine intestinal tumors. (A) Immunohistochemistry with an anti-Kaiso antibody 6F in colonic tumor and matched normal mucosa from *Muc2*$^{-/-}$ mice at the indicated magnification factors. (B) Western blots performed with anti-Kaiso and antiactin antibodies in two pairs of *Muc2*$^{-/-}$ intestinal tumors compared to matched normal mucosa.

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*$^{-/y}$ *Apc*$^{Min/+}$ offspring. A significant increase in the survival of the *Kaiso*$^{-/y}$ *Apc*$^{+/Min}$ mice compared to *Kaiso*$^{+/y}$ *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 though 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 detected 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 *wt* 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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