

A Family of Human Zinc Finger Proteins That Bind Methylated DNA and Repress Transcription

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In vertebrates, densely methylated DNA is associated with inactive transcription. Actors in this process include proteins of the MBD family that can recognize methylated CpGs and repress transcription. Kaiso, a structurally unrelated protein, has also been shown to bind methylated CGCGs through its three Krüppel-like C₂H₂ zinc fingers. The human genome contains two uncharacterized proteins, ZBTB4 and ZBTB38, that contain Kaiso-like zinc fingers. We report that ZBTB4 and ZBTB38 bind methylated DNA in vitro and in vivo. Unlike Kaiso, they can bind single methylated CpGs. When transfected in mouse cells, the proteins colocalize with foci of heavily methylated satellite DNA and become delocalized upon loss of DNA methylation. Chromatin immunoprecipitation suggests that both of these proteins specifically bind to the methylated allele of the *H19/Igf2* differentially methylated region. ZBTB4 and ZBTB38 repress the transcription of methylated templates in transfection assays. The two genes have distinct tissue-specific expression patterns, but both are highly expressed in the brain. Our results reveal the existence of a family of Kaiso-like proteins that bind methylated CpGs. Like proteins of the MBD family, they are able to repress transcription in a methyl-dependent manner, yet their tissue-specific expression pattern suggests nonoverlapping functions.

In mammalian genomes, the regulation of transcriptional activity relies on a complex combinatorial interplay of transcription factors, but also on epigenetic mechanisms. The latter establish a transcriptional landscape that is transmitted from a cell to its progeny (19). Even though the epigenetic state is essentially stable throughout cell generations, it is far from static. The epigenome can indeed be remodelled throughout the life of a cell, for instance, during differentiation or senescence (12, 37). DNA methylation is one of the epigenetic mechanisms that regulate gene expression.

The methylation of promoter regions causes a strong and heritable transcriptional inhibition of the corresponding genes (3). An explanation for this phenomenon came with the milestone discovery that some proteins recognize the methylation marks and shut down transcription (21, 35). These proteins are characterized by a specific affinity for methylated versus non-methylated CpGs and are collectively termed MBPs (methyl-DNA-binding proteins). In vertebrates, proteins containing the MBD (methyl-DNA-binding domain) constitute a large and well-studied family of proteins that bind single methylated CpGs (16). The MBD is not the only protein fold that can permit recognition of methylated DNA; for instance, the protein Kaiso uses a three-zinc-finger motif to bind methylated CGCGs (39). The zinc fingers of Kaiso have a dual specificity in vitro, as they can bind either DNA sequences containing

methylated CGCG or the consensus Kaiso binding site (KBS), TCCTGCNA (8). Experiments in *Xenopus laevis* have shown that Kaiso does bind both classes of sequence elements in vivo. Indeed, it binds gene promoters containing the KBS and transmits both canonical and noncanonical Wnt signals (25, 38), but it also binds a large number of methylated promoters to repress transcription, especially before the mid-blastula transition (43). In human cells, Kaiso also binds some methylated promoters (46), as well as some KBS-containing promoters (41, 44).

DNA methylation is an essential phenomenon (29), yet none of the MBPs identified so far are required for viability (14, 15, 47), raising the possibility that other MBPs remain to be found. In an attempt to discover new MBPs, we performed a BLAST search on the human genome for proteins containing Kaiso-like zinc fingers and identified two such proteins: ZBTB4 and ZBTB38. We report that both proteins bind methylated DNA in vitro and in vivo. Unlike Kaiso, ZBTB4 and ZBTB38 can bind sequences containing a single methylated CpG. Ectopic expression in mouse cells shows specific enrichment of both proteins to highly methylated sequences. We also observed that ZBTB4 and ZBTB38 seem to be present at the methylated paternal allele of the *H19/Igf2* differentially methylated region. We next showed that ZBTB4 and ZBTB38 are methyl-dependent transcriptional repressors. Finally, we determined their expression pattern and found that both genes are highly expressed in the brain.

MATERIALS AND METHODS

Plasmids. Plasmid construction followed standard molecular biology procedures. When PCR amplification was required we used Phusion polymerase (Finnzymes). All the constructs generated were sequenced to verify lack of mutation. Human cDNA clones were obtained from the Mammalian Gene Collection via

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TABLE 1. Primers

Primer	Sequence
GF48	TAATGCGATTAGTGAATTCGCGCGCTGGCTACCGGCGAT
GF50	TCATTATGTGATAATGCCGCGCGTCGTGATTAGCGCCGT
GF88	GTGCAGCGCGTCGTAATC
GF89	GATTACGACGCGCTGCAC
GF90	GTGCAGCGMGTCTGTAATC
GF91	GATTACGAMGCGCTGCAC
GF110	CATCTGGTMAGCTGGGGAA
GF109	TTCCCCAGCTGACCAGATG
GF118	TTCCCCACMTGGCCAGATG
GF117	CATCTGGCMAGGTGGGGAA
GF134	GTGCTTCCCGCCAATAACG
GF135	CGTTATTGGCGGGAAGCAC
GF128	GTGCTTCCTGCCAATAACG
GF129	CGTTATTGGCAGGAAGCAC
PAD184	AGGAAGTACCCCTGCCGCTA
PAD185	TTGTAGCCTCCATTGGGTGT
PAD190	CTGAAGGCAAGATGGGTAC
PAD191	GCCTATGTCCTTCGCGTACT
DMD56/1	GTGCAACAAGGGAACGGATGC
DMD78/1	AACCGCAATTTTGGTCACCT

RZPD (www.rzpd.de). We used human *ZBTB4* (clone IRAKp961P0289Q2) and human *Kaiso* (clone IRAKp961D2172Q2). A full-length cDNA clone of *ZBTB38* was obtained by PCR on a commercial cDNA preparation of Du145 cells (BD Biosciences). Green fluorescent protein (GFP) and red fluorescent protein (RFP) fusions were obtained by cloning into pEGFP-C2 (Clontech) and a derivative in which enhanced GFP (EGFP) was replaced by mRFP1. For in vitro transcription and translation, cDNAs were cloned into pCITE-4a (Novagen).

Gel retardation assay. Zinc finger domains ranged from residues 455 to 639 for Kaiso, 268 to 460 for ZBTB4, and 418 to 612 for ZBTB38. Protein fragments were produced in vitro using TNT rabbit reticulocyte lysate (Promega) and pCITE-4a (Novagen) constructions as DNA templates. A fragment of the *lacZ* gene was amplified with primers GF048 and GF050 and was used as a probe. The sequence of this fragment is TAATGCGATTAGTGAATTCGCGCGCTGGCTACCGGCGATGAGCGAACGCGTAACGCGAATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCGGCATTATCACATAATGA. Methylation was carried out using SssI (NEB) according to the manufacturer's instructions. Four pmol of DNA probes was labeled using polynucleotide kinase (NEB) and 20 μ Ci [γ -³²P]ATP. Assembly of complexes was carried out in 25 μ l by adding 12.5 μ l 2 \times binding buffer (50 mM HEPES, pH 7.5, 200 mM KCl, 2 mM EDTA, pH 8.0, 20 mM MgCl₂, 0.2% NP-40, 2 mM dithiothreitol, 10% glycerol), 1 μ g bovine serum albumin, 1.2 μ g yeast genomic DNA, 3 μ l lysate, and 0.2 pmol (*lacZ*) or 0.08 pmol (KBS) of labeled probe. Mixes were incubated for 1 h at 4°C, and reactions were run on a 6% polyacrylamide–Tris-acetate-EDTA gel at 10 V/cm for 4 h. When used, competing double-stranded oligonucleotides were added to a final concentration of 3.3 nM (1 \times) or 6.6 nM (2 \times) when using KBS as a probe and 0.08 μ M (10 \times) or 0.8 μ M (100 \times) when using *lacZ*. Single-stranded oligonucleotides corresponding to nonmethylated CpG (GF88/GF89), hemimethylated CpG (GF88/GF91), fully methylated CpG (GF90/GF91), methylated CpA (GF110/GF109), methylated CCTGG (GF117/GF118), mutated matrilysin sequence (GF134/GF135), or wild-type matrilysin sequence (GF128/GF129) were boiled in H₂O–50 mM NaCl and then annealed by cooling slowly.

Primers. Primers used are listed in Table 1.

Generation of antibodies. To obtain antisera directed against mouse and human proteins, two rabbits were immunized with the pair of peptides CERAGV ERTQKGDVG and CGKSFHHPKRLQTHE (ZBTB4) or CSDQDSTDKPWR PYY and MTVMSLSRDLKDDFC (ZBTB38). Three immunizations were performed (day 0, 21, and 42), and a first bleed was collected at day 53. A booster shot was given at day 63, and then a second bleed was taken at day 74. The titer of antibodies directed against the peptides was determined by enzyme-linked immunosorbent assay. The first and second bleeds were performed similarly, both for ZBTB4 and ZBTB38.

Microscopy and immunofluorescence. Twenty-four hours after transfection, GFP-expressing cells were washed with phosphate-buffered saline (PBS), fixed for 10 min at room temperature with 2% paraformaldehyde (PFA), permeabilized for 5 min at 4°C in PBS–0.5% Triton X-100, stained for 3 min with 0.3 μ g/ml 4',6'-

diamidino-2-phenylindole (DAPI) in PBS, and mounted in Vectashield (Vector Laboratories Inc.). Images were acquired on a Zeiss LSM-510 microscope.

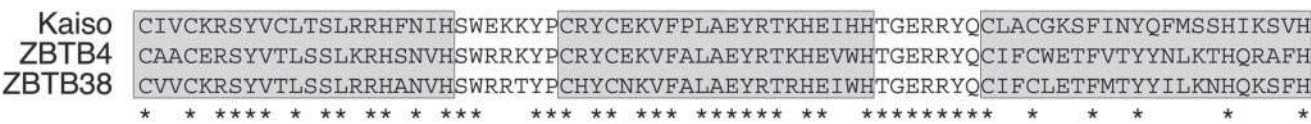
Yeast two-hybrid assay. Interactions between zinc fingers were tested by a GAL4-based system in *Saccharomyces cerevisiae*. Baits and prey were cloned, respectively, into pBGDU(C)1 (20) and pACT3.1 (45). Bait and prey plasmids were introduced, respectively, in strains PJ69-4a and PJ69-4 α (20). Transformants were crossed on yeast extract-peptone-dextrose, and diploids were selected on plates, then grown in liquid medium to saturation, and 10- μ l drops were spotted on selective synthetic complete medium without histidine, supplemented with 1 mM 3-amino-triazole, and on nonselective synthetic complete medium without leucine and uracil. Plates were then incubated at 30°C for 3 days before scoring.

Methylation-dependent repression assay. A repression test was performed as described previously (39). The lower concentration of the effector plasmid was equal to 50 ng, and the higher concentration was 125 ng.

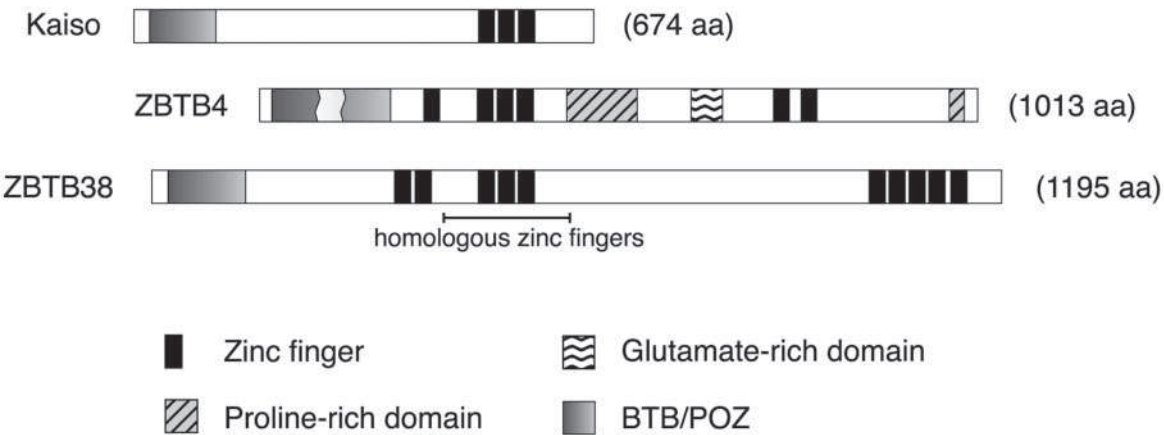
Chromatin immunoprecipitation assay. We crossed female C57 Black mice with male Sd7 mice (13). F₁ animals were sacrificed, and the brains were dissected. Approximately 5 milligrams of tissue was used for each immunoprecipitation. Chromatin was prepared on brain extracts as described on the Upstate website (<http://www.upstate.com>). Chromatin was immunoprecipitated overnight at 4°C on a rotating platform with 20 μ g anti-CTCF antibodies (Upstate reference 06-917), 100 μ g anti-Kaiso (39), anti-ZBTB4, or anti-ZBTB38 serum. After de-cross-linking, the DNA was amplified by PCR with primers DMD56/1 and DMD78/1 under the following conditions: 95°C for 5 min and 25 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s. We used a polymorphism at position 57 in the PCR product. The sequence in the *Mus musculus domesticus* strain is GGCC (which creates an HaeIII site), whereas it is GGAC in *M. musculus spretus*. The PCR products were fractionated on a 2% agarose gel. The bands were excised, and the DNA was purified, then digested with HaeIII, and again run on a 2% agarose gel which was stained with ethidium bromide (0.5 μ g/ml in running buffer). The larger, uncut band represents product originated from the paternal allele, and the shorter product comes from the maternal allele. The following controls were carried out in parallel: immunoprecipitation with no antibodies, immunoprecipitation with no specific antibodies (ZBTB4 preimmune serum), and PCR in the absence of template.

Northern blotting and quantitative reverse transcription-PCR (RT-PCR). A premade mouse tissue Northern blot assay was purchased from Sigma. The full-length mouse *ZBTB4* cDNA was used to probe the membrane. The blot was rehybridized with a *GAPDH* cDNA. Mouse cDNAs prepared from different tissues were purchased from Clontech. *ZBTB4* cDNAs were amplified with the primers PAD184 and PAD185, each on a different side of an intron. Quantitation was done by real-time PCR on a LightCycler (Roche). The housekeeping gene used as a reference was *RPS29*, amplified with primers PAD190 and PAD191. The amount of *ZBTB4* cDNA was normalized to the amount of the

A



B



C

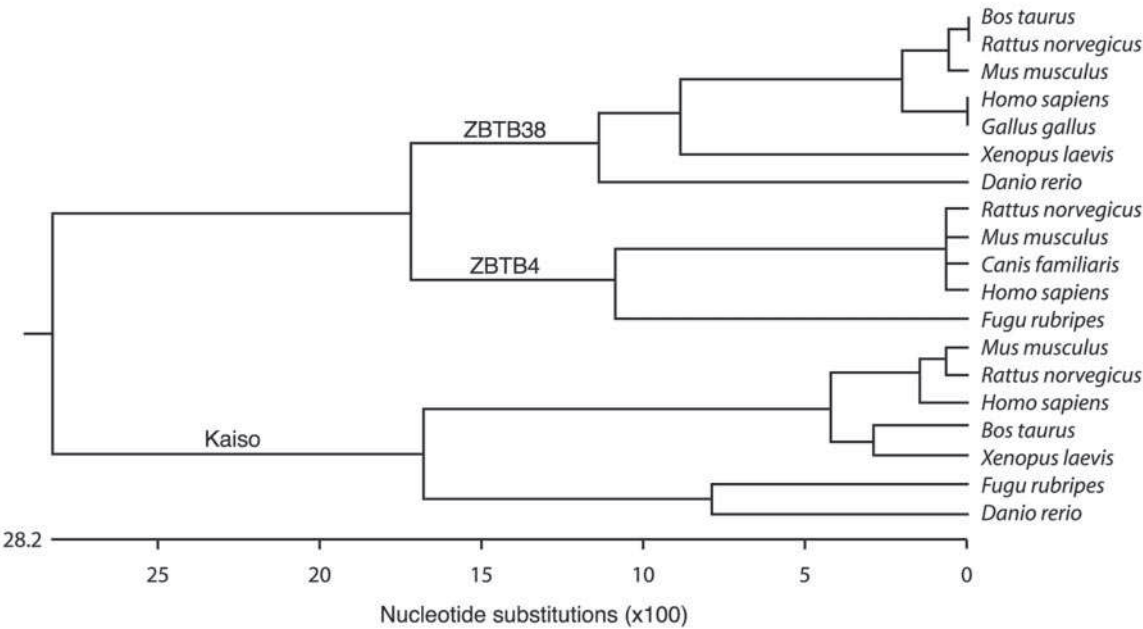


FIG. 1. The human proteins ZBTB4 and ZBTB38 contain Kaiso-like zinc fingers. A. Alignment of a region of human Kaiso with ZBTB4 and ZBTB38. The three zinc fingers are boxed. Residues that are identical between the three proteins are indicated by asterisks. B. Organization of human Kaiso, ZBTB4, and ZBTB38. The proteins were centered on the three conserved zinc fingers. The region used for in vitro DNA-binding assays is underlined. The BTB/POZ domain of ZBTB4 contains an insertion (wavy lines). C. Phylogenetic tree for the Kaiso, ZBTB4, and ZBTB38 gene families. Accession numbers for all aligned genes are available on request.

RPS29 cDNA. The result of an experiment performed in duplicate is shown below in Fig. 7C. It was confirmed in two independent repeats.

In situ hybridization on mouse brain sections. Female 2-week-old mice were anesthetized, and their tissues were fixed by intracardiac perfusion with PBS and then PFA. The brain was dissected, postfixed with PFA, and embedded in bovine

serum albumin-gelatin, and 250- μ m sections were cut with a Vibratome. The sections were then processed for hybridization with a riboprobe as described previously (24). The probe was synthesized by in vitro transcription of a fragment of the mouse ZBTB4 cDNA containing nucleotides 1938 to 2949. The sense and antisense probes were tested in parallel in the same experiment.

RESULTS

Two human proteins have Kaiso-like zinc fingers. Kaiso binds methylated DNA through a three-zinc-finger motif (8, 39); therefore, we reasoned that other human proteins with a similar motif might also be methyl-binding proteins. A BLAST search based on a 78-amino-acid sequence of human Kaiso identified two human proteins with Kaiso-like zinc fingers: ZBTB4 and ZBTB38. ZBTB4 is uncharacterized, while ZBTB38 is the human orthologue of the rat protein Zenon, which was recently cloned in a one-hybrid screen using the tyrosine hydroxylase gene promoter as a target sequence (24). ZBTB4 has 62% identity to Kaiso over the region used for BLAST, while ZBTB38 has 65% identity (Fig. 1A). The next best match to Kaiso was KIAA1559, which is only 38% identical. Many other proteins also have 30 to 35% identity to Kaiso over the region examined, due to the conservation of the zinc finger motif. This sequence analysis suggests that Kaiso, ZBTB4, and ZBTB38, but no other human proteins, have a related triple-zinc-finger domain. Sequence conservation between the proteins is highest for the first two zinc fingers, the third being less conserved. The three zinc fingers of ZBTB4 and ZBTB38 are 79% identical and therefore are closer to one another than either is to Kaiso.

All three proteins have an N-terminal BTB/POZ domain. The BTB/POZ domain of ZBTB38 is the closest relative of Kaiso's BTB/POZ in the databases, with 47% identity over 100 amino acids. The BTB/POZ domain of ZBTB4 is interrupted by a stretch of 60 residues, mainly serines and alanines (Fig. 1B). In mouse and rat the insertion contains fewer amino acids, whereas the dog protein has no insertion. BTB/POZ domains have a conserved three-dimensional structure (1, 34). The insertion in ZBTB4 is located between α -helix 3 and β -sheet 4 and might not alter the dimerization surface. There is little sequence identity between Kaiso, ZBTB4, and ZBTB38 outside of the BTB/POZ domain and the three zinc fingers mentioned above. ZBTB4 and ZBTB38 contain additional zinc fingers, but those are not similar to the methyl-DNA-binding motif of Kaiso. Finally, motif prediction algorithms also identified two proline-rich domains and one glutamate-rich domain in ZBTB4.

Examination of the available genome sequences showed that all vertebrates contain Kaiso and at least one gene orthologous to ZBTB4 or ZBTB38. For example, *Fugu rubripes* seems to have only a ZBTB4 orthologue. As the genome sequences of *Bos taurus*, *Canis familiaris*, and *Gallus gallus* are not yet complete, it is possible that additional genes of this family are present in those organisms. In accordance with the identity scores, the phylogenetic tree built on the Kaiso-like zinc fingers shows that Kaiso diverged earlier than the separation of ZBTB4 and ZBTB38 (Fig. 1C). The phylogenetic tree based on comparison of the full-length proteins confirmed this trend (not shown).

Kaiso, ZBTB4, and ZBTB38 bind methylated DNA in vitro. A portion of Kaiso containing the three zinc fingers (amino acids 455 to 639 in the human protein) binds methylated DNA in vitro (8). We tested whether the homologous regions in ZBTB4 and ZBTB38 (outlined in Fig. 1B) also recognize methylated DNA. We synthesized in vitro the conserved zinc fingers of Kaiso, ZBTB4, and ZBTB38. We then tested the affinity of these proteins for methylated DNA by gel retarda-

tion assay (Fig. 2A). The probe was a 161-bp CG-rich fragment from the *lacZ* gene that was either unmethylated or fully methylated in vitro by the bacterial methyltransferase SssI. The zinc fingers of Kaiso, ZBTB4, and ZBTB38 all failed to bind the nonmethylated DNA probe. In contrast, all three recognized the methylated probe. Multiple complexes were observed. This was likely due to the fact that each molecule of probe contains several methylated CpGs: the bands of increasing molecular weight probably correspond to the DNA probe complexed to an increasing number of protein molecules. From these results we conclude that the Kaiso-like zinc fingers of ZBTB4 and ZBTB38 have a specific affinity for methylated DNA.

The three zinc fingers of Kaiso can bind the symmetrically methylated sequence CGCG, but also the consensus sequence TCCTGCNA (8), called the KBS. We sought to determine whether ZBTB4 and ZBTB38 could also bind the KBS. This was achieved by gel retardation experiments using a labeled KBS probe (Fig. 2B). As expected, Kaiso bound the KBS. We found that ZBTB4, but not ZBTB38, could also bind the KBS. Using a mutant KBS competitor (TCCCGCCA), we checked that the binding of Kaiso and ZBTB4 was specific, as the mutant KBS had poorer competing ability than the wild-type KBS (not shown). From this we conclude that the zinc fingers of ZBTB4, like those of Kaiso, have a bimodal specificity and that they can bind the sequence TCCTGCNA. We then sought to evaluate the relative affinity of ZBTB4 for methylated DNA and for the KBS. Complexes formed between ZBTB4 and a labeled KBS were incubated with excess unlabeled KBS or with the same molar amount of unlabeled oligonucleotide containing a single methylated CpG (Fig. 2B). Methylated DNA was a better competitor than the KBS, showing that ZBTB4 has higher in vitro affinity for methylated DNA than for a KBS.

Next we examined more precisely the capacity of ZBTB4 and ZBTB38 to bind methylated DNA. We carried out gel retardation assays with the methylated probe, in the presence of excess unlabeled competitor oligonucleotides bearing different methylated sequences. A series of oligonucleotides contained a single CpG that was unmethylated, hemimethylated, or symmetrically methylated. We also tested oligonucleotides containing a single methylated CpA or a single methylated CCTGG, which have been shown to be present in small amounts in human cells (40) (32). ZBTB4 and ZBTB38 did not bind unmethylated oligonucleotides, nor oligonucleotides containing methylated CpA or CCTGG (Fig. 2C and D). In contrast, binding of ZBTB4 and ZBTB38 to the labeled probe was efficiently competed by oligonucleotides containing a single symmetrically methylated CpG. In addition, ZBTB4 showed some affinity for hemimethylated CpGs, yet this was much lower than for symmetrically methylated CpG, since the hemimethylated competitor did not fully displace the complexes, even at 100-fold excess. These data show that ZBTB4 and ZBTB38 bind methylated CpGs, but no other methylated sequence. They also point to an important difference between Kaiso and its relatives, as Kaiso requires at least two consecutive methylated CpGs for binding (39), while ZBTB4 and ZBTB38 can both bind single methylated CpGs.

Ectopically expressed ZBTB4 and ZBTB38 are targeted to regions containing methylated DNA. In the nucleus of mouse cells, the pericentric regions of different chromosomes aggregate to form structures called chromocenters (27). The chro-

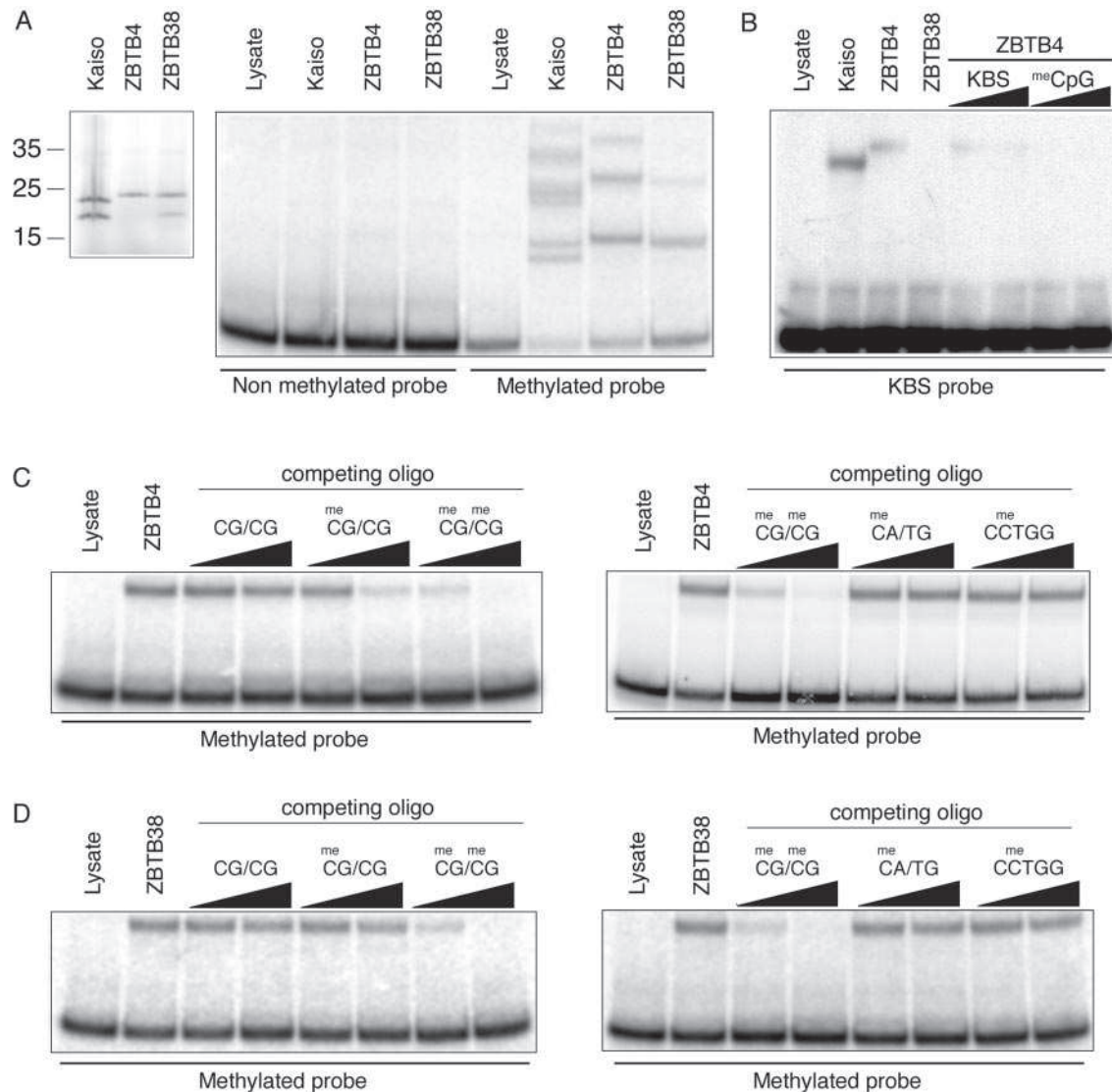


FIG. 2. ZBTB4 and ZBTB38 bind methylated CpGs in vitro. A. Left panel: the zinc fingers of human Kaiso, ZBTB4, and ZBTB38 were expressed in rabbit reticulocyte lysate in the presence of radioactive methionine and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular masses in kilodaltons are indicated. The faster-migrating bands arise from internal initiation. The proteins were then incubated with a labeled 161-bp probe containing multiple CG, CGCG, and CGNCG sequences and resolved on a 6% polyacrylamide gel. The probe was either left unmethylated or methylated in vitro with *Sss*I. Kaiso, ZBTB4, and ZBTB38 form complexes only with the methylated probe. The different bands correspond to complexes involving different numbers of proteins bound per DNA molecule. B. Gel retardation assay with a KBS. Kaiso and ZBTB4 bind the KBS, but ZBTB38 does not. The complex between ZBTB4 and the KBS was competed by unlabeled KBS or by equal molar amounts (onefold and twofold concentration relative to the probe) of an oligonucleotide containing a single methylated CpG. C. Complexes between the zinc fingers of ZBTB4 and the methylated probe were assembled in the presence of oligonucleotides containing the indicated sequences (10-fold and 100-fold molar excess relative to the probe). The oligonucleotide containing a single symmetrically methylated CpG efficiently displaces the complex, whereas an unmethylated oligonucleotide does not. A hemimethylated oligonucleotide has an intermediate effect. D. Results of an experiment carried out with ZBTB38 as for panel C. Only the oligonucleotide containing a single symmetrically methylated CpG displaces the complex.

nocenters contain the major satellite repeats, which are heavily methylated. In addition, these structures are readily detectable by microscopy, as they stain very brightly with DAPI. We asked whether ZBTB4 and ZBTB38, like MeCP2 (28) and MBD1 (22), would localize to the chromocenters when expressed in mouse cells.

We transfected NIH 3T3 cells with cDNAs encoding human ZBTB4 or ZBTB38 and performed immunofluorescence with

antibodies specific for these proteins (Fig. 3A). In both cases, we detected a strong signal colocalizing with the chromocenters. There was little signal in the rest of the nucleus, suggesting that most transfected ZBTB4 or ZBTB38 is recruited to the chromocenters of mouse cells. We then constructed plasmids expressing fusions of ZBTB4 and ZBTB38 to the red fluorescent protein mRFP1 (4). We transfected the constructs in NIH 3T3 cells and observed a clear colocalization

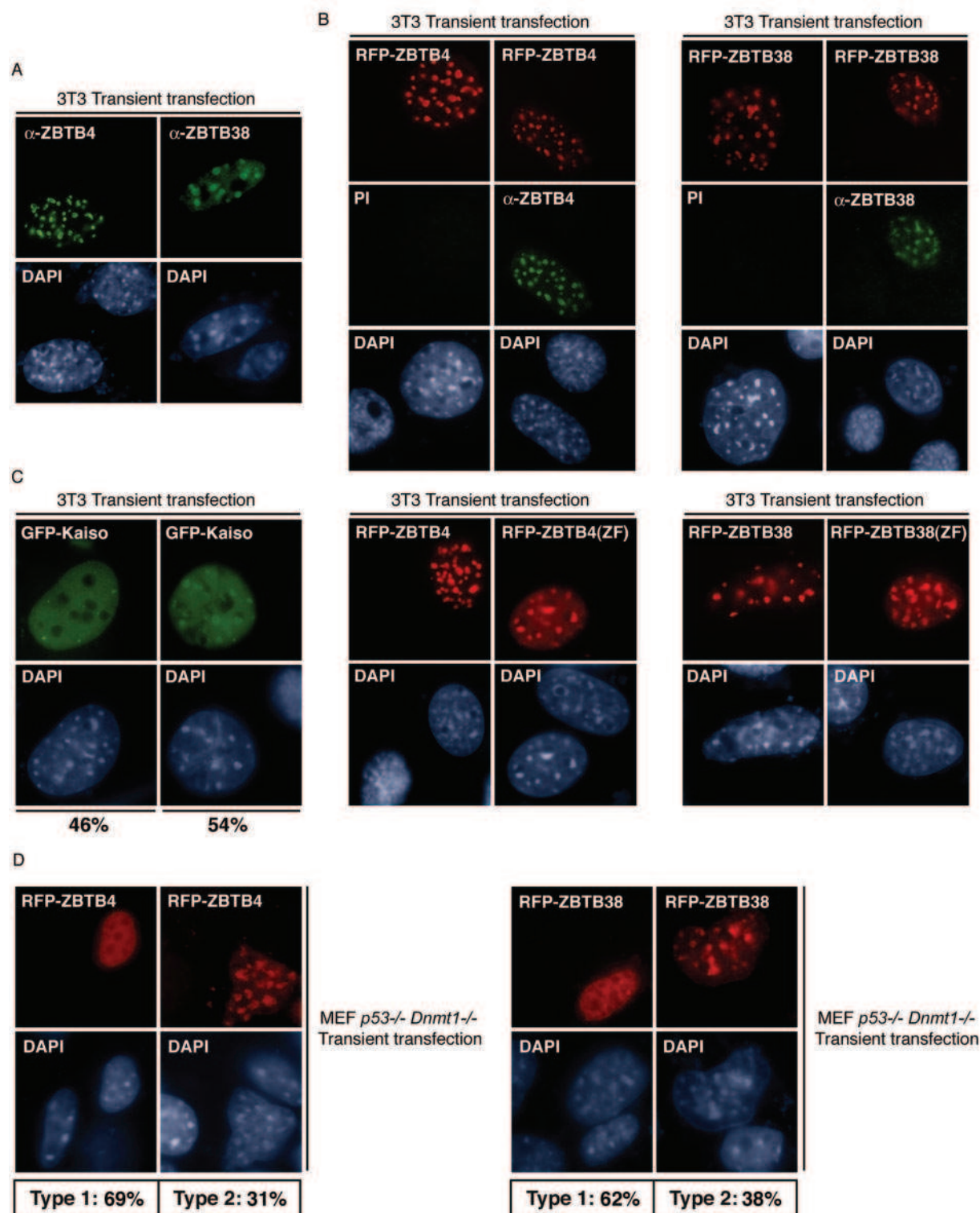


FIG. 3. ZBTB4 and ZBTB38 are recruited to methylated DNA in cells. A. Characterization of the antibodies directed against ZBTB4 and ZBTB38. NIH 3T3 cells (which do not detectably express ZBTB4 or ZBTB38) were transfected with the indicated human cDNAs, used for immunofluorescence, and counterstained with DAPI. Images were taken at 100 \times magnification. Nontransfected cells show no detectable signal. B. RFP-ZBTB4 shows the same localization pattern as ZBTB4 and is recruited to the chromocenters. 3T3 cells were transfected with an expression vector encoding a fusion of mRFP1 to ZBTB4 and then used for immunofluorescence. The red fluorescence colocalizes with the green signal observed after immunofluorescence with anti-ZBTB4 serum and with the DAPI-dense chromocenters (in blue). Cells treated with the preimmune serum (PI) show no green signal. RFP-ZBTB38, like ZBTB38, is also recruited to the chromocenters. C. The localization of GFP-Kaiso in the nucleus of transfected mouse cells is different from that of ZBTB4 or ZBTB38. The zinc fingers of ZBTB4 and ZBTB38 are sufficient for recruitment to the chromocenters. 3T3 cells were transfected with plasmids encoding the indicated fusions of fluorescent proteins to full-length

of the red fluorescence with the DAPI-bright chromocenters (Fig. 3B). This proves that the fluorescent tag does not interfere with the recruitment of the proteins to methylated DNA. Next we tested whether the three zinc fingers that bind methylated DNA in vitro could direct the proteins to the methylated chromocenters. We fused the Kaiso-like zinc fingers of ZBTB4 and ZBTB38 to mRFP1 and, again, we observed foci of red fluorescence that colocalized with the DAPI-dense chromocenters (Fig. 3C). This proves that the Kaiso-like zinc fingers are sufficient for recruitment to the chromocenters. For comparison purposes, we also transfected NIH 3T3 cells with a plasmid expressing human Kaiso fused to GFP (Fig. 3C). We observed two types of motifs: in about half of the cells the nucleus showed a diffuse GFP staining with a few bright dots. In the rest of the cells the GFP signal was present in the whole nucleus with a faint enrichment at the chromocenters. The mouse major satellite repeats do not contain the sequence CGCG; therefore, it seems unlikely that Kaiso is recruited to the chromocenters through direct binding to methylated DNA. Even though we cannot fully explain why there was an enrichment to the chromocenters in some cells, these data support the fact that Kaiso has a different in vivo specificity from ZBTB4 and ZBTB38.

Finally, we wanted to test whether the pattern observed depends on the presence of methylated DNA in the chromocenters. We transfected the fluorescent fusion proteins in mouse cells that are impaired for DNA methylation (22) (a kind gift from Howard Cedar). In these mouse embryo fibroblast cells, the catalytic domain of the maintenance DNA methyltransferase Dnmt1 is deleted, so that the overall methylation level is drastically reduced. However, DNA methylation is not completely abrogated, due to the residual activity of the de novo methyltransferases Dnmt3a and Dnmt3b. The mutation of *Dnmt1* is lethal in wild-type differentiated cells but is viable in the absence of p53. Therefore, the *Dnmt1* mutation was made in a *p53*^{-/-} background, and *p53*^{-/-} mouse embryo fibroblasts containing Dnmt1 were used as a control of the experiment. Both mRFP-ZBTB4 and mRFP-ZBTB38 colocalized with the chromocenters in 100% of the transfected *p53*^{-/-} cells (not shown). In contrast, in a majority of *p53*^{-/-} *Dnmt1*^{-/-} cells, the localization of mRFP-ZBTB4 and mRFP-ZBTB38 was diffuse in the nucleus (Fig. 3D). In the remaining cells a fraction of the proteins localized to the chromocenters with variable degrees of diffuse nuclear signal. We also observed incomplete delocalization of GFP-MeCP2 in the *p53*^{-/-} *Dnmt1*^{-/-} cells. As previously pointed out by other investigators (36), this variability is probably due to varied levels of demethylation in the cell population. This shows that mutation of *Dnmt1*, and the consequent demethylation of the major satellite repeats, inhibits recruitment of ZBTB4 or ZBTB38 to the chromocenters. We cannot formally rule out the possibility that ZBTB4 and ZBTB38 are recruited to the chromocenters indirectly, via another heterochromatin-binding protein. However, these data, together with our in vitro

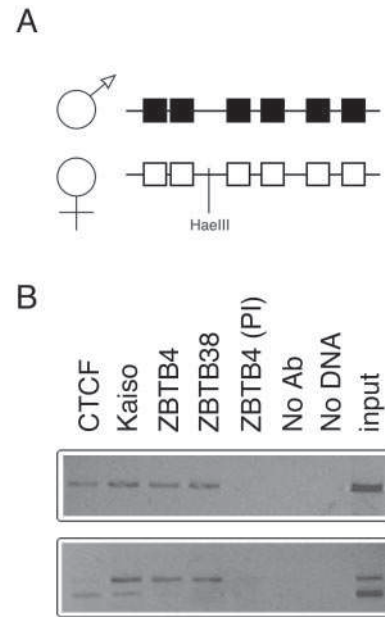


FIG. 4. ZBTB4 and ZBTB38 bind the methylated allele of the *H19/Igf2* DMR. A. Schematic showing the structure of the imprinted locus. The paternal allele of the DMR is fully methylated (black boxes), whereas the maternal allele is unmethylated (white boxes) and contains an HaeIII restriction site. B. Chromatin immunoprecipitations using the indicated antibodies were performed on mouse brain extracts as indicated in the text. Top panel: the immunoprecipitated DNA was amplified with primers that amplify both alleles. Bottom: the PCR products seen above were digested with HaeIII to identify the parental origin of the amplified DNA. CTCF interacts predominantly with the maternal unmethylated allele, whereas ZBTB4 and ZBTB38 interact mostly with the paternal methylated allele. Input, 0.5% of the input chromatin.

results, strongly argue that ZBTB4 and ZBTB38 bind methylated DNA in vivo.

Endogenous ZBTB4 and ZBTB38 bind methylated DNA in vivo. We next asked whether the endogenous ZBTB4 and ZBTB38 proteins would bind a specific methylated locus. The parental origin of imprinted genes determines their methylation status and consequently their level of expression (11). The *H19/Igf2* locus on mouse chromosome 7 contains two reciprocally imprinted genes whose expression depends on a differentially methylated region (DMR) methylated only on the paternal allele. To be able to distinguish alleles, we crossed female *M. musculus domesticus* mice to male Sd7 mice (13) (an *M. musculus domesticus* strain containing the distal portion of *Mus spretus* chromosome 7 [a gift from Wolf Reik]). We first mapped single-nucleotide polymorphisms within the part of the DMR that had the highest density of CpGs and identified a single-nucleotide polymorphism that creates an HaeIII restriction site in the *M. musculus domesticus* strain (Fig. 4A). We isolated chromatin from the brain of hybrid mice and then

Kaiso, ZBTB4, and ZBTB38 or to the zinc fingers of ZBTB4 and ZBTB38. D. ZBTB4 and ZBTB38 are removed from the chromocenters in a majority of methylation-deficient cells. The fluorescent fusion proteins were expressed in *p53*^{-/-} *Dnmt1*^{-/-} mouse embryo fibroblasts. One hundred twenty cells were scored for total loss (left column) or partial loss (right column) of signal colocalizing with the chromocenters. Both ZBTB4 and ZBTB38 colocalize with the chromocenters in 100% of control *p53*^{-/-} cells (not shown).

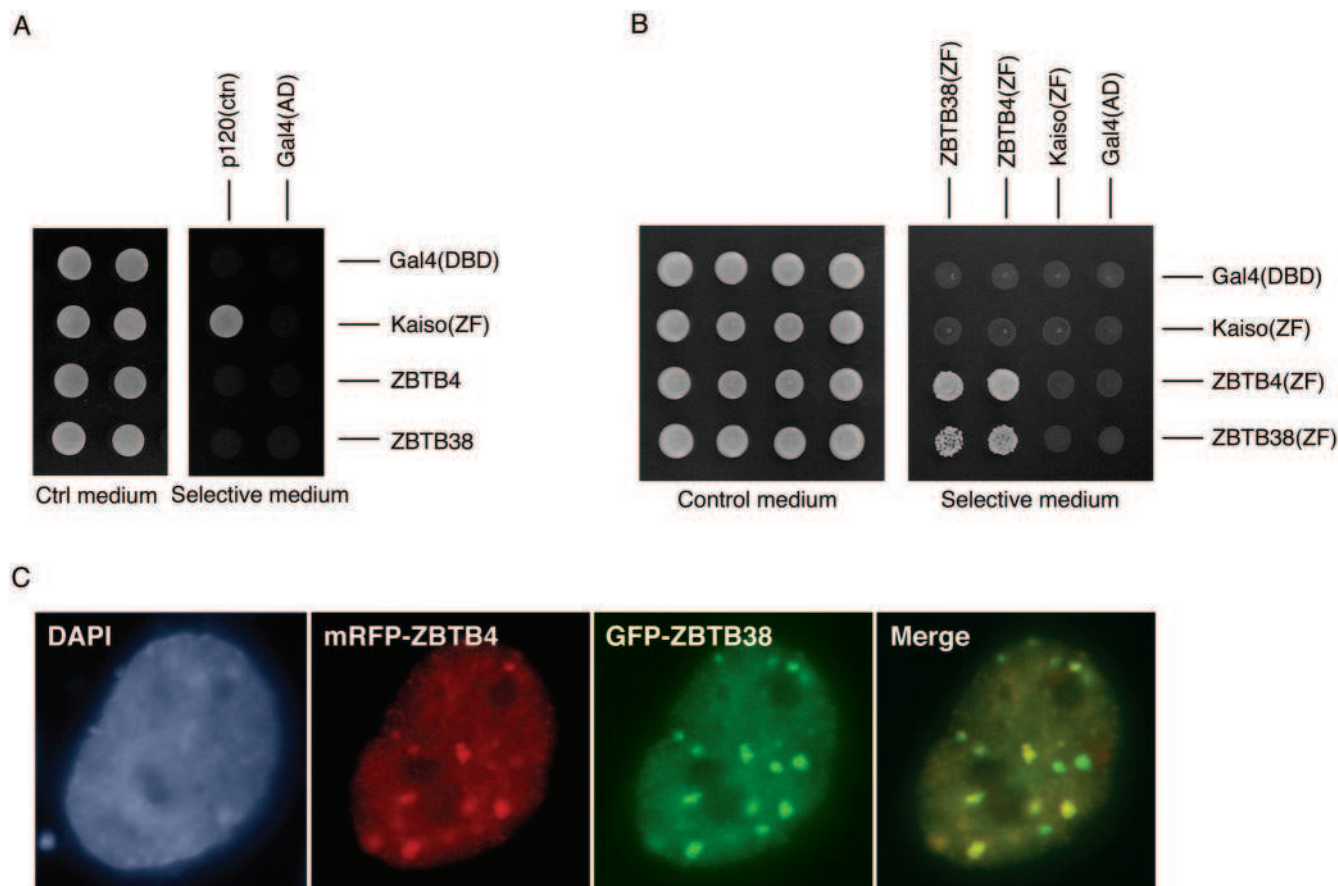


FIG. 5. ZBTB4 and ZBTB38 interact with each other, but not with Kaiso or p120 catenin. A. Kaiso, but not ZBTB4 or ZBTB38, interacts with p120-catenin. B. The zinc finger domain of ZBTB4 and ZBTB38 can homo- and heterodimerize. The indicated proteins or protein domains were fused to the Gal4 DNA-binding domain (DBD) or activation domain (AD) and introduced in the reporter strain. The transformed cells were spotted on control medium (selecting for both plasmids) or on selective medium (selecting for the interaction between the proteins). C. ZBTB4 and ZBTB38 form dots in transfected HeLa cells. Some of the dots show clear colocalization, whereas others seem to contain only ZBTB38.

immunoprecipitated the protein-DNA complexes with antibodies directed to CTCF, Kaiso, ZBTB4, and ZBTB38. We next performed PCR on the immunoprecipitated DNA with primers that amplify the DMR. The resulting product was digested with HaeIII and analyzed on a gel (Fig. 4B). CTCF is known to bind the unmethylated maternal DMR. As expected, the CTCF antibody precipitated DNA of maternal origin. In contrast, an antiserum directed against Kaiso precipitated mostly paternal DNA. The antisera directed against ZBTB4 and ZBTB38 both precipitated the methylated paternal DMR but not the unmethylated maternal DMR. It is presently unknown if the binding of ZBTB4 and ZBTB38 to the methylated allele influences the expression of *H19* and *Igf2*. Nevertheless, this result suggests that ZBTB4 and ZBTB38, like Kaiso, bind methylated DNA in vivo.

Kaiso and ZBTB4/ZBTB38 have distinct binding partners.

The zinc fingers of Kaiso interact with p120-catenin (7) to regulate targets of the Wnt pathway (38). We sought to determine if ZBTB4 and ZBTB38 also interact with p120-catenin. To address this question, we used a Gal4-based two-hybrid assay in which interaction activates *HIS3* and permits growth on selective medium without histidine (20). As expected, the zinc finger domain of Kaiso interacted with p120-catenin in

this system (Fig. 5A), whereas ZBTB4 and ZBTB38 failed to interact. Next, we tested homotypic and heterotypic interactions between the zinc finger domains. We observed that the zinc finger domains of ZBTB4 and ZBTB38 can homo- and heterodimerize (Fig. 5B) but do not interact with the zinc fingers of Kaiso. This probably explains the observation that Zenon, that rat orthologue of ZBTB38, can homodimerize independently of the BTB/POZ domain (24).

The interaction observed in the two-hybrid assay suggested that ZBTB4 and ZBTB38 might colocalize in the nucleus. We thus cotransfected HeLa cells with mRFP-ZBTB4- and GFP-ZBTB38-expressing plasmids. We observed that in the majority of transfected cells, both proteins form punctate patterns (Fig. 5C). The overlap of these patterns was variable, as some dots seemed to contain only one protein, whereas others contained both. Unlike mouse cells, human cells such as HeLa do not have chromocenters. The nature of the nuclear structures in which ZBTB4 and ZBTB38 concentrate is currently under investigation. Taken together, these data show that ZBTB4 and ZBTB38 interact and colocalize. In contrast, they do not interact with Kaiso or p120-catenin, suggesting that they are involved in distinct cellular functions.

ZBTB4 and ZBTB38 are methyl-dependent transcriptional repressors. Since Kaiso and several other BTB/POZ zinc finger proteins are transcriptional repressors (6), we set out to determine whether ZBTB4 and ZBTB38 were repressors and whether repression was specific to methylated DNA. We used an assay in which ZBTB4 and ZBTB38 expression constructs were cotransfected with methylated or nonmethylated reporter plasmids. The reporter gene used was luciferase expressed from a simian virus 40 promoter, in a plasmid that contains 48 CGs and 11 CGCGs. Since the Mbd2 protein is abundant in cells and strongly represses the transcription of methylated reporter genes, we used *Mbd2*^{-/-} cells, in which methylated templates maintain a detectable level of expression (15).

The different expression constructs were cotransfected along with the methylated and the unmethylated reporter, and the ratio of these values was plotted. In the absence of exogenous Kaiso, ZBTB4, or ZBTB38, the activity of the methylated reporter was 25% that of the nonmethylated reporter (Fig. 6A). When increasing amounts of Kaiso expression vectors were cotransfected, this ratio fell to 12% and 6%, reflecting the fact that Kaiso represses transcription of the methylated plasmid. Upon transfection of ZBTB4, the methylated plasmid had less than 1% of the activity of the unmethylated plasmid cotransfected with the same amount of ZBTB4. This proves that ZBTB4 is a potent repressor of the methylated reporter. ZBTB38 behaved similarly to Kaiso: cotransfection with a methylated plasmid reduced the activity to 6% of control values. From these results we conclude that ZBTB4 and ZBTB38 are methyl-dependent transcriptional repressors.

We next wanted to delineate the regions responsible for this activity. Deleting the POZ domain of ZBTB4 (construct 1) or its C-terminal half (construct 4) did not affect its repressive effect (Fig. 6C). In contrast, expression of a truncated form of ZBTB4 lacking the Kaiso-like zinc fingers (construct 2) was inactive. However, this deleted protein failed to enter the nucleus. We thus added the nuclear localization signal of Kaiso to the sequence (construct 3) and verified that the resulting protein was nuclear (not shown). This nuclear protein was still devoid of repressive activity on the methylated plasmid, suggesting that the Kaiso-like zinc fingers are necessary for methyl-dependent repression. A construct containing only the Kaiso-like zinc fingers and their neighboring regions (construct 5) was almost as efficient as full-length ZBTB4 for repression. In the case of ZBTB38, deletion of the POZ domain (construct 6) resulted in loss of repression (Fig. 6D). Deleting the C-terminal half of the protein did not affect repression (construct 7), suggesting that methyl-dependent repression involves the Kaiso-like zinc fingers, the POZ domain, and possibly the first two zinc fingers, located between these two domains.

ZBTB4 and ZBTB38 have different expression patterns. The expression profile of Zenon, the rat homologue of ZBTB38, has been described in detail (24). The gene is transcribed in the brain and in neuroendocrine tissues. We investigated the expression pattern of ZBTB4 in mouse tissues. Northern blotting on adult mouse tissues revealed four species of transcripts with estimated sizes of 5.5, 6.0, 6.7, and 8.3 kb (Fig. 7A), which likely arise by alternative splicing of the six predicted exons of mouse ZBTB4. Transcripts were detected in all tissues, with high expression levels in the brain, lung, kidney, muscle, and heart, an intermediate level in placenta, liver, spleen, and thy-

mus, and lowest expression in the testis. We also performed RT-PCR analysis on cDNAs from mouse tissues (Fig. 7B). For this we used a primer pair that spans an intron and that amplifies the cDNAs containing the zinc finger region. The results obtained on cDNAs from adult tissues were in good general agreement with the Northern blot assay, except for the muscle where the amount of transcripts is significantly higher when detected by Northern blotting. A possible explanation may be that some splice variants are not amplified by the primer pair we chose. In addition, RT-PCR revealed that transcripts were undetectable at the four embryonic stages that we tested. To get more precise information on the cell types that express ZBTB4 in the central nervous system, we performed in situ hybridization on mouse brain sections (Fig. 8). We observed staining in many areas which corresponded to neuronal populations but were not limited to a single class of neurotransmitters. We did not detect ZBTB4 expression in glial cell populations. The amount of ZBTB4 messenger was highest in the hippocampus, a region that also expresses Mbd1, Mbd2, and MeCP2 (23). Intense staining was also seen in several structures involved in olfaction: the olfactory bulb, piriform cortex, and habenular nuclei. ZBTB4 was also expressed in other specific areas, including the arcuate nuclei, the motor nuclei of the brainstem, and the granular layer of the cerebellum. Our data suggest that ZBTB4 controls gene expression in different types of neurons. More specifically, it may be involved in olfactory, motor, and hippocampal functions. The fact that *Mbd1*^{-/-} mutant mice have specific defects in hippocampal functions (47) suggests that the epigenetic control of gene expression may be of special importance in that structure.

DISCUSSION

We have identified ZBTB4 and ZBTB38, two human proteins related to Kaiso. These proteins share three zinc fingers similar to those of Kaiso and a similar architecture, including an N-terminal BTB/POZ domain. ZBTB4 and ZBTB38 are more related to one another than they are to Kaiso, and this gene family probably resulted from two gene duplication events of a common ancestor, the first yielding a precursor of Kaiso and a ZBTB4/ZBTB38 precursor which then diverged after a second duplication. We failed to detect a fourth Kaiso relative in the human genome, indicating either that the Kaiso precursor was not duplicated or that the other gene in the pair was lost. Our results show that all three proteins bind methylated DNA. This argues that this was also a property of their common ancestor and predicts that, in addition to Kaiso, some Kaiso-like proteins that bind methylated DNA should be present in all vertebrates.

This study of Kaiso-related proteins gives us some insight on how Kaiso may regulate target genes. The linker sequence most frequently found in human proteins between two adjacent zinc fingers is TGEKP, in which the glycine residue caps the preceding helix and stabilizes binding to DNA (26). The linker between the first and second zinc fingers of Kaiso is SWEKK; it is clearly divergent from the canonical sequence and does not include a glycine. A possible explanation would be that the first zinc finger of the domain is not involved in DNA binding, but in protein-protein interactions. Indeed, the first zinc finger of Kaiso is well conserved in ZBTB4 and ZBTB38, yet it is dispensable for binding DNA (8). This situ-

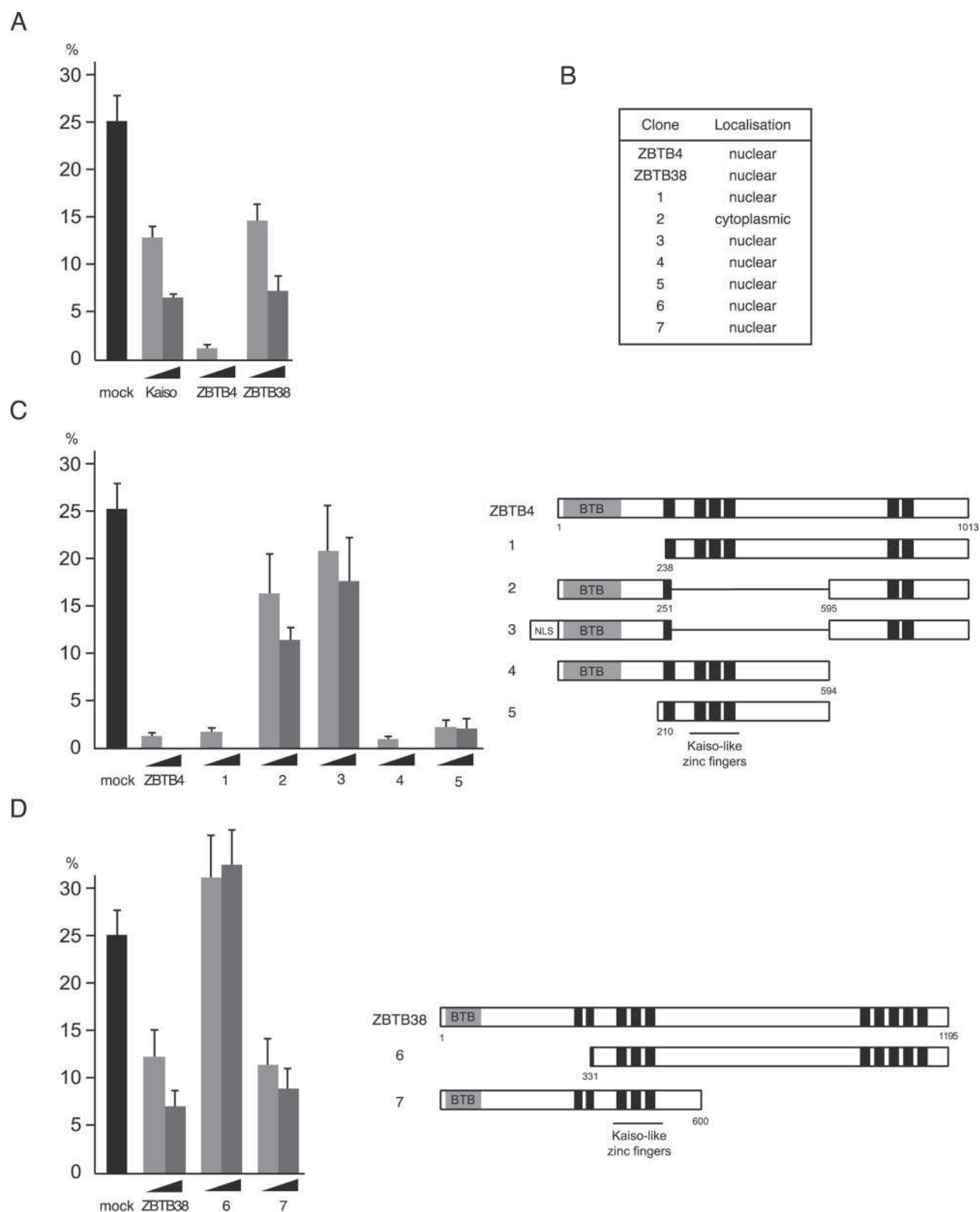


FIG. 6. ZBTB4 and ZBTB38 repress transcription in vivo in a methyl-CpG-dependent manner. A. Methyl-CpG-dependent repression by Kaiso, ZBTB4, and ZBTB38. The protein expression constructs (lower concentration [gray bars] and higher concentration [shadowed bars]) were cotransfected with a simian virus 40-luciferase reporter into *Mbd2*^{-/-} mouse cells. The percent activity of the methylated plasmid is plotted [(methylated reporter expression)/(nonmethylated reporter expression) $\times 100$]. Results are the averages of at least three experiments. B. Subcellular localization of the constructs used in the deletion study. C. The Kaiso-like zinc fingers of ZBTB4 are required for repression. The indicated truncated derivatives of ZBTB4 were transfected as for panel A. D. The BTB/POZ domain and Kaiso-like zinc fingers of ZBTB38 are required for repression. The experiment was performed as for panel C.

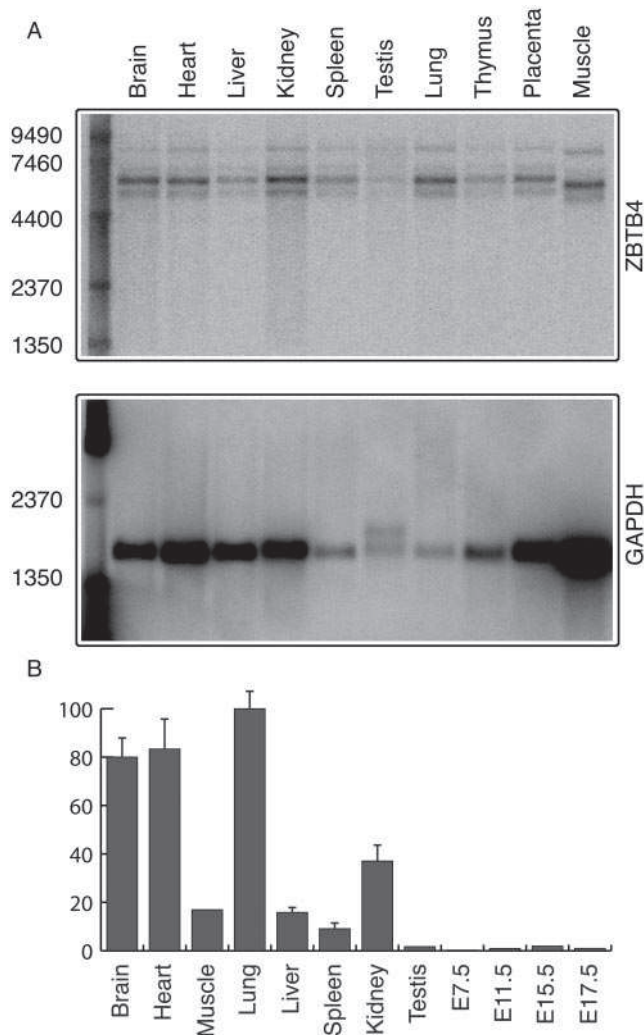


FIG. 7. ZBTB4 is expressed in several adult tissues, but not in embryos. A. Northern blotting on RNAs extracted from adult mouse tissues. Top panel: the blot was hybridized with a full-length cDNA of *ZBTB4*. Lower panel: the blot was hybridized with a standardizing cDNA of *GAPDH*. B. RT-PCR on RNAs extracted from the indicated adult or embryonic tissues. The amount of *ZBTB4* mRNA was quantified by real-time PCR after reverse transcription. It was normalized to the amount of *RPS29* signal.

ation would parallel that of the protein GATA-1, in which one of the zinc fingers is used to interact with the cofactor FOG-1 (31). We observed that ZBTB4 and ZBTB38 can bind a single methylated CpG, whereas Kaiso cannot: it requires at least two consecutive CpGs for binding (39). It might be that two molecules of Kaiso each recognize a CpG and that their interaction stabilizes binding. Regulation of this hypothetical dimerization might be a mechanism to regulate the binding of Kaiso to DNA. An important question for the future will be to identify the determinants that allow Kaiso, ZBTB4, and ZBTB38 to bind methylated DNA. The examination of conserved residues between the proteins will be helpful in this prospect.

Three lines of evidence suggest that Kaiso, ZBTB4, and ZBTB38 have different biological functions. First, they have different protein partners. Kaiso interacts with the p120-catenin to regulate Wnt target genes (38), but ZBTB4 and ZBTB38 do not

interact with this protein in a two-hybrid assay (Fig. 5). Also, ZBTB4 and ZBTB38 interact with each other, but do not interact with Kaiso. Second, the proteins have different DNA-binding specificities. ZBTB4 and ZBTB38 can bind single methylated CpGs, whereas Kaiso needs two such methylated CpGs in a row. Kaiso and ZBTB4 recognize the Kaiso binding site, TCCTGCNA, and ZBTB38 binds the E-box CACCTG (24). Hence, they have distinct sets of potential binding sites. Finally, the three genes have different expression patterns. ZBTB38 mRNA is restricted to the brain and neuroendocrine organs, while ZBTB4 has a broader distribution but seems to be particularly expressed in the brain. Kaiso on the other hand is widely expressed but shows the lowest expression in the brain (7).

Kaiso is a complex transcriptional regulator that targets both methylated DNA and nonmethylated consensus sequences (8, 39). It seems to be also the case for ZBTB4 and ZBTB38. Furthermore, Zenon, the rat homologue of ZBTB38, activates transcription when bound to the E-box in the tyrosine hydroxylase gene promoter (24), while it represses transcription when bound to methylated DNA in our assay. This raises the possibility that ZBTB4 may as well be a repressor or an activator depending on the context. The fact that the proteins inhibit transcription of a reporter gene when bound to methylated DNA suggests that they might be bona fide regulators of methylated sequences. Proving this possibility will require the identification of endogenous genes that are repressed by these proteins upon methylation. In mammals, they might also contribute to the regulation of parentally imprinted genes. Parental imprinting plays a key role in controlling the growth of the placenta, an organ in which ZBTB4 is present. Interestingly, recent studies suggest that expression of imprinted genes in the brain, and especially in the hippocampus, may impact on behavior (9, 10). Since ZBTB4 is highly expressed in that region, it is possible that it plays a role in this particular aspect of imprinting. Our chromatin immunoprecipitation results suggest that both ZBTB4 and ZBTB38 bind the methylated allele of the *H19/Igf2* DMR locus, yet further analyses will be needed to determine if this binding plays any role in the transcriptional regulation of these genes.

There is an increasing number of reports confirming that methylated genes are transcriptional targets for the MBPs (5, 17, 18, 33). However, deletion of *Mbd1* (47), *Mbd2* (15), *MeCP2* (14), and *Kaiso* (A. Prokhortchouk et al., submitted for publication) and even the combined deletion of *Mbd2* and *MeCP2* (14) do not significantly impair mouse development. This contrasts with the embryonic lethality observed when DNA methylation is impaired by a mutation of *Dnmt1* (30). One possible explanation for this discrepancy is that MBPs are not the sole mechanism of DNA methylation-driven repression. Models have been proposed arguing that methylation of cytosines acts in part by prohibiting the binding of activating transcription factor. One such example is that methylation of the differentially methylated region in the *H19/Igf2* locus prevents binding of CTCF, thereby maintaining the imprinted status of the locus (2). An alternative explanation to the discrepancy would be the existence of extensive functional redundancy within the MBP family. One last possible explanation would be that the genome contains additional, currently unidentified MBPs. Our search for new MBPs was driven by this last consideration.

While database searches of the human genome identified

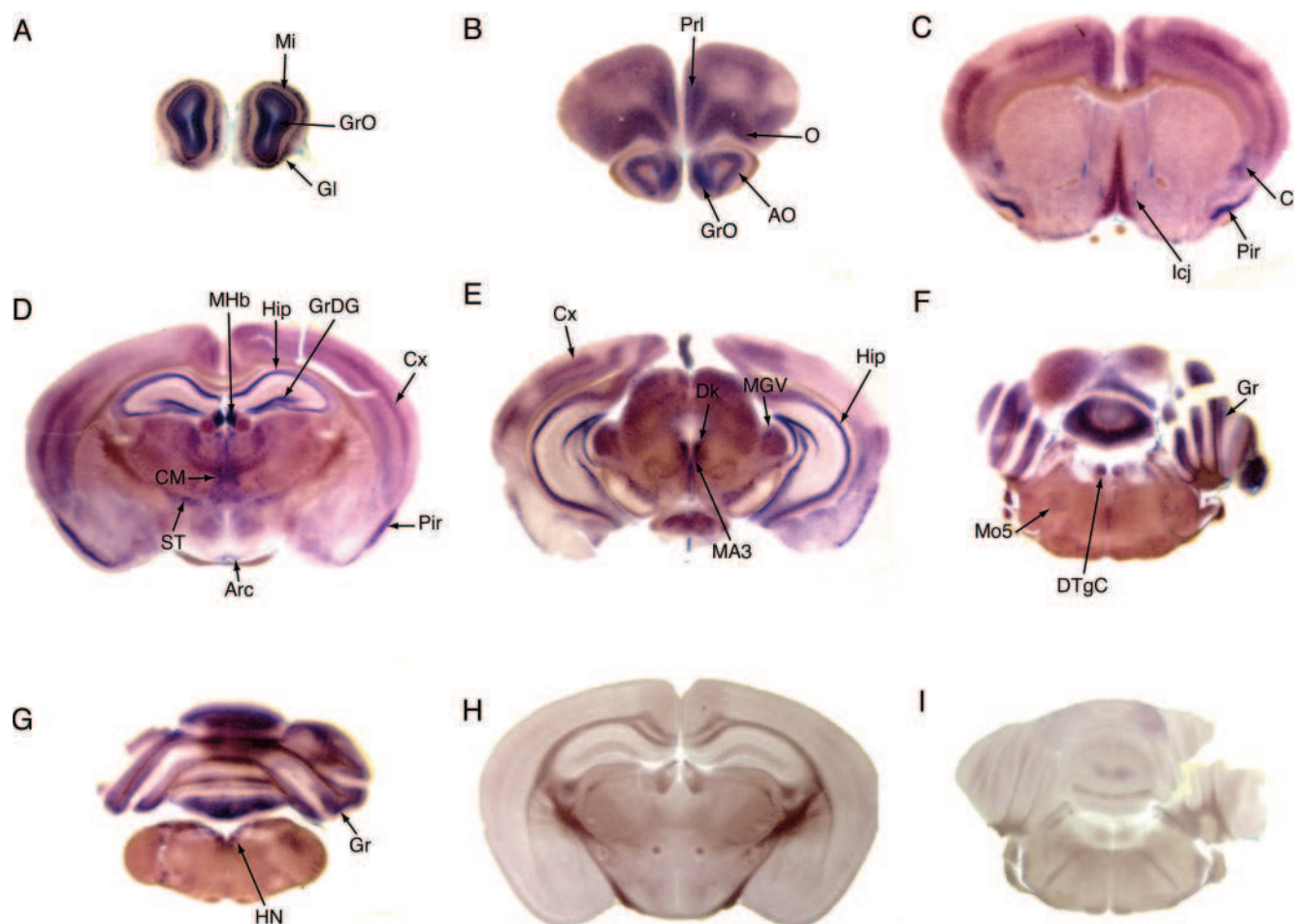


FIG. 8. ZBTB4 is expressed by several neuronal populations in the brain. Sections of an adult mouse brain were hybridized with a ZBTB4 riboprobe. (A to G) A series of anterior to posterior sections hybridized with the antisense probe. (H and I) Two control sections hybridized with the sense probe. (A) GrO, granular layer of the olfactory bulb; Gl, glomerular layer of the olfactory bulb; Mi, mitral layer of the olfactory bulb. (B) AO, anterior olfactory nucleus; O, orbital cortex; Prl, prelimbic cortex. (C) Cl, claustrum; ICj, island of Calleja; Pir, piriform cortex. (D) Arc, arcuate nuclei; CM, central medial thalamic nucleus; Cx, cortex; GrDG, granulate layer of the dentate gyrus; Hip, hippocampus; MHb, medial habenular nuclei; ST, subthalamic nuclei. (E) Cx, cortex; Dk, nuclei of Darkschewitsch; Hip, hippocampus; MA3, medial accessory oculomotor nucleus; MGv, medial geniculate nucleus, ventral portion. (F) DTgC, dorsal trigeminal nucleus, central portion; Gr, granular layer of the cerebellum; Mo5, motor trigeminal nucleus. (G) Gr, granular layer of the cerebellum; HN, hypoglossal nucleus.

many proteins containing the MBD (42), only MBD1, MBD2, MBD4, and MeCP2 bind methylated DNA. Here we show that at least three zinc finger proteins bind methylated DNA in vitro and in vivo: Kaiso, ZBTB4, and ZBTB38. In the hundreds of other zinc finger-containing proteins encoded by the human genome, some might also have this ability. In addition, the genome may contain yet other proteins that bind methylated DNA through different protein folds. The existence of multiple proteins that all recognize the same seemingly simple signal—methylated CpG—is intriguing and suggests that the DNA methylation signal may have complex and subtle consequences at different genomic loci and in different cell types. The identification of the genomic targets of specific MBPs and the study of the mechanisms by which they are recruited to these loci should help us address this question.

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ADDENDUM IN PROOF

The mouse homolog of ZBTB38 has been recently cloned and named CIBZ (N. Sasai, E. Matsuda, E. Sarashina, Y. Ishida, and M. Kawaichi, *Genes Cells* **10**:871–885, 2005).

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