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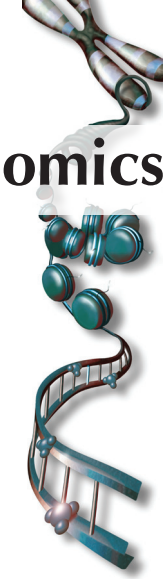

Tagging methyl-CpG-binding domain proteins reveals different spatiotemporal expression and supports distinct functions

Aim: DNA methylation is recognized by methyl-CpG-binding domain (MBD) proteins. Multiple MBDs are linked to neurodevelopmental disorders in humans and mice. However, the functions of MBD2 are poorly understood. We characterized *Mbd2* knockout mice and determined spatiotemporal expression of MBDs and MBD2–NuRD (nucleosome remodeling deacetylase) interactions. **Experimental procedures:** We analyzed behavioral phenotypes, generated biotin-tagged MBD1 and MBD2 knockin mice, and performed biochemical studies of MBD2–NuRD. **Results:** Most behavioral measures are minimally affected in *Mbd2* knockout mice. In contrast to other MBDs, MBD2 shows distinct expression patterns. **Conclusion:** Unlike most MBDs, MBD2 is ubiquitously expressed in all tissues examined and appears dispensable for brain functions measured in this study. We provide novel genetic tools and reveal new directions to investigate MBD2 functions *in vivo*.

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DNA methylation is a chemical modification to cytosine nucleotides that is essential for mammalian viability and development [1–3]. It occurs mostly at CpG dinucleotides (mCG) and has historically been associated with gene repression [1]. Recent studies have found that methylation also occurs in a non-CpG context and has a complex role in the regulation of gene expression [4,5]. The mechanism by which DNA methylation is interpreted into transcriptional output is not fully understood, but involves protein 'readers' that bind to methylated sites [6]. These readers include the methyl-CpG-binding domain (MBD) family of proteins, comprised of MeCP2 and MBDs 1–6 [6,7]. The MBD is a highly conserved domain originally identified for its ability to specifically bind mCG sites [8]. Most MBD proteins, with the exception of MBD3, MBD5 and MBD6, bind to DNA in a methylation density-dependent manner when expressed in embryonic stem (ES) cells [9].

Biochemical studies support the role of MBD proteins in mediating transcriptional repression through the recruitment of various corepressor complexes [10–12]. MBD2 or MBD3 are incorporated into mutually exclusive complexes with the Mi2/nucleosome remodeling and deacetylase (NuRD) complex, while MeCP2 associates with the repressive Sin3A and NCoR/SMRT complexes [10,13–16]. MBD1 interacts with several heterochromatin-associated proteins, including repressive histone methyltransferases [17–20]. Despite this biochemical evidence, very few methylated loci have been identified as specific, direct targets of transcriptional regulation by the MBD proteins [21–23]. Alternative functions such as maintenance of genomic stability or heterochromatin formation have been proposed for the MBD [6].

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Additionally, the *in vivo* interactions of MBD2 and the NuRD complex are not well defined. There is evidence that the NuRD/MBD2 complex is localized to transcriptionally active domains in addition to mCG dense loci [9]. This finding supports the model that the MBD proteins, and corepressor complexes in general, act as dynamic modulators of transcriptional activity rather than simple repressors, but many questions remain regarding the functions of these proteins *in vivo* [24].

Genetic studies of MBD proteins in humans and animal models have demonstrated a critical role for this protein family in the brain [22,25–29]. Mutations in several MBD proteins have been identified in individuals with autism spectrum disorder (ASD), although further work is needed to determine if these mutations are causative [25,26]. MeCP2 and MBD5 in particular are linked to the specific neurological diseases Rett syndrome and 2q23.1 microdeletion syndrome, respectively [27,30], both of which are closely recapitulated in mouse models with deletion or mutation of each gene [31,32]. *Mbd1* knockout mice also exhibit behavioral phenotypes that mimic features of ASD such as learning and memory deficits, reduced sociability, and changes in anxiety-related phenotypes [22,29]. Furthermore, MBD3 and multiple components of the MBD-associated corepressor complexes, such as NuRD, Sin3A and NCoR/SMRT, also have critical roles in the brain [33–37]. Given the similarities between the MBD proteins, it has been postulated that MBD2 may also have a role in brain function [6]. Although a nurturing deficit has been reported in *Mbd2* knockout mice [38], the extent to which loss of MBD2 leads to other behavioral phenotypes related to brain function has yet to be determined.

The role of MBD family proteins outside of the brain has been less well studied. In order to examine MBD functions *in vivo*, it is necessary to identify the specific tissues or cell types where the MBD proteins are expressed. MBD expression at the transcriptional level has been assessed in several tissues for MeCP2, MBD1 and MBD2 [7,39]. However, a closer analysis of MeCP2 expression showed that MeCP2 protein levels do not correlate with transcript abundance in mouse or human tissues, indicating that significant post-transcriptional regulation may be occurring [40]. Phenotypic data must also be carefully evaluated to determine the cell-type-specific functions of MBD proteins. For example, MeCP2 loss-of-function phenotypes in non-neuronal tissues may be occurring due to non-cell-autonomous effects that are neuronal in origin, as was recently shown in a study of muscle [41]. A comprehensive analysis of MBD protein expression in tissues of interest has not been possible due to limitations of currently available antibodies, especially for MBD2.

In this study, we sought to explore the functions of MBD2 *in vivo* in the context of the other MBD family proteins. Given the well-defined neuronal functions of MeCP2 and MBD1 and the recently described role of NuRD in the brain [22,31,34], we carried out behavioral characterization of *Mbd2* knockout mice similar to studies of *Mecp2* and *Mbd1* knockout mice [22,31]. We found that *Mbd2* knockout mice are comparable to wild-type and heterozygous littermates on most measures, but exhibit a few subtle differences in locomotor activity, nest building and body weight, at the examined age. To investigate the underlying causes of the distinct behavioral and physiological outcomes of *Mecp2*, *Mbd1* and *Mbd2* knockout mice, we generated knockin mouse lines with endogenous MBD2 or MBD1 tagged with biotin. We used these mice to systematically compare the expression patterns of the MBD proteins and found that, in contrast to MBD1, MeCP2 and MBD3 that are enriched in the brain, MBD2 is highly expressed in non-neuronal tissues. We also used these mice to show that MBD2 interacts with the NuRD complex ubiquitously across several tissues. Therefore, MBD2 is distinct from other MBD proteins in its spatiotemporal expression patterns and apparent lack of impact on neuronal functions. These findings raise the possibility that MBD2 plays a more important role in peripheral tissues than in the brain.

Experimental procedures

Detailed experimental procedures are included in the Supplementary Information.

Results

Behavioral characterization of *Mbd2* knockout mice

Given that genetic studies of humans and mice have linked several MBD proteins to ASD and neurodevelopmental disorders, we hypothesized that MBD2 may also have a critical role in brain function and that loss of MBD2 would result in behavioral phenotypes similar to those observed in *Mecp2* or *Mbd1* knockout mice [6,22,28,32]. Although an *Mbd2* knockout mouse (*Mbd2*$^{-/-}$) has been generated, the only reported phenotype is a pup nurturing and retrieval deficit [38] and a full phenotypic characterization has not been reported. In this mouse, exon 2 of *Mbd2* is replaced with a promoterless 7kb β-*Geo LacZ* cassette, removing most of the conserved MBD [38]. Small amounts of read-through transcript are present, but no full-length protein of either MBD2 isoform (MBD2a and MBD2b) is detectable, indicating this is a null allele [38]. We focused our analysis on behavioral phenotypes that are affected in mouse models of ASDs, such as *Mecp2* or *Mbd1* knockout mice, and assessed motor function,

anxiety-related behaviors, sociability, and learning and memory [22,31,42].

Changes in locomotor activity have been observed in *Mecp2* knockout mice [31], so we quantified the locomotor activity of *Mbd2*$^{-/-}$ mice in a home-cage-like environment. We found that *Mbd2*$^{-/-}$ mice show significantly reduced locomotor activity compared with wild-type (*Mbd2*$^{+/+}$) and heterozygous (*Mbd2*$^{+/-}$) littermates (Figure 1A). We then asked if the observed hypoactivity could be attributed to motor impairments, as motor function is also affected in *Mecp2* knockout mice [31]. We tested motor coordination and learning in a rotarod task, in which mice were challenged to stay on an accelerating rod for 16 total trials over 4 days. All genotypes showed comparable improvement in this task over each trial and testing day, indicating that motor coordination and learning is not impaired in *Mbd2*$^{-/-}$mice (Figure 1B). Anxiety-related phenotypes are altered in both *Mecp2* and *Mbd1* knockout mice [22,31]. Therefore, we used a zero maze to assess anxiety, a task that takes advantage of a mouse's innate preference for enclosed spaces. Mice that spend increased time in the open arm of the maze are inferred to have decreased anxiety-related behavior [43]. We found *Mbd2*$^{-/-}$mice displayed a preference for the closed arm that was equivalent to control littermates, indicating that anxiety-related behavior is not affected by the loss of MBD2 (Figure 1C).

We next assessed whether *Mbd2*$^{-/-}$ mice exhibited several autistic-like phenotypes that have been shown to be affected in mice lacking MeCP2 or MBD1 [22,31]. Impaired social interaction is a common feature of ASDs and is recapitulated in numerous mouse models [42,44]. Mice are inherently social animals and most mice prefer to interact with other mice [44]. We tested sociability of *Mbd2*$^{-/-}$mice using the social approach test in a three-chambered apparatus (Figure 1D). This assay tests the preference of the test mouse to inter-

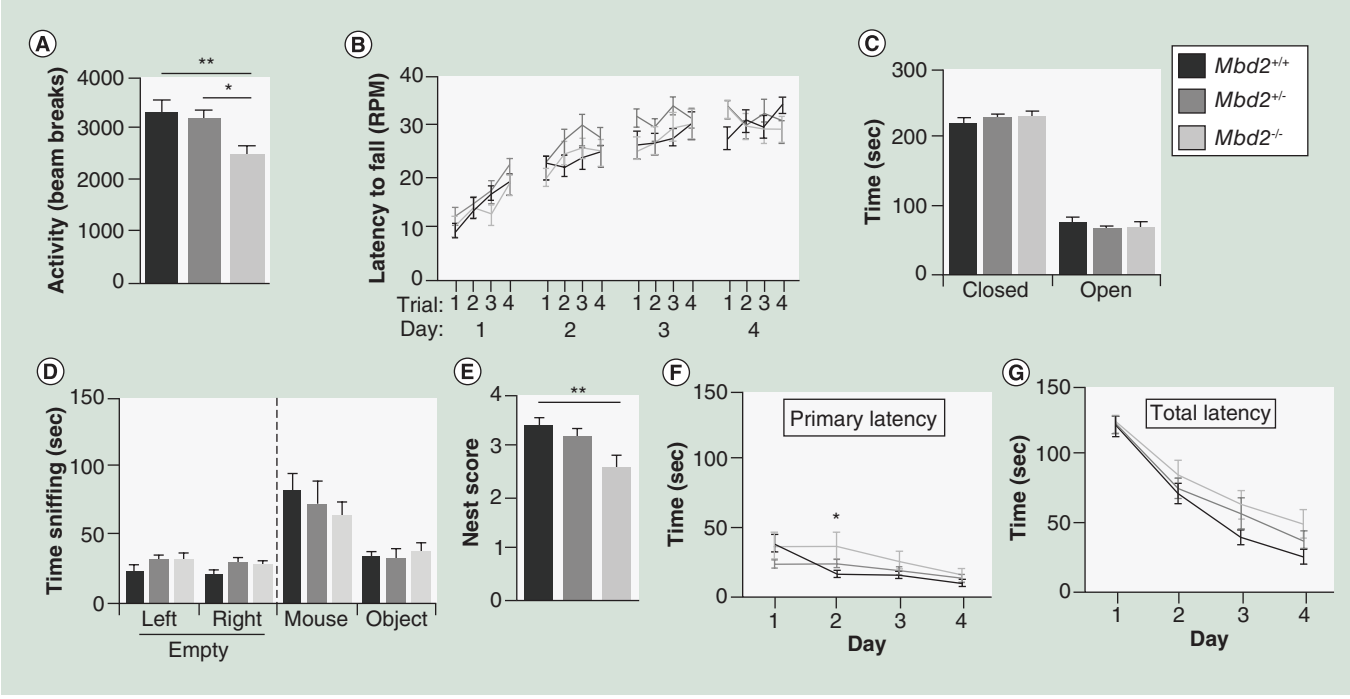

Figure 1. Behavioral characterization of *Mbd2*$^{-/-}$ mice compared with wild-type and heterozygous littermates. (A) *Mbd2*$^{-/-}$mice are hypoactive as measured by the number of infrared beam breaks in a home-cage-like environment over 60 min (n = 14 per genotype, **p < 0.01, *p < 0.05, one-way ANOVA with Tukey's test). **(B)** *Mbd2*$^{-/-}$mice perform similarly to littermates in the rotarod task indicating unaffected motor coordination or learning (n = 14 per genotype, two-way ANOVA). **(C)** *Mbd2*$^{-/-}$ mice have unaltered anxiety-related behavior in a zero maze assay (n = 14 per genotype, one-way ANOVA of time in open arm). **(D)** Sociability of *Mbd2*$^{-/-}$ mice was assessed in the social approach test in a three-chambered apparatus. All genotypes show equal preference between two empty cylinders (left and right) during the habituation phase. All genotypes show equivalent increased preference for a social stimulus (mouse) versus a nonsocial stimulus (object) (*Mbd2*$^{+/+}$and *Mbd2*$^{+/-}$ n = 13, *Mbd2*$^{-/-}$ n = 14, two-way ANOVA). **(E)** *Mbd2*$^{-/-}$ mice have significantly impaired nest-building behavior (*Mbd2*$^{+/+}$ n = 22, *Mbd2*$^{+/-}$ n = 19, *Mbd2*$^{-/-}$ n = 18, **p < 0.01, one-way ANOVA with Tukey's test). **(F & G)** Spatial learning and memory were assessed using a Barnes maze paradigm. *Mbd2*$^{-/-}$ mice show a slight but significant delay to initially reach a target escape hole on the second training day (primary latency, F), but equivalent time to enter the escape hole (total latency, G) during 4 days of training (*Mbd2*$^{+/+}$ n = 22, *Mbd2*$^{+/-}$ n = 19, *Mbd2*$^{-/-}$ n = 18, *p < 0.05, two-way ANOVA with Tukey's test). All data are presented as mean ± standard error of the mean.
RPM: Revolutions per minute.

act with a novel social stimulus (unfamiliar mouse) or novel object. In the first habituation phase of the test, *Mbd2*$^{+/+}$, *Mbd2*$^{+/-}$ and *Mbd2*$^{-/-}$ mice showed no significant difference in time spent sniffing two empty cylinders. At the start of the second phase of the test, a novel stimulus mouse and novel object were introduced to the cylinders. We found no significant difference between *Mbd2*$^{+/+}$, *Mbd2*$^{+/-}$ and *Mbd2*$^{-/-}$ littermates in the amount of time spent sniffing the social stimulus mouse, suggesting sociability is not affected by the loss of MBD2. We next assayed nest building as an alternative test that is commonly used to assess home-cage behaviors related to ASD-like phenotypes in mice [42]. We found that *Mbd2*$^{-/-}$ mice show impaired nest-building activity in contrast to wild-type and heterozygous littermates (Figure 1E). Together, these results indicate that *Mbd2*$^{-/-}$ mice show impairments in nest building, but the reduced home-cage locomotor activity (Figure 1A) complicates the interpretation of this nest-building phenotype.

Deficits in learning and memory have been found in numerous mouse models of ASDs and have also been observed in mice lacking MeCP2 or MBD1 [22,31,42]. To determine if *Mbd2*$^{-/-}$ mice have a similar phenotype, we tested spatial learning and memory using the Barnes maze paradigm over four consecutive training days. *Mbd2*$^{-/-}$mice showed a slight but significant increase in the time needed to initially reach the target hole on the second training day (primary latency, Figure 1F), but equal time to enter the target hole and complete the trial on all testing days (total latency, Figure 1G) compared with wild-type and heterozygous littermates. The increase in primary latency time on the second training day is unlikely to reflect significant changes in learning and memory because *Mbd2*$^{-/-}$ mice are comparable to control littermates on all other measures in this assay. In summary, we found that *Mbd2* knockout mice are equivalent to wild-type and heterozygous littermates on most measures tested, with the exception of locomotor activity and nest building. These results are in notable contrast to mice lacking MeCP2 or MBD1, which show significant alterations in locomotor activity, sociability, anxiety-related behaviors and deficits in learning and memory [22,31]. Therefore we conclude that unlike most MBD proteins, MBD2 does not have a major impact on brain functions that underlie many key behavioral phenotypes relevant to ASD.

Mbd2$^{-/-}$ mice have reduced body weight & decreased food intake

Although *Mbd2*$^{-/-}$ mice are viable and fertile as previously reported [38], we noticed that these mice have reduced reproductive ability. We also weighed *Mbd2*$^{+/+}$, *Mbd2*$^{+/-}$ and *Mbd2*$^{-/-}$ male and female littermates and found that *Mbd2*$^{-/-}$ males and females have subtle but significantly lower body weights beginning at 4 and 6 weeks of age, respectively (Figure 2A & B). Loss of MeCP2 and MBD1 both affect brain weight, as well [29,31]. Therefore, we tested if brain weight is affected in *Mbd2*$^{-/-}$ mice and compared these values to total body weight (Figure 2A). We observed that *Mbd2*$^{-/-}$male mice have slightly but significantly decreased brain weight, but have increased brain weight as a percentage of body weight compared with littermates (Figure 2C & D). This finding suggests that the low brain weight of *Mbd2*$^{-/-}$ mice is likely due to the loss of overall body mass. To further explore the low body weight observed in *Mbd2*$^{-/-}$mice, we next measured daily food intake for each genotype. We found that male *Mbd2*$^{-/-}$ mice consume less food per day compared with wild-type and heterozygous littermates (Figure 2E). However, when food consumption is normalized to body weight, *Mbd2*$^{-/-}$mice consume comparable amounts of food for their body size as littermates (Figure 2F). These findings are in contrast to mice with *Mecp2* mutations, which show increasing phenotypic severity with age in addition to significantly reduced body and brain weight [31].

Although *Mbd2*$^{-/-}$ mice are comparable to control littermates on most measures, we next sought to determine if the observed changes in locomotor activity, body weight and impaired nesting behavior are linked to any alterations in biogenic amine levels. Genetic or pharmacological disruption of biogenic amine levels, particularly striatal dopamine, is associated with many of these phenotypes, including low body weight, hypophagia, hypoactivity, and impairments in nesting and pup nurturing [45,46]. We performed HPLC analysis to measure the abundance of essential monoamines and their metabolites in the cortex and striatum of wild-type and *Mbd2*$^{-/-}$ littermates (Figure 3A & B). These include norepinephrine (NE), dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). We found that none of the measured biogenic amine levels in the cortex and striatum are altered in *Mbd2*$^{-/-}$ mice compared with wild-type littermates. Therefore, the loss of MBD2 is unlikely to affect monoamine signaling in the brain, in contrast to the essential role of MeCP2 in regulating these systems [47].

Generation of knockin mice expressing biotin-tagged MBD2 or MBD1

Given that previous studies linked MeCP2 and MBD1 to brain function in both humans and mice [22,25,28,31], it is surprising that mice lacking MBD2 behave largely similar to wild-type and heterozygous littermates.

MBD2 likely has critical functions in other tissues and systems, such as the gastrointestinal (GI) and immune systems [48–50], that may contribute to the hypoactivity, impaired nesting and nurturing [38], and low body weight phenotypes observed in *Mbd2* knockout mice. To better understand MBD2 functions and localization in other tissues, we first sought to characterize MBD2 interactions with NuRD in different tissues and where and when MBD2 is expressed *in vivo* in comparison to other MBD proteins. However, we were impeded by the lack of reliable antibodies that specifically recognize MBD2 (Figure 4C). In addition, even with reliable antibodies, the variable immunoreactivity of each antibody makes comparison of the expression levels for different MBD proteins difficult. To overcome these limitations, we took a genetic approach and developed a knockin mouse line tagging MBD2 with a biotinylation consensus sequence derived from the *Escherichia coli* biotin ligase, BirA (Figure 4A). The tag was introduced to the C-terminus of MBD2 and consists of two parts: a tobacco etch virus (TEV) protease cleavage site and an avidin-binding site upon biotinylation. We labeled this tag as the 'Tavi' tag and the tagged allele as $Mbd2^{Tavi}$. We also generated a separate transgenic mouse line with *BirA* constitutively expressed from the *Rosa26* locus ($R26^{BirA/+}$) [Johnson *et al.*, Unpublished Data]. Therefore, $MBD2^{Tavi}$ protein can be specifically biotinylated in the presence of BirA (Figure 4C). This set of transgenic mice allows us to biotinylate endogenous MBD2 proteins constitutively, and supports streptavidin-mediated detection of protein expression.

Two previously described isoforms of MBD2, MBD2a and MBD2b [7], carry the Tavi tag (Figure 4A). These isoforms arise from alternative translation start sites from the same transcript that is expressed in adult somatic tissues [7]. MBD2a, but not MBD2b, includes a N-terminal GR repeat region. Post-translational methylation of the GR repeat has been shown to affect MBD2a function by reducing its affinity for DNA and the NuRD complex [51]. $MBD2^{Tavi}$ also includes a transcriptional repression domain that interacts with the NuRD complex [52]. Another isoform, MBD2c, is expressed exclusively in the testes and ES cells [7,53]. MBD2c has an alternatively spliced C terminus that does not include the Tavi tag and is not depicted in Figure 4A. Mice heterozygous or homozygous for $Mbd2^{Tavi}$ are viable and fertile similar to littermate controls. In contrast to $Mbd2^{-/-}$ mice that have reduced body weight (Figure 2A), $Mbd2^{Tavi/Tavi}$ mice with or without BirA expression show equivalent body weight to wild-type and $R26^{BirA/+}$ littermates (Figure 4B) and we have not observed any gross phenotypic abnormalities during routine handling. Therefore, biotinylation of $MBD2^{Tavi}$ likely does not alter MBD2 function.

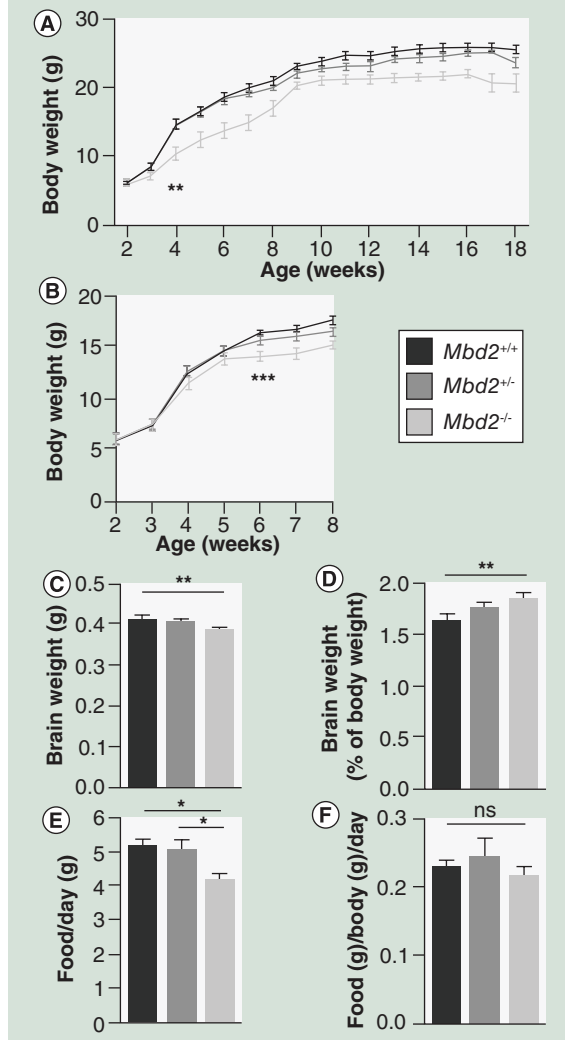

Figure 2. $Mbd2^{-/-}$ mice show decreased body and brain weight associated with reduced food intake. (A) Male $Mbd2^{-/-}$ mice have decreased body weight compared with $Mbd2^{+/+}$ and $Mbd2^{+/-}$ littermates (n = 10 per genotype, $p < 0.01$, interaction, two-way ANOVA). $Mbd2^{-/-}$ weight was significantly decreased at 4 weeks continuing to 18 weeks postnatal (**$p < 0.01$, two-way ANOVA with Tukey's test). **(B)** Female $Mbd2^{-/-}$ mice have decreased body weight (n = 10 per genotype, $p < 0.001$, interaction, two-way ANOVA). $Mbd2^{-/-}$ weight was significantly decreased at 6 weeks continuing to 8 weeks postnatal (***$p < 0.001$, two-way ANOVA with Tukey's test). **(C)** 13-week-old male $Mbd2^{-/-}$ mice have significantly decreased brain weight ($Mbd2^{+/+}$ and $Mbd2^{+/-}$ n = 11, $Mbd2^{-/-}$ n = 12, **$p < 0.01$, one-way ANOVA with Tukey's test). **(D)** 13-week-old male $Mbd2^{-/-}$ mice have significantly increased brain weight as a percentage of body weight ($Mbd2^{+/+}$ and $Mbd2^{+/-}$ n = 11, $Mbd2^{-/-}$ n = 12, **$p < 0.01$, one-way ANOVA with Tukey's test). **(E & F)** Eight-week-old male $Mbd2^{-/-}$mice consume significantly less food per day on an unrestricted diet of standard chow, but **(F)** consume equivalent food normalized to body weight ($Mbd2^{+/+}$and $Mbd2^{+/-}$, n = 5, $Mbd2^{-/-}$ n = 8, *$p < 0.05$, ns: Not significant, one-way ANOVA with Tukey's test). All data are presented as mean ± standard error of the mean.

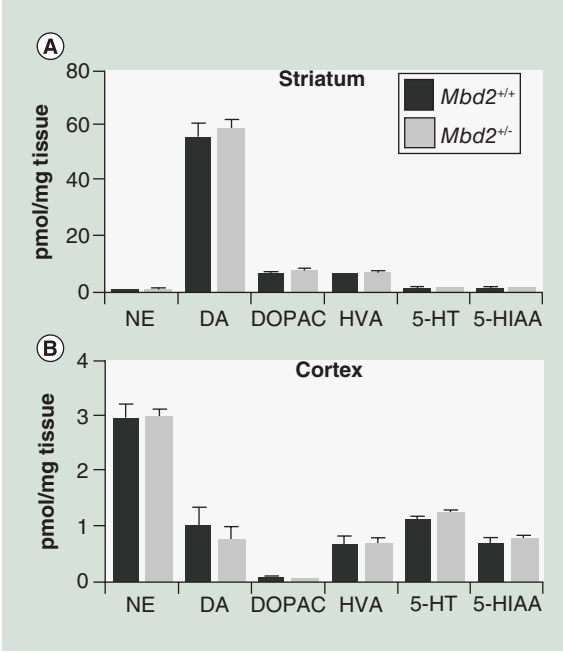

Figure 3. *Mbd2*$^{-/-}$ mice have equivalent biogenic amine content to wild-type littermates. HPLC analysis showed equivalent levels of biogenic amines and their metabolites in the cortex **(A)** and striatum **(B)** between 12-week-old male *Mbd2*$^{+/+}$ and *Mbd2*$^{-/-}$ mice (*Mbd2*$^{+/+}$ n = 6, *Mbd2*$^{-/-}$ n = 8, unpaired two-tailed *t*-test with Holm-Sidak multiple comparison correction). All data are presented as mean ± standard error of the mean. 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: Serotonin; DA: Dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: Homovanillic acid; NE: Norepinephrine.

We then used fluorophore-conjugated streptavidin to examine MBD2^{Tavi} expression by western blot (Figure 4C). A representative commercial antibody to MBD2 (and others, data not shown) recognizes several unknown proteins that do not differ between wild-type (WT) and *Mbd2*$^{-/-}$ (KO) adult brain lysate (denoted by *), demonstrating invalidity of the stated antibody. In contrast, streptavidin detects three biotinylated MBD2^{Tavi} isoforms with high specificity in *Mbd2*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$mice, but not in mice expressing BirA without MBD2^{Tavi} (*R26*$^{BirA/+}$). In addition to the predicted MBD2a^{Tavi} and MBD2b^{Tavi} isoforms, we observed a third MBD2 isoform slightly smaller than MBD2a^{Tavi} that has been previously detected but not described in detail [13]. This isoform has the C-terminal Tavi tag and is biotinylated in the presence of BirA. We designated this novel isoform as MBD2d. It includes exon 6 where the Tavi tag is inserted and may occur through alternative splicing or translation start site usage (Figure 4A). Direct comparison of proteins detected by the MBD2 antibody versus streptavidin (Figure 4C, merged image) demonstrates the specificity and robustness of this biotin-tagging approach to identify MBD2 isoforms.

In order to directly compare the expression levels of MBD2 to MBD1 *in vivo*, we employed the same approach and generated knockin mice expressing MBD1^{Tavi} (Figure 5A). Murine *Mbd1* has three reported alternatively spliced isoforms that have two or three CxxC zinc finger DNA-binding domains in addition to a transcriptional repression domain [54]. MBD1a and MBD1b both carry the Tavi tag, but MBD1c has an alternatively spliced C-terminus that does not include the Tavi tag (Figure 5A). *Mbd1*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$ mice are viable and fertile with no gross phenotypic abnormalities observed during routine handling. Using western blots, we found that an antibody against MBD1 detects several cross-reacting proteins in postnatal day 7 (P7) brain lysate, comparing *Mbd1* knockout (KO) to wild-type mice (Figure 5B, denoted by *) [29]. All three isoforms of MBD1, MBD1a^{Tavi}, MBD1b^{Tavi} and untagged MBD1d, are visible in *R26*$^{BirA/+}$and *Mbd1*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$mice when compared with *Mbd1* knockout mice using this antibody. In contrast to *R26*$^{BirA/+}$mice and *Mbd1* knockout mice, streptavidin detects two specific proteins corresponding to biotinylated MBD1a^{Tavi} and MBD1b^{Tavi} in lysate from *Mbd1*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$mice. Streptavidin also detects a 75kDa endogenously biotinylated protein that is close in size to untagged MBD1d (denoted by *). The merged image demonstrates that streptavidin specifically detects the two biotin-tagged MBD1^{Tavi} isoforms that are also recognized by the MBD1 antibody (yellow bands) (Figure 5B). In summary, this biotin-tagging approach represents a powerful tool to study multiple isoforms of endogenous MBD proteins, overcoming antibody limitations.

Distinct spatiotemporal expression patterns of endogenous MBD2 in contrast to other MBD proteins

Previously published studies support a role for MBD2 in tissues or organs outside the brain, in notable contrast to MeCP2 and MBD1 [48–50]. To better understand where and when MBD2 functions *in vivo*, we next analyzed the expression patterns of MBD2, MBD1, MeCP2 and MBD3 across eight different tissues and two developmental time points, P7 and P42. We took advantage of the Tavi-tagged alleles of MBD2 and MBD1 to reliably determine the expression level of each protein and compared their expression side-by-side using quantitative western blots. This approach allowed us to obtain a comprehensive view of endogenous MBD spatiotemporal expression patterns at the protein level.

Using MBD2^{Tavi} mice with constitutively expressed BirA (*Mbd2*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$), we analyzed the expression of three Tavi-tagged isoforms of MBD2, MBD2a, MBD2b and MBD2d, using two complementary

reagents. First, we used an antibody raised against the biotinylation consensus sequence for BirA (labeled Avi) to detect the Tavi tag of MBD2^{Tavi}. We also used streptavidin to detect biotinylated MBD2^{Tavi}. In order to exclude cross-reacting proteins recognized by the Avi antibody and also exclude endogenously biotinylated proteins, we first used these reagents with protein lysate from *R26*$^{BirA/+}$animals that do not express any Tavi alleles (Figure 6A & B). The Avi antibody detects several cross-reacting proteins, most notably a 50 kDa protein that is highly expressed in the brain at both P7 and P42 and a weaker cross-reacting band at 37 kDa

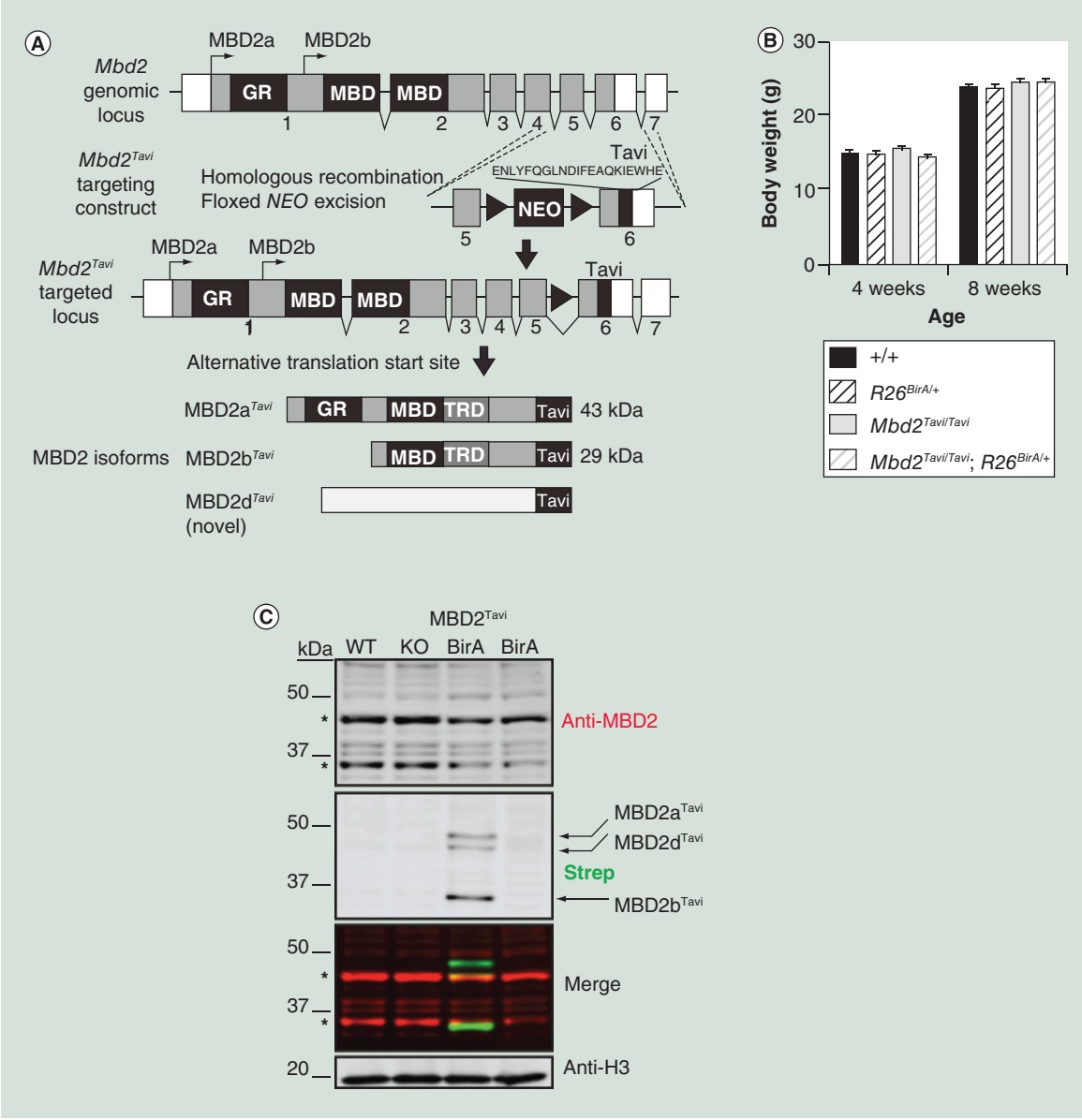

Figure 4. Development of *Mbd2*^{Tavi} knockin mice. (A) Targeting strategy for generating *Mbd2*^{Tavi} knock in mice. The Tavi tag is inserted into exon 6. Two documented isoforms from alternative translation start sites, MBD2a^{Tavi} and MBD2b^{Tavi}, are expressed. A third previously undescribed isoform, MBD2d, is also Tavi-tagged and expressed. **(B)** *Mbd2*$^{Tavi/Tavi}$ (n = 8) and *Mbd2*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$ (n = 7) mice have equivalent body weight to WT (+/+, n = 7) and *R26*$^{BirA/+}$ (n = 5) mice (two-way ANOVA). Data are presented as mean ± standard error of the mean. **(C)** Detection of MBD2 isoforms in the brain. An antibody against MBD2 detects cross-reacting bands in WT and *Mbd2*$^{-/-}$ (KO) brain lysate. Three isoforms of MBD2^{Tavi} are specifically biotinylated and detected by streptavidin in *Mbd2*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$, but not *R26*$^{BirA/+}$, brain lysate. Cross-reacting and endogenously biotinylated proteins are denoted by *.
GR: GR repeat domain; KO: Knockout; MBD: Methyl-CpG-binding domain; NEO: Neomycin selection cassette; TRD: Transcriptional repression domain; WT: Wild-type

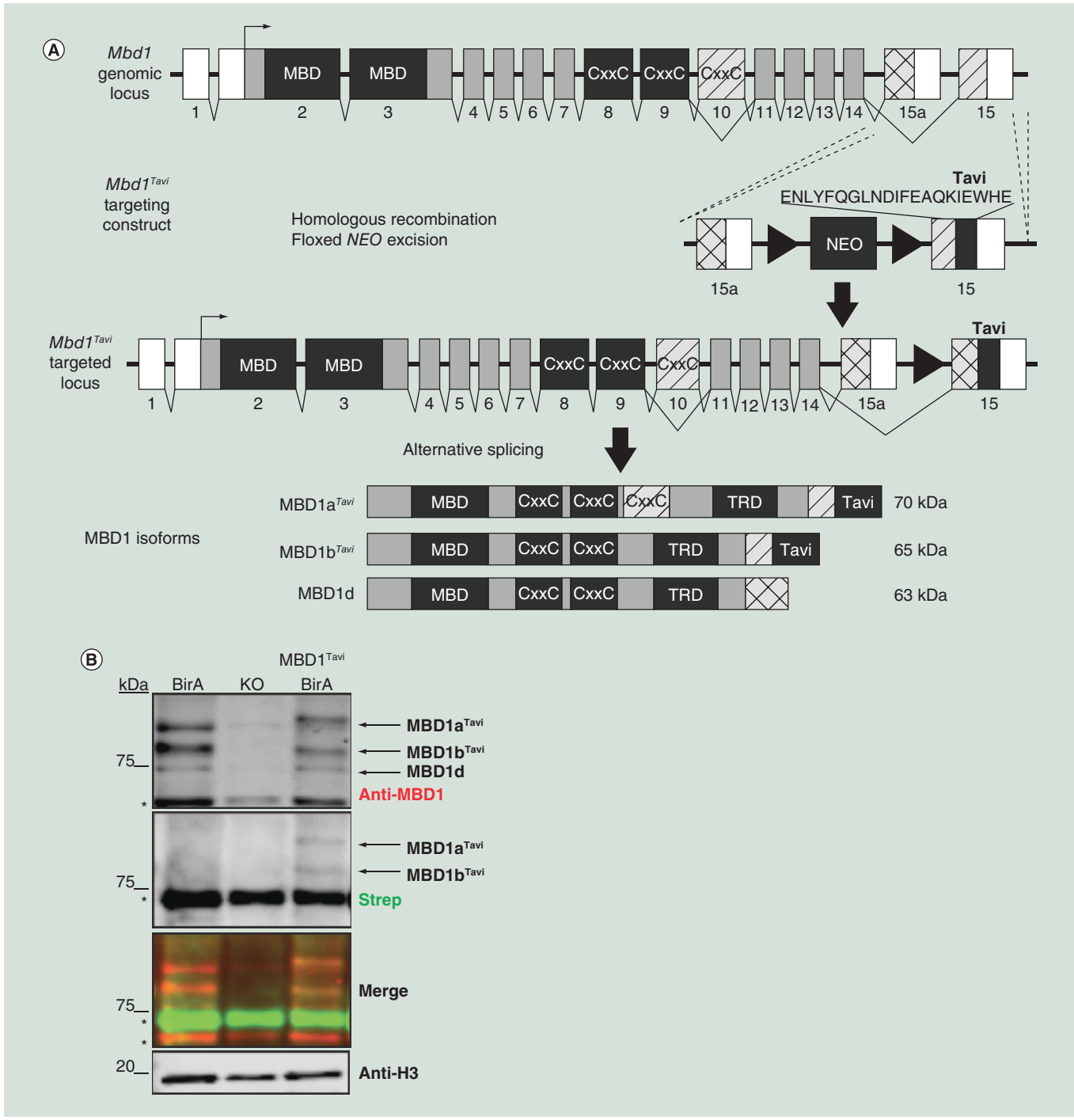

Figure 5. Development of *Mbd1*^{Tavi} knockin mice. (A) Targeting strategy for generating *Mbd1*^{Tavi} knockin mice. The Tavi tag is inserted into exon 15, which is included in MBD1a^{Tavi} and MBD1b^{Tavi}, but not in the alternatively spliced isoform MBD1d. **(B)** An antibody against MBD1 detects three MBD1 isoforms that are present in *R26*$^{BirA/+}$ and *Mbd1*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$, but not *Mbd1*$^{-/-}$ (KO), P7 brain lysate. Two MBD1^{Tavi} isoforms are biotinylated and detected by streptavidin specifically in lysate from *Mbd1*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$ mice (merged image, yellow bands). Cross-reacting and endogenously biotinylated proteins are denoted by *.
CxxC: CxxC zinc finger DNA-binding domain; KO: Knockout; MBD: Methyl-CpG-binding domain; NEO: Neomycin selection cassette; TRD: Transcriptional repression domain.

at P7. Several endogenous biotinylated proteins are also detected by streptavidin in the molecular size around MBD2, particularly a 37 kDa protein in the heart, liver, kidney and small intestine at P42 (Figure 6A & B, denoted by *). Western blot analysis of protein lysate from *Mbd2*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$ animals showed that all three

isoforms of MBD2 are partially obscured by the cross-reacting bands detected by the Avi antibody, particularly in the brain (Figure 6C & D). However, MBD2a^{Tavi} and MBD2b^{Tavi} are clearly detected by streptavidin in multiple tissues at both P7 and P42. By examining the merged images from the streptavidin and Avi antibody signals (yellow bands), we concluded that the three isoforms of MBD2^{Tavi} are expressed highly in multiple tissues including the heart, lung, liver, kidney and colon at P7 and P42. MBD2d^{Tavi} appears to be expressed at a lower level than the 2a and 2b isoforms in all tissues examined, particularly at P42. These isoforms are expressed in the brain, but at a lower level compared with other tissues. Interestingly, all MBD2^{Tavi} isoforms are not expressed in spleen or small intestine at P7, but become upregulated in these tissues at P42 (Figure 6C & D).

We next sought to place MBD2 spatiotemporal expression patterns into the context of the MBD family. Therefore, we surveyed MBD1 and MeCP2 expression in the same set of tissues and time points included in our MBD2 study. First, we identified cross-reacting proteins detected by the Avi antibody and endogenously biotinylated proteins by analyzing protein lysate from *R26*$^{BirA/+}$ animals (Figure 7A & B). At the molecular size around MBD1, the Avi antibody detected only a minor cross-reacting band at 75 kDa in the kidney at P42 (denoted by *). Several endogenous biotinylated proteins are detected by streptavidin, particularly a 75 kDa protein with significant expression in all tissues examined at P7 and P42 (denoted by *) (Figure 7A & B). We then used *Mbd1*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$ mice to perform western blots with streptavidin and the Avi antibody to

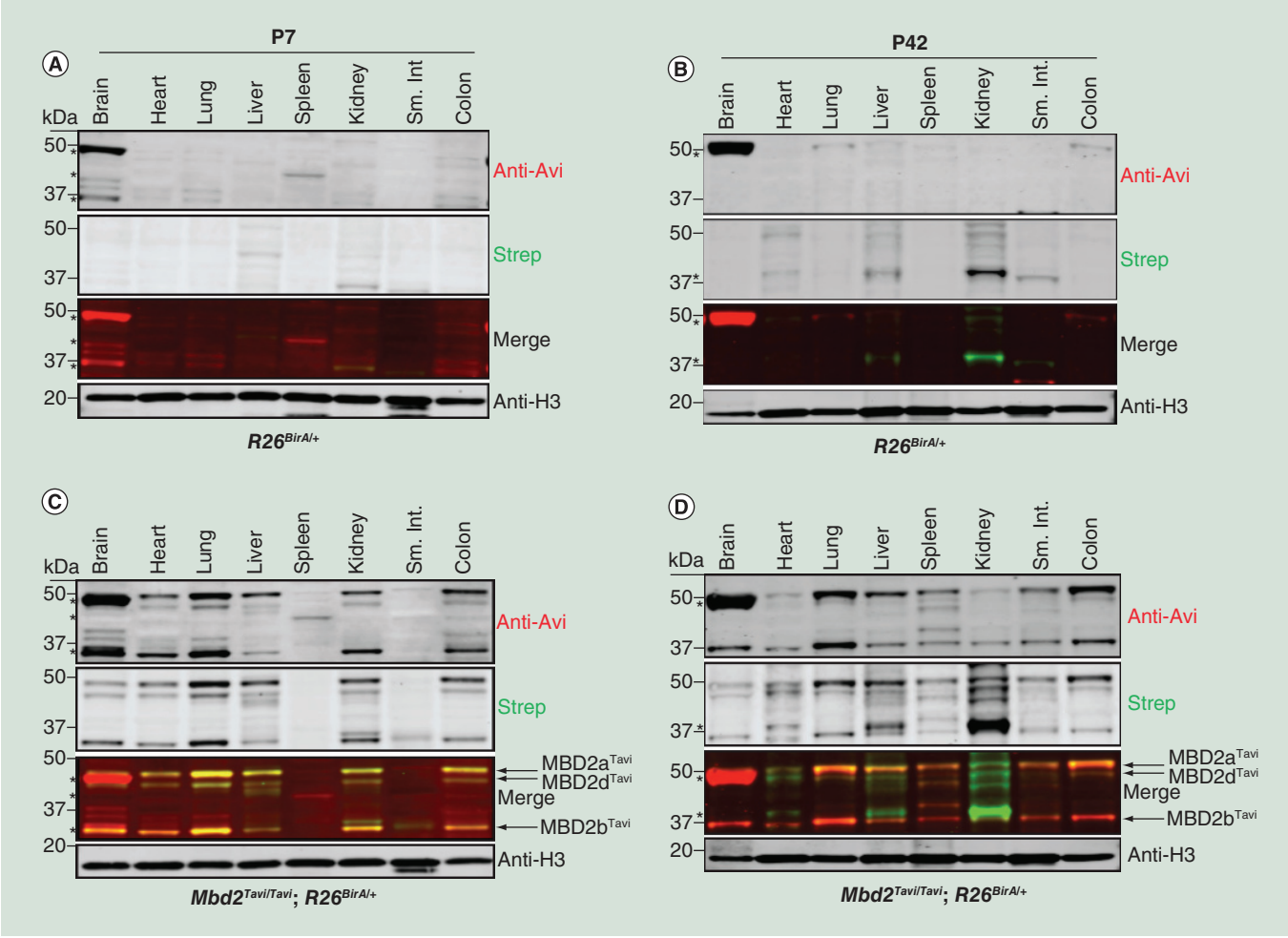

Figure 6. Spatiotemporal expression of MBD2^{Tavi} at postnatal days 7 and 42. (A & B) Western blot analysis with lysate from *R26*$^{BirA/+}$ mice reveals cross-reacting proteins detected by the Avi antibody and endogenously biotinylated proteins detected by streptavidin. **(C & D)** Three isoforms of MBD2^{Tavi} (MBD2a^{Tavi}, MBD2b^{Tavi} and MBD2d^{Tavi}) are biotinylated and detected by streptavidin and the Avi antibody. All isoforms of MBD2^{Tavi} are highly expressed throughout the body at P7, but are not expressed in spleen and small intestine. All isoforms of MBD2^{Tavi} are highly expressed throughout the body at P42, with upregulation in spleen and small intestine compared with P7. Cross-reacting and endogenously biotinylated proteins are denoted by *.
Sm. Int.: Small intestine.

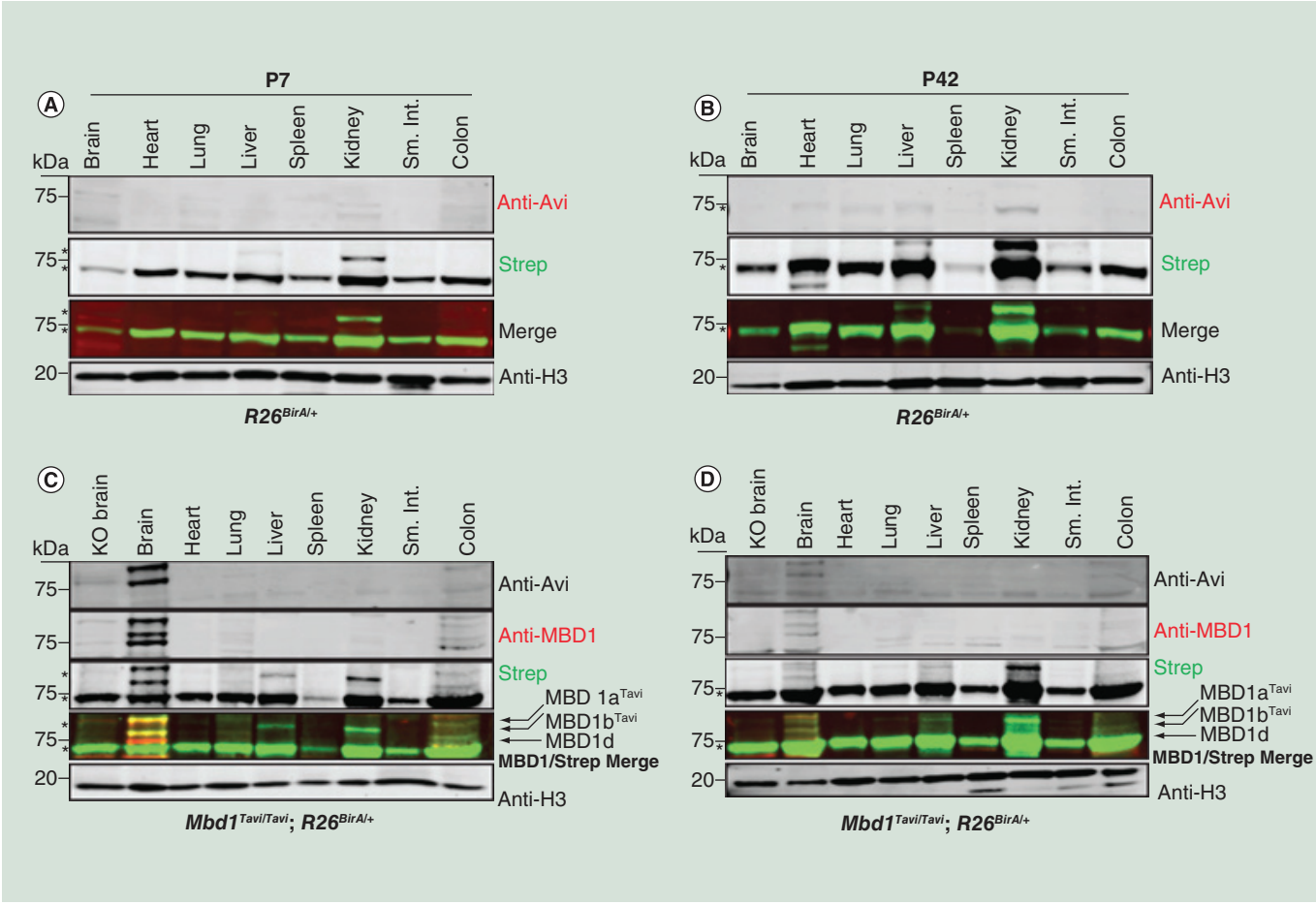

Figure 7. Spatiotemporal expression of MBD1^{Tavi} at postnatal days 7 and 42. (A & B) Western blot analysis with lysate from *R26*$^{BirA/+}$ mice reveals cross-reacting proteins detected by the Avi antibody and endogenously biotinylated proteins detected by streptavidin. **(C & D)** Two isoforms of MBD1^{Tavi} (MBD1a^{Tavi} and MBD1b^{Tavi}) are biotinylated and detected by the Avi antibody and streptavidin. An antibody against MBD1 also detects MBD1d, compared with *Mbd1* knockout brain lysate (KO brain). All three isoforms are highly expressed in the brain and show downregulation at P42 compared with P7. Cross-reacting and endogenously biotinylated proteins are denoted by *.
KO: Knockout; Sm. Int.: Small intestine.

detect biotinylated MBD1^{Tavi} expression (Figure 7C & D). Streptavidin and the Avi antibody both detect biotinylated MBD1a^{Tavi} and MBD1b^{Tavi}. In order to examine the untagged MBD1d isoform, we also performed western blots with an antibody against MBD1 and included brain lysate from *Mbd1* knockout mice to specifically identify MBD1 isoforms (Figure 7C & D). The MBD1 antibody detects the untagged MBD1d isoform in addition to the two biotinylated Tavi-tagged isoforms (MBD1 antibody and streptavidin merged images, note red unbiotinylated MBD1d band). We found that at P7, the three MBD1 isoforms are all highly expressed in the brain, consistent with a previously reported role for MBD1 in neural stem cells [55]. Notably, MBD1 is barely detectable in non-neuronal tissues at P7. In addition, the expression of all MBD1 isoforms is significantly reduced in P42 brains compared with P7 while remaining nearly undetectable in other tissues.

Finally, we assessed MeCP2 and MBD3 expression in P7 and P42 wild-type animals using antibodies against MeCP2 and MBD3 (Figure 8). The specificity and reliability of these antibodies allowed us to complete the expression survey. Consistent with previous findings [40], we found that MeCP2 is highly expressed in the brain at P7 and P42, but is also expressed relatively highly in the lung and colon. Developmentally, MeCP2 expression is upregulated in the heart and liver but downregulated in the lung, kidney and colon in adult tissues, while remaining consistently high in the brain (Figure 8A & B). Parallel analysis of MBD3 expression in wild-type animals revealed that MBD3 is highly expressed in the brain with lowly detectable expression only in the colon at P7 (Figure 8C). Similarly to MBD1 (Figure 7C & D), MBD3 is significantly downregulated at adult ages in the brain while remaining lowly expressed in the colon and undetectable in other

tissues (Figure 8D). This result suggests that MBD3 may be acting at early developmental time points in the brain, similar to MBD1 but opposed to MBD2.

Our side-by-side comparison of MBD expression levels reveals that MBD2 is distinct from other MBD proteins in that it is highly expressed across multiple non-neuronal tissues. By contrast, MBD1, MeCP2 and MBD3 are highly enriched in the brain, consistent with their well-described neuronal functions [22,28,33]. Our biotin-tagging approach also allows for direct quantitative comparison of MBD2^{Tavi} and MBD1^{Tavi} protein expression by measuring the streptavidin signal, an approach that is not possible with different antibodies. We quantified the relative expression of MBD2a^{Tavi} and MBD1a^{Tavi} by normalizing the streptavidin signal to the H3 antibody signal. We performed this analysis in the brain and two additional representative tissues: the lung, where MBD1 is undetectable, and colon, where MBD1 is lowly expressed (average of three representative western blots) (Figure 9). This analysis demonstrated that MBD2a^{Tavi} is expressed significantly higher in the lung and colon compared with the brain at both developmental time points, P7 and P42 (Figure 9A). In contrast, MBD1a^{Tavi} is expressed significantly higher in the brain compared with the lung and colon at P7 and P42 (Figure 9B). We also assessed the temporal expression changes for MBD2a^{Tavi} and MBD1a^{Tavi} specifically in the brain (Figure 9C). We found that MBD2a^{Tavi} expression levels in the brain do not significantly change from P7 to P42, but MBD1a^{Tavi} is significantly downregulated at P42 compared with P7. In P42 brains, though the expression of MBD2a^{Tavi} is significantly higher than MBD1a^{Tavi}, it is relatively lower than MeCP2 (Figure 9C & Figure 8B). Together, the distinct spatiotemporal expression patterns of MBD2 in comparison to MBD1 and MeCP2 appear to underlie the distinct behavioral phenotypes observed in each individual MBD knockout mouse model.

Streptavidin-mediated pulldown of biotinylated MBD2^{Tavi} reveals constitutive interaction with the NuRD complex across tissues *in vivo*

Our analysis of MBD2^{Tavi} expression patterns showed that it co-expresses with other MBDs in several tissues, including the brain, lung and colon (Figures 6–9). It is possible that in the brain the other MBD proteins are predominant and may be able to compensate for the loss of MBD2 in *Mbd2* knockout mice. This model may explain why loss of MBD2 results in only mild behavioral phenotypes (Figure 1). However, the interactions between these proteins in other tissues where MBD2 is the predominantly expressed MBD protein are unclear. In particular, the functions of NuRD in tissues such as the lung where MBD2 is expressed but MBD3 is undetectable may be primarily MBD2-dependent.

Interactions between MBD2 and NuRD have only been examined using cell culture systems and the dynamics of these interactions *in vivo* have not been fully explored. Therefore, in order to verify MBD2 and corepressor interactions *in vivo*, we performed streptavidin-mediated co-pulldown followed by western blot experiments with MBD2^{Tavi} using brain, lung and colon lysate from *Mbd2*$^{Tavi/Tavi}$; *R26*$^{BirA/+}$ and *R26*$^{BirA/+}$ adult

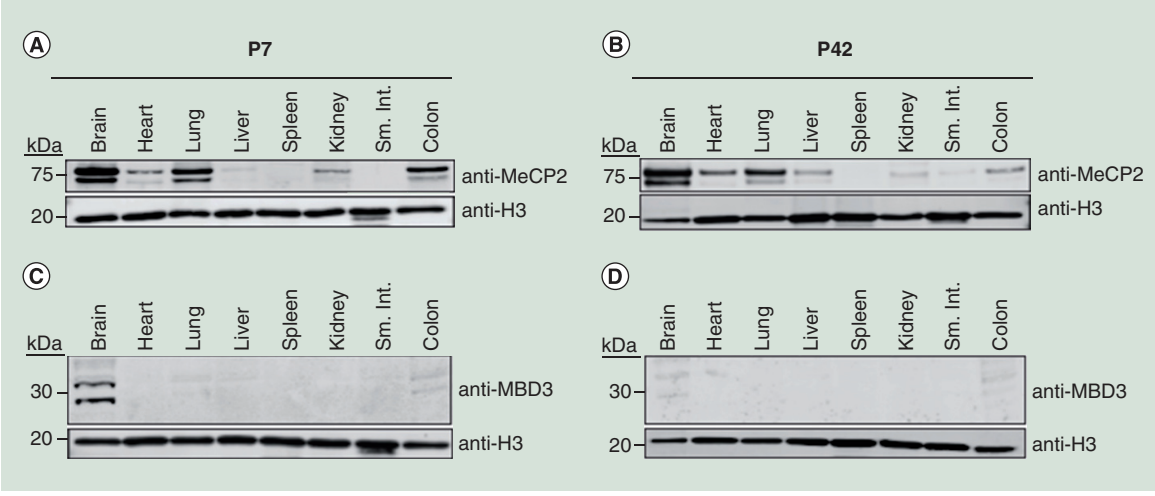

Figure 8. Spatiotemporal expression of MeCP2 and MBD3 at postnatal days 7 and 42. (A & B) MeCP2 is highly expressed in the brain at P7 and P42 as detected by an antibody against MeCP2 using lysate from wild-type animals. MeCP2 is also expressed in other tissues including the lung and colon. **(C & D)** MBD3 is highly expressed as a doublet band in the brain at P7 with lowly detectable expression at P42. MBD3 is also lowly detectable in the colon at P7 and P42.
Sm. Int.: Small intestine.

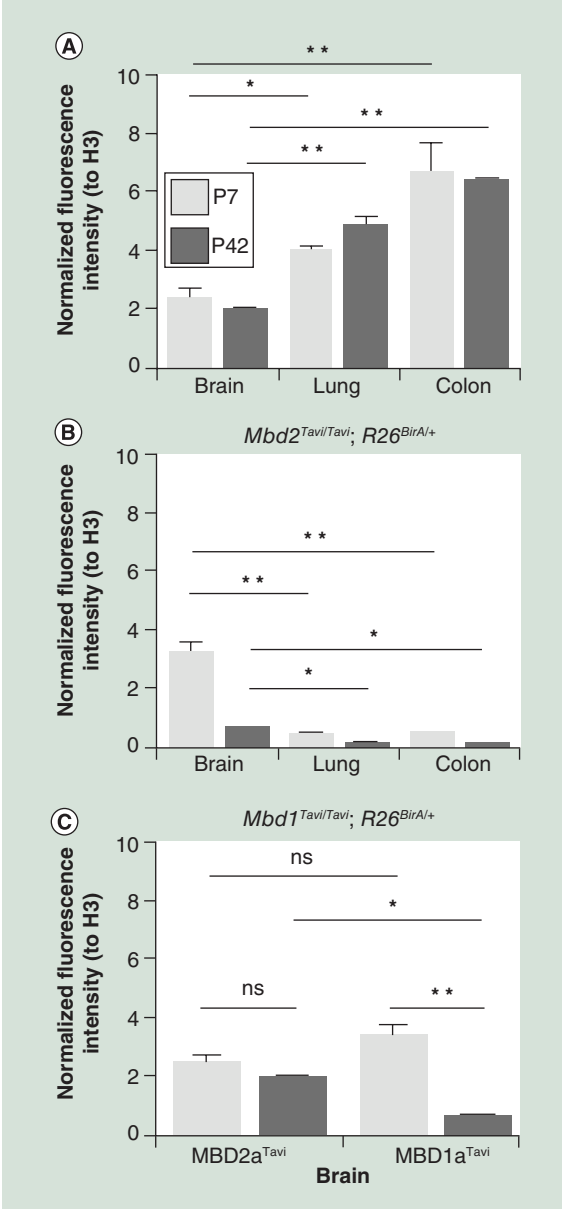

Figure 9. Quantification of MBD2a^{Tavi} and MBD1a^{Tavi} expression levels. Expression levels of biotinylated MBD2a^{Tavi} and MBD1a^{Tavi} were determined by normalizing the fluorescence levels of streptavidin to H3 (average of three representative western blots). **(A)** MBD2a^{Tavi} is expressed significantly higher in the lung and colon compared with the brain at P7 and P42 (*p < 0.05, **p < 0.01, two-way ANOVA with Tukey's test). **(B)** MBD1a^{Tavi} is expressed significantly higher in the brain compared with the lung and colon at P7 and P42 (*p < 0.05, **p < 0.01, two-way ANOVA with Tukey's test). **(C)** MBD2a^{Tavi} expression in the brain does not significantly change from P7 to P42, but MBD1a^{Tavi} is significantly downregulated in the brain at P42 compared with P7. At P42, MBD2a^{Tavi} is expressed significantly higher than MBD1a^{Tavi} in the brain (ns: Not significant, *p < 0.05, **p < 0.01, two-way ANOVA with Holm-Sidak multiple comparison test). All data are presented as mean ± standard error of the mean.

mice. This approach also allowed us to determine if there are tissue-specific interactions between the MBD proteins and their binding partners. We focused our analysis on the NuRD protein complex components that have been investigated in cultured cells [9,10,56].

In the brain and colon input samples, a nonspecific band at 50 kDa that is recognized by the Avi antibody partially obscures MBD2a^{Tavi} (Figure 10A & C). The absence of this nonspecific band in lung lysate (Figure 10B) and in the streptavidin-pulldown lanes for all tissues further verifies the specificity of our biotin-tagging approach. The merged Avi-antibody (red) and streptavidin (green) signals (yellow bands) further verify the biotinylation of MBD2^{Tavi}. We found that MBD2^{Tavi} had a strong association with components of the NuRD complex, including HDAC1, HDAC2, and MTA2, in the brain, lung and colon. To test the specificity of our pulldown approach, we examined MBD2^{Tavi} interaction with LaminB1 and found no association, as expected [9]. The essentially identical result observed in three separate tissues where MBD2 is highly expressed (Figure 6) suggests that these interactions are ubiquitous and may not have tissue or cell type specificity. Interestingly, these interactions also appear to be unchanged regardless of MBD3 expression, which is lowly expressed in the adult brain but undetectable in the lung or colon (Figure 8D). In these tissues MBD2 is likely the predominant MBD protein in association with NuRD. Together, these results support that MBD2 may act primarily in non-neuronal tissues to mediate NuRD complex-related activity.

Discussion

In this study we show that MBD2, relative to MeCP2 and MBD1, has less impact on brain function and has widespread expression and interactions with NuRD in peripheral tissues. We also describe a novel biotin-tagging system for MBD2 and MBD1 and demonstrate the utility of this system for the study of MBD functions *in vivo*. We found that MBD2 is highly expressed in many tissues and that MBD2 expression increases significantly in spleen and small intestine in adult mice. In contrast, MBD1, MeCP2 and MBD3 are primarily expressed in the brain, reflecting their well described neuronal functions [28–29,33]. This study provides new genetic tools and identifies new areas for future studies of MBD function *in vivo*.

We present the first behavioral characterization of *Mbd2*$^{-/-}$ mice, with emphasis on phenotypes affected by loss of related proteins MeCP2 or MBD1 [22,31]. Unexpectedly, we found that loss of MBD2 only results in a few subtle behavioral phenotypes. Most behavioral measures were unaffected in *Mbd2*$^{-/-}$ mice compared with wild-type and heterozygous littermates, including

motor ability, anxiety, sociability, and spatial learning and memory (Figure 1). *Mbd2*$^{-/-}$ mice are hypoactive in a home-cage environment and have impaired nesting behavior (Figure 1A & E). We also found that loss of MBD2 results in subtle, but significantly decreased body weight (Figure 2A & B). Although *Mbd2*$^{-/-}$ mice eat less food per day, they consume equivalent food normalized to body weight as wild-type littermates (Figure 2E & F). These findings support the conclusion that low body weight is established early in *Mbd2*$^{-/-}$ mice after weaning, after which *Mbd2*$^{-/-}$mice stabilize and continue to eat a sufficient amount of calories to maintain their body weight at steady state (Figure 2A).

In addition, we did not find any significant alterations in the levels of monoamines and their metabolites in *Mbd2*$^{-/-}$mice, unlike those observed in *Mecp2* or *Mbd1* knockout mice (Figure 3) [22,47].

In summary, *Mbd2*$^{-/-}$ mice show subtle changes in activity, body weight and nesting, at the age examined in this study. These phenotypes are multigenic and complex in nature and therefore it is challenging to determine the precise origins of these phenotypes [57–59]. These phenotypes have been observed in several mouse models of ASD or other neurodevelopmental disorders, but they are usually accompanied by significant deficits in other behavioral measures, supporting

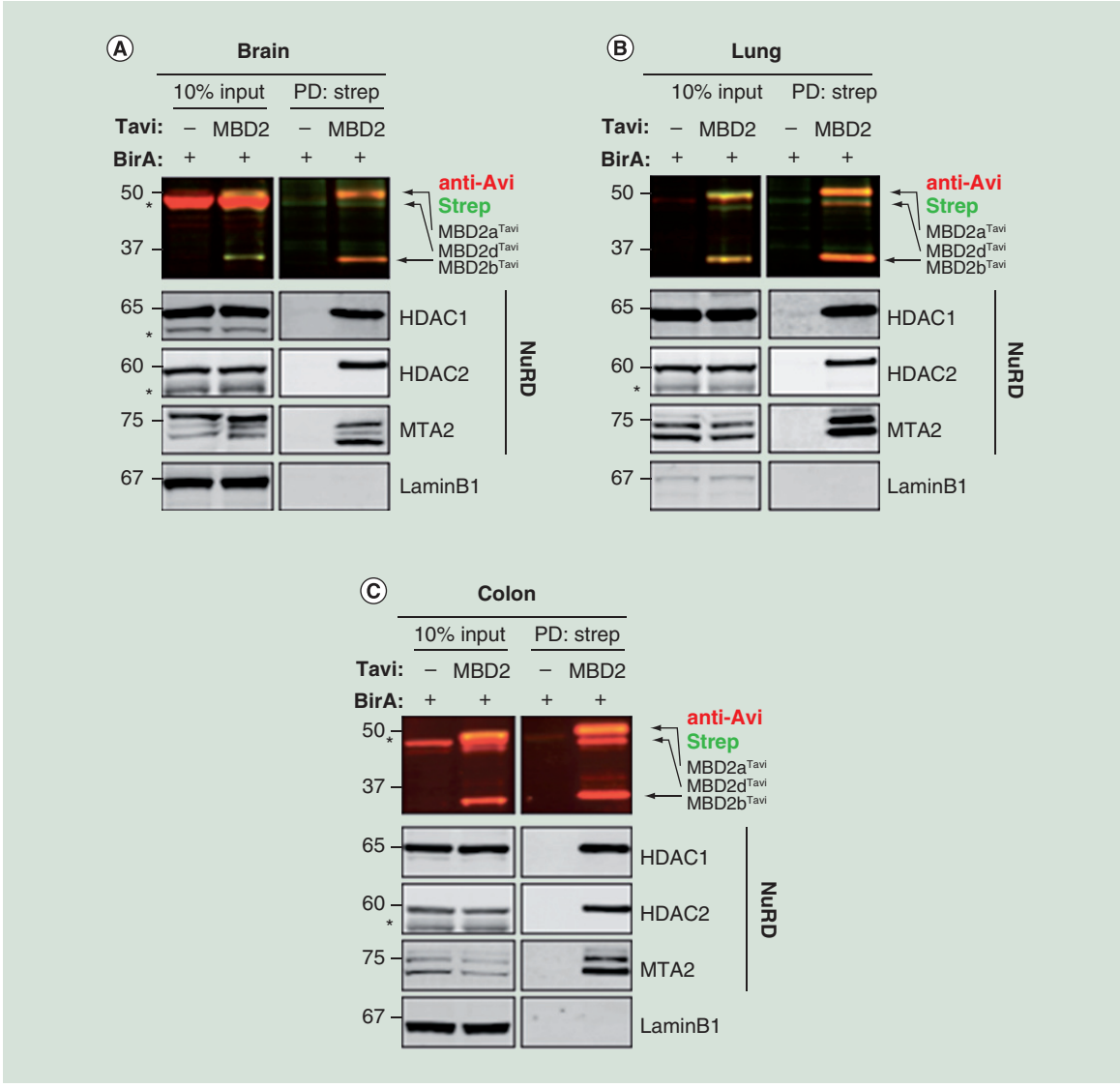

Figure 10. MBD2^{Tavi} interacts with the NuRD complex similarly across different tissues. Three isoforms of biotinylated MBD2^{Tavi} are specifically pulled down by streptavidin in **(A)** brain, **(B)** lung and **(C)** colon lysate and are detectable by the Avi-antibody and streptavidin (top panels). Streptavidin pulldown of MBD2^{Tavi} in each tissue co-precipitates components of the NuRD complex including HDAC1, HDAC2 and MTA2, with no interaction detected with LaminB1. Cross-reacting bands are denoted by *.
NuRD: Nucleosome remodeling and deacetylase complex; Strep: Streptavidin pulldown.

a neuronal origin [42,43]. Because *Mbd2*$^{-/-}$ mice show these phenotypes in the absence of additional changes in behavior or monoamine signaling, we conclude that they are not neuronal in origin and that the absence of MBD2 does not significantly impact neuronal functions. These findings are surprising in the context of the other MBD family proteins, which have been linked to brain-related functions in humans and mice [25,26,28,29,32,33].

To investigate possible MBD2 functions outside of the brain, we used a biotin-tagging approach (Figure 4 & Figure 5) to identify *in vivo* interactions with the NuRD complex in different tissues (Figure 10) and analyze the different spatiotemporal expression patterns of the MBD proteins (Figures 6–9). We observed that MBD2 likely interacts with components of the NuRD complex in a ubiquitous fashion, based on the representative results of three different tissues (Figure 10). The biotin-tagging strategy allowed us to perform a side-by-side, quantitative comparison of MBD2 and MBD1 expression, while avoiding the use of unreliable antibodies for MBD2. We were able to identify multiple isoforms of MBD2 and MBD1, including one MBD2 isoform that was not reported previously. Our spatiotemporal expression survey (Figures 6–9) demonstrates that MBD2 is distinct from MBD1, MeCP2 and MBD3 as it is highly expressed in many tissues instead of being primarily expressed in the brain. Previous studies have detected expression at the transcript level of *Mbd1*, *Mbd2* and *Mbd3* in the tissues examined here in adult mice, in contrast to the protein expression data we present. This finding is reminiscent of the reported disparities in *Mecp2* transcript and protein levels [40] and further illustrates the necessity of examining MBD protein expression at the post-translational level to guide further studies into MBD protein functions.

Our finding that MBD3 expression is undetectable in the tissues examined, with the exception of the colon, while MBD2 appears to be ubiquitously expressed raises new questions about the *in vivo* dynamics of the NuRD complex and MBD2 or MBD3. Biochemical evidence has shown that MBD2 and MBD3 are mutually exclusive in the NuRD complex, and it is apparent that the NuRD complex is essential in many tissues and biological processes including development and tumorigenesis [14,60]. Our expression data on MBD2 and MBD3 suggest that in adult tissues MBD2 must be the primary MBD protein component in the NuRD complex, as MBD3 is not detectable. However, this scenario again raises the question of why *Mbd2* knockout mice show relatively subtle phenotypes. Our expression data for MBD3 also indicate that MBD3 may be essential at early time points, particularly in the brain. This hypothesis is supported by mouse genetic studies showing that constitutive *Mbd3* knockout is embryonic lethal, while brain-specific *Mbd3* conditional knockout mice show perinatal lethality with neuronal deficits [33,38].

Our finding that MBD2 expression increases significantly in the spleen and small intestine from P7 to P42 is intriguing in the context of previous studies on the role of MBD2 in immunity and the small intestine (Figure 6C & D) [48–50,61–63]. Precise temporal control of methylation and demethylation is essential for the development and differentiation of both T cells [64] and intestinal epithelial crypt cells [65], indicating that MBD2 may contribute to the interpretation of DNA methylation in these tissues. For example, MBD2 has a role in T-cell development that is partially dependent on the methylation status of critical genes such as *Foxp3* [50,66], but also has a role in the innate immune system to indirectly affect T-cell activation and differentiation [49]. Interestingly, both MeCP2 and MBD1 have also been implicated in innate and adaptive immunity [67–69] indicating that MBD family proteins may have a wider role in these systems. In the intestine, loss of MBD2 is linked to altered gene expression [61]. MBD2 has also been identified as a potential target for cancer therapeutic intervention. Loss of MBD2 is protective against tumorigenesis in a mouse model of colorectal cancer by downregulating Wnt signaling [48,62]. In addition, MBD2 contributes to silencing of aberrantly hypermethylated tumor suppressor genes linked to colon cancer [70].

It is possible that MBD2 functions in non-neuronal systems such as the GI tract underlie the reduced body weight and other phenotypes observed in *Mbd2*$^{-/-}$ mice. The gastrointestinal system also has an essential role in immune system homeostasis [71]. Therefore, dysfunction in these two systems upon constitutive loss of MBD2 could have a synergistic effect on body weight or food intake phenotypes. In addition, body weight is inexorably linked to energy homeostasis [57], which could in turn affect activity levels to produce the hypoactivity and nesting deficits we observed in *Mbd2*$^{-/-}$ mice. Additional characterization of any metabolic phenotypes in *Mbd2*$^{-/-}$ mice may clarify the complexities of these phenotypes. Future studies of *Mbd2* conditional knockout mice would also aid in the isolation of tissue-specific MBD2 functions.

There are several possible explanations for why the absence of MBD2 did not result in significant changes in behavior or brain-related functions at the age examined. It has been proposed that the MBD family proteins may be functionally redundant because transcriptional repression is generally maintained in single MBD knockout models [72]. Our findings support the model of functional redundancy with regard to the

role of MBD2 in the brain. It is possible that MeCP2, which is expressed very highly in the brain and is essential for brain function [28,73], is the predominant MBD protein and is able to compensate for loss of MBD2. Alternatively, MBD3/NuRD may be the principal form of NuRD in the brain, as it is expressed in this tissue (Figure 8) and other studies have shown that it affects synaptic connectivity in addition to regulating cortical neuronal differentiation [33,34]. A related explanation for absence of significant MBD2-dependent brain function is that a brain-specific, alternate form of NuRD that interacts with MBD3, but not MBD2, may be acting predominantly in the brain *in vivo* [36].

Despite the possibility of functional redundancy for MBD2 in the brain, biochemical and genetic evidence suggests that MBD2 has functions distinct from the other MBD proteins. For example, MBD2/NuRD and MBD3/NuRD have different DNA-binding capabilities, which is reflected in their distinct genome-wide binding profiles [9,74,75]. Genetic evidence shows that loss of each MBD protein produces distinct phenotypes in mice, emphasizing the unique role for each MBD protein [22,28,38]. Furthermore, loss of both MBD2 and MeCP2 decreases survivability compared with loss of MeCP2 alone [76], indicating that MBD2 has other functions for which MeCP2 is unable to compensate. One example of MBD2-specific regulation occurs at the BRCA1–NBR2 locus in HeLa cells where MBD2, but not MeCP2 or MBD1, is highly expressed and binds to a constitutively methylated regulatory region [77]. An *Mbd1* and *Mbd2* double knockout mouse would help clarify these questions, but this has been difficult to achieve because the two genes are <4 Mb apart on the same chromosome. Further study of *Mbd2* knockout mice combined with *Mbd1* knockout or conditional *Mbd3* deletion will further elucidate the role of MBD2 in the context of the other MBD proteins.

An essential question, the MBD protein field that remains undetermined, is defining the precise functions of these proteins. Surprisingly, attempts to identify specific methylated loci that show direct transcriptional regulation by the MBD proteins have been mostly unsuccessful. Ablation of the MBD proteins, including MeCP2, MBD2 or MBD3, produces subtle transcriptional changes without a clear tendency for aberrant upregulation of genes that would be expected according to models of MBD proteins being transcriptional repressors [74,78]. A possible explanation for this discrepancy is that there are multiple and perhaps redundant mechanisms for transcriptional regulation. These include but are not limited to steric hindrance, where methylation simply blocks binding of other factors, and the establishment of repressive chromatin marks, both of which may occur independently of the MBD proteins [1]. For example, small amounts of aberrant *Xist* transcript are detectable in the absence of MBD2, but not other MBD proteins. However, other silencing mechanisms including HDAC activity were intact in *Mbd2* null cells [21]. Moreover, recent studies demonstrated that DNA methylation can be associated with active transcription possibly through the binding of activating transcription factors to methylated DNA, many with developmental or cell-type specificity [79]. Furthermore, the MBD proteins, including MBD2 and NuRD, are localized at many transcriptionally active sites [9,74]. These findings argue for a more dynamic picture of methylation-mediated transcriptional regulation than simple repression by the MBD proteins and their corresponding corepressor proteins [24].

Conclusion

In summary, our study describes the distinct loss-of-function phenotype, spatiotemporal expression patterns and corepressor interactions for MBD2 relative to other related MBD proteins. We found that, in contrast to MBD1, MeCP2 and MBD3, MBD2 is widely expressed in non-neuronal tissues and appears to have minimal impact on brain function and behavior that we measured. We also determined that MBD2 interacts with the NuRD complex similarly across tissues. Our findings provide new insights into the functions of MBD2 *in vivo* and also provide genetic tools to investigate the MBD family proteins in the context of neurodevelopmental disease, immunity and cancer.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi-2015-0004

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Behavioral characterization of *Mbd2* knockout mice

- Methyl-CpG-binding domain 2 (MBD2) functions *in vivo* are poorly understood, but other related methyl-CpG-binding domain (MBD) proteins are linked to brain function and neurodevelopmental disorders.
- *Mbd2* knockout mice show subtle behavioral phenotypes compared with wild-type and heterozygous littermates, including hypoactivity and deficits in nesting behavior.
- These phenotypes are in contrast to mice with loss of related MBD proteins, which have significant neurological and behavioral deficits.

***Mbd2*$^{-/-}$ mice have reduced body weight & decreased food intake**

- *Mbd2* knockout mice show reduced body weight and food intake but no changes in biogenic amine levels compared with littermates.

Generation of knockin mice expressing biotin-tagged MBD2 or MBD1

- We generated mouse lines in which endogenous MBD2 or MBD1 are biotin-tagged.
- We used these tagged mice to analyze MBD protein spatiotemporal expression in order to clarify the cell types and time points in which MBD2 may be acting *in vivo*.

Distinct spatiotemporal expression patterns of endogenous MBD2 in contrast to MBD1, MeCP2 & MBD3

- Unlike MBD1, MeCP2 and MBD3, which are enriched in the brain, MBD2 is highly expressed throughout the body in multiple tissues.

Streptavidin-mediated pulldown of biotinylated MBD2^{Tavi} reveals constitutive interaction with the NuRD complex across tissues *in vivo*

- Streptavidin-mediated pulldown of biotinylated MBD2^{Tavi} shows MBD2 interacts with the nucleosome remodeling and deacetylase component similarly across tissues.

Conclusion

- The distinct expression patterns and knockout phenotypes of MBD2 compared with those of related MBD proteins suggest that MBD2 has unique functions amongst the MBD family of proteins with minimal function in the brain.
- This study provides novel mouse lines and reveals new directions to characterize MBD2 function *in vivo*.

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