Affinity for DNA Contributes to NLS Independent Nuclear Localization of MeCP2

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INTRODUCTION

Eukaryotic cells are defined by having their genetic material contained within a membrane-bound organelle known as the nucleus. The nuclear pore complex (NPC) is a large protein channel embedded in the nuclear membrane that mediates transport of macromolecules between the nucleus and the cytoplasm. Small proteins are able to diffuse passively through the NPC, whereas larger factors require a nuclear localization signal (NLS)—a short motif that binds to proteins mediating active transport through the NPC. One such factor thought to require NLS-dependent transport into the nucleus is the chromatin-associated protein, MeCP2.

MeCP2 has been subjected to intense study since the discovery that mutations in this factor cause Rett syndrome (RTT)—a severe neurological disorder affecting approximately 1 in 10,000 girls. The original identification of MeCP2 was based on the affinity of its methyl-CpG binding domain (MBD) for DNA containing methylated CpG dinucleotides. RTT-causing missense mutations cluster within the MBD and impair the ability of MeCP2 to interact with chromatin, so MBD-mediated binding to DNA is likely to be essential for MeCP2 function. Analysis of brain regions from MeCP2-deficient mice revealed a slight relative increase in the expression levels of many genes with the greatest number of methylated cytosines, suggesting that MeCP2 functions as a weak but global transcriptional repressor.

In this study, we reveal a redundancy in the mechanism by which MeCP2 is localized to the nucleus. We report that nuclear localization of MeCP2 is not exclusively dependent on its canonical NLS. Rather, we find that either an intact NLS or chromatin binding is sufficient for nuclear retention. Consistent with NLS-independent nuclear localization of MeCP2, we find that disease progression in a mouse model of Rett syndrome is not impacted by a mutation that abolishes binding of the MeCP2 NLS to the nuclear import factors Kpna3 and Kpna4. Taken together, our results reveal a functional redundancy between DNA binding and NLS in targeting MeCP2 to the nucleus. This may rationalize the absence of pathogenic mutations in the NLSs of MeCP2 and may also be considered as an explanation for the scarcity of disease-causing mutations in the NLS motifs of other factors.

RESULTS

The NLS of MeCP2 Interacts with KPNA3 and KPNA4

Previously, we affinity purified MeCP2 from the brains of MeCP2-Egfp knockin mice and identified the nuclear import factors...
Kpna3 and Kpna4 as binding partners by mass spectrometry (Lyst et al., 2013). Subsequently, others mapped the KPNA3/KPNA4 binding site on MeCP2 to its previously identified NLS (Baker et al., 2015; Nan et al., 1996). This bipartite NLS is located between the MBD and the NCOR1/NCOR2 interaction domain (NID) of MeCP2 (Figure 1A).

To validate the mass spectrometry data and to ensure that these interactions are not artifacts of the EGFP tag, we performed immunoprecipitation from wild-type mouse brain nuclear extracts using an antibody against MeCP2 and revealed bound Kpna3/Kpna4 by western blotting. These proteins were absent in control immunoprecipitations performed using extracts from Mecp2-null mice, demonstrating the specificity of this assay. MeCP2 binding to Kpna3/Kpna4 was still readily detectable even using stringent washes with 300 mM sodium chloride, suggesting that this interaction is robust (Figure 1B). Next, we tested whether the MeCP2 NLS is sufficient to bind directly to the nuclear import machinery. To this end, we bacterially expressed and purified the MeCP2 NLS as a glutathione S-transferase (GST) fusion and immobilized this protein on glutathione Sepharose (Figure 1C). This protein was able to pull down recombinant 3×FLAG-tagged Kpna4 produced by in vitro translation in rabbit reticulocyte lysate. GST alone, on the other hand, showed no binding to Kpna4 in this assay (Figure 1D). Together with previous observations, our data strongly suggest that the MeCP2 NLS interacts directly with the nuclear import machinery in vivo.

The MBD of MeCP2 Is Sufficient for Nuclear Localization
Previous studies have shown that MeCP2 fused to EGFP localizes primarily to the nucleus, even in the presence of truncation mutations that disturb the originally defined NLS (Baker et al., 2013, 2015; Kumar et al., 2008; Schmiedeberg et al., 2009). Possible explanations for these observations could be residual activity of the canonical NLS or the presence of an unidentified NLS in MeCP2, which is not affected by these truncation mutations. An alternative hypothesis—not mutually exclusive with these models—is that MeCP2 might pass through nuclear pore complexes in a NLS-independent manner and then be retained in the nucleus due to its affinity for DNA.

To investigate these scenarios, we introduced a deletion mutation into the MBD—the primary DNA binding domain of MeCP2. This DNA binding mutant was then combined with truncation mutations, which either preserve or disrupt the NLS. The resulting proteins were then expressed in NIH 3T3 cells as fusions with EGFP. MeCP2 G273X, which has a wild-type MBD but was mutated in the context of the R255X truncation into the MBD—the primary DNA binding domain of MeCP2, localized correctly in the nucleus due to its affinity for DNA.

Retention of MeCP2 DNA Binding Correlates with NLS Independent Nuclear Localization of MeCP2
To probe further the relationship between nuclear retention and affinity for DNA, we extended the deletion mutant in the MBD to include the adjacent DNA binding motif, AT-hook 1 (Figure 1A).
AT-hook 1 is a sequence-specific DNA binding motif that contributes to chromatin binding by MeCP2 (Lyst et al., 2016). When this deletion was combined with G273X, the NLS of MeCP2 was nevertheless able to direct efficient nuclear localization (Figure 2A, bottom left). However, in combination with R255X, this mutation led to increased cytoplasmic accumulation of MeCP2 (Figure 2A, bottom right). Notably, in the absence of the NLS, simultaneous inactivation of the MBD and AT-hook 1 leads to a greater degree of cytoplasmic localization than that caused by abrogation of the MBD alone (Figure 2B). Therefore, the data support the conclusion that only inactivation of both the NLS and the ability to bind to DNA results in substantial cytoplasmic accumulation of MeCP2 (Figure 2B).

A Role for DNA Binding in Nuclear Localization of MeCP2 in Human Neurons
Given that RTT is a neurological disorder, we next asked whether this redundancy between the NLS and the DNA binding domains of MeCP2 could be observed in a more physiologically relevant neuronal culture system. In particular, we employed the human LUHMES cell line, which can be efficiently differentiated into mature post-mitotic dopaminergic neurons in vitro (Scholz et al., 2011). Using a lentiviral expression system to produce EGFP-MeCP2 in these cells, we observed that both MeCP2 G273X and MeCP2 R255X as well as MeCP2 G273XΔMBDΔAT-hook 1 were all found in the nucleus as assessed by co-localization with DAPI-stained DNA (Figure S1, upper three panels). MeCP2 R255XΔAT-hook 1, on the other hand, the only mutant protein with the DNA binding domains of MeCP2 inactivated together with its NLS, showed minimal co-localization with DAPI-stain nuclear DNA (Figure S1, bottom panels). Strikingly, the majority of the EGFP signal was found in aggregates outside of the nucleus, suggesting that, in this system, the DNA binding domains of MeCP2 play a key role in directing NLS-independent nuclear localization of MeCP2. We therefore conclude that the redundancy between the NLS and the DNA binding domains of MeCP2, which we first observed in mouse fibroblasts, is a general phenomenon that also holds true in human neurons.

MeCP2 R270X Retains a Residual Interaction with KPNA3/KPNA4
A previous study reported that the R270X mutation in MeCP2 abolishes its binding to KPNA3 but preserves a residual interaction with KPNA4 (Baker et al., 2015). The authors proposed that this weak binding to KPNA4 was likely to explain the nuclear localization of MeCP2 R270X. In our hands, as assessed by co-immunoprecipitation with exogenously expressed EGFP-MeCP2 in HeLa cells, both KPNA3 and KPNA4 show strongly reduced binding to the R270X mutation. However, both still retain a residual interaction compared with the more severe R255X truncation, which does not bind detectably to KPNA3 or
NLS Inactivation Does Not Influence MeCP2 Level or Localization in Mice

An earlier study found that the MeCP2 R270X mutation gives rise to more severe neurological deficits than the MeCP2 G273X mutation in mice. These phenotypic differences were attributed to the presence of an AT-hook in this region of the protein (AT-hook 2; Figure 1A) rather than any effects of the overlapping NLS, due to the nuclear localization of MeCP2 R270X (Baker et al., 2013). However, despite proper nuclear localization of MeCP2 R270X, loss of a non-canonical function of importin interaction contributing to the phenotypic deficits in these animals could not be excluded.

To assess the functional relevance of the MeCP2 NLS, we generated mutant mice designed to distinguish the effect of AT-hook 2 from that of importin binding. The first animal model carried a truncation mutation of MeCP2, where every amino acid from G273 is removed and replaced with a linker followed by EGFP. This essentially recapitulates an earlier model (Baker et al., 2013), except that the mutation is knocked into the endogenous locus rather than being expressed as a transgene. A second line was constructed similarly, except that four basic residues in the N-terminal part of the NLS were mutated to alanine. These residues do not overlap with AT-hook 2 (Figure 1A), and so this mutation allowed us to separate the phenotypic consequences of inactivating the NLS or this AT-hook (Figure 1A). We designed these two lines as Mecp2G273X-EGFP and Mecp2G273X-NLS-EGFP.

We first checked the expression levels of MeCP2 in these mice. As assessed by western blotting, total MeCP2 was slightly increased above that in wild-type controls, closely mirroring the situation when full-length MeCP2 is tagged with EGFP (Figure 3A; Brown et al., 2016). To confirm that the MeCP2G273X-NLS-EGFP mutation abolished the interaction of MeCP2 with Kpna3/Kpna4, we performed immunoprecipitation using an antibody against EGFP followed by western blotting with antibodies against Kpna3 and Kpna4. Whereas Kpna3 and Kpna4 bind robustly to MeCP2G273X-EGFP, no interaction could be detected with MeCP2G273X-NLS-EGFP, even though mild buffers containing only 150 mM sodium chloride were used (Figure 3B).

Next, we investigated the subcellular localization of MeCP2G273X-EGFP and MeCP2G273X-NLS-EGFP. Imaging of EGFP in CA1 hippocampal neurons in brain sections from these mice revealed that both mutant proteins localize correctly to DAPI dense foci, demonstrating that the NLS is not necessary for proper nuclear localization of MeCP2 in vivo (Figure 3C). To confirm the exclusively nuclear localization of these proteins, we prepared whole-cell and cytoplasmic extracts from these brains. Western blotting revealed that, unlike the control protein, gamma-tubulin, MeCP2 was found only in the whole-cell extract and was completely absent from the cytoplasmic fractions (Figure 3D). From these observations, we conclude that, in the brain, MeCP2G273X-EGFP remains strictly nuclear, even when the interaction of its NLS with Kpna3/Kpna4 is lost. The dispensability of the MeCP2 NLS that we have uncovered in tissue culture systems therefore appears to be also applicable in the most physiologically relevant tissue for this protein—the brain.

MeCP2 NLS Inactivation Does Not Influence the RTT-like Phenotype in Mice

As the Mecp2G273X-EGFP and Mecp2G273X-NLS-EGFP mice express MeCP2 to similar levels, we used these animals to assess the phenotypic consequences of destroying the interaction of MeCP2 with Kpna3/Kpna4. Like other RTT models, both lines initially developed grossly normally before succumbing to various neurological deficits. In order to make an objective comparison of the phenotypic severity in these two lines, we monitored the survival times and body weights of cohorts of male mice hemizygous for the Mecp2 gene. Consistent with the previous report on the G273X mutation, our Mecp2G273X-EGFP line displayed a median survival time of 36 weeks (Baker et al., 2013). This is substantially longer than the median survival time of 8 weeks reported for Mecp2-null mice (Baker et al., 2013; Chen et al., 2001; Guy et al., 2001). Our Mecp2G273X-NLS-EGFP mice also survived longer than null animals with a median age at death of 41 weeks. This is comparable to survival of the Mecp2G273X-EGFP animals, with no statistically significant difference detected between the two groups (Figure 4A). Also consistent with previous findings on Mecp2-null mice (Guy et al., 2001), we observed that animals from both lines were underweight when compared to wild-type controls (Figure S2A). However, in keeping with the similar survival curves of Mecp2G273X-EGFP and Mecp2G273X-NLS-EGFP mice, we did not detect a significant difference between the body weights of these two lines (Figure S2B).

To further characterize the Mecp2G273X-EGFP and Mecp2G273X-NLS-EGFP mice, we employed a phenotypic scoring system designed to assay several characteristic features of the RTT-like phenotype, including activity, gait, hind limb claspimg, tremor, breathing, and general condition (Guy et al., 2007). Both models initially displayed a rapid onset of symptoms before remaining relatively stable from approximately 15 weeks. Strikingly, the trajectory of disease progression was extremely similar for both lines (Figure 4B), and we were unable to detect any statistically significant difference between the two groups at age 15 weeks—the last time point at which all animals were still alive (Figure S2C).

Finally, we investigated the molecular and histological phenotypes present in our mutant mice. To this end, we analyzed the expression levels of a panel of genes previously reported to be consistently downregulated (Sst, Gabbr1, and Tspan17) or upregulated (Flrt3 and Arhgap10) across multiple brain regions and in several mouse models of RTT (Baker et al., 2013; Ben-Shachar et al., 2009; Mellén et al., 2012; Zhao et al., 2013). When compared to wild-type controls, both of our mouse lines showed subtle but statistically significant changes in the expression levels of these genes. However, for no tested genes were we able to detect any gene expression changes between our Mecp2G273X-EGFP and Mecp2G273X-NLS-EGFP animals.
To assess the histological phenotypes of our MeCP2 mutant mouse lines, we examined the brain weights and cortical thicknesses of these animals. Mouse models of RTT have previously been shown to have smaller brains with reduced cortical thicknesses (Chen et al., 2001; Robinson et al., 2012). Consistent with these reports, we found that both the MeCP2G273X-EGFP and MeCP2G273X,DNLS-EGFP animals displayed reduced brain weights and reduced thickness of the primary motor cortex when compared to wild-type controls. However, we were unable to detect any changes in these features when we compared the two mutant lines with one another (Figures 4D and 4E).

In summary, by monitoring survival, weight, phenotypic severity score, gene expression profiles, and cortical thickness, we were not able to observe any additional deficits in MeCP2G273X,DNLS-EGFP mice compared with MeCP2G273X-EGFP controls. We conclude that importin binding does not substantially affect the course of the pathology in these models of RTT and that the NLS of MeCP2 does not therefore strongly contribute to the function of this protein.

**DISCUSSION**

MeCP2 is a nuclear factor whose ability to recruit the NCOR1/NCOR2 co-repressor complex to chromatin is essential for proper brain function. Given that MeCP2 resides in the nucleus, the absence of disease-causing mutations in its NLS may at first appear inconsistent with the fact that MeCP2 inactivation leads to Rett syndrome. We have resolved this paradox by uncovering a functional redundancy in the mechanism by which MeCP2 is targeted to the nuclear compartment. An alternative explanation for the lack of RTT-causing mutations in the NLS would be if spontaneous mutations inactivating the MeCP2 NLS do not...
However, this appears unlikely, given the demonstration that a single amino acid substitution in the NLS can abolish its interaction with the nuclear import machinery (Baker et al., 2015).

The NLS of MeCP2 was originally defined by expressing fragments of MeCP2 as beta-galactosidase fusion proteins and assaying their localization in mouse fibroblasts (Nan et al., 1996). In this system, the NLS was necessary for MeCP2 to be partitioned in the nucleus. At first sight, these data contradict our finding that the MeCP2 NLS is dispensable for its nuclear localization. A likely explanation for the discrepancy is that beta-galactosidase, a large 464-kDa homo-tetramer, fused to MeCP2 might obligatorily require active transport through the nuclear pore complex. According to this hypothesis, smaller MeCP2 fusions with EGFP would be capable of NLS-independent entry into the nucleus, where they are then retained by their affinity for DNA. Although we cannot formally exclude the possibility that the DNA binding domains of MeCP2 interact with the nuclear import machinery to mediate active transport of this protein into the nucleus, we consider this less likely. First, we find no evidence for these domains interacting with the nuclear import machinery (Figure 2C). Second, two independent DNA binding domains, the MBD and AT-hook 1, act synergistically to direct nuclear localization. These two domains are structurally unrelated, but each has an affinity for a subset of nucleotide sequence motifs that is frequent in the genome.

Given that the MeCP2 NLS appears not to be required for localization of MeCP2 in the nucleus, a question arises as to the function of its interaction with KPNA3 and KPNA4. The NLS is a highly conserved region of MeCP2, and so its inactivation is likely to be detrimental, even if only on evolutionary timescales. One possibility is that importin binding to MeCP2 reflects a non-canonical role of these proteins. Importins have been proposed to function as chaperones for certain factors (Jäkel et al., 2002), and KPNA3/KPNA4 binding to MeCP2 could also reflect such a role. Alternatively, the selection pressure on the MeCP2 NLS may arise due to an extremely subtle effect on the nuclear targeting of this protein. For example, the kinetics of MeCP2 nuclear import might be perturbed or an extremely low

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**Figure 4.** MeCP2<sup>G273X</sup>-EGFP Mice Appear Unaffected by a Mutation Inactivating the MeCP2 NLS

(A) Survival curves for MeCP2<sup>G273X</sup>-EGFP (n = 8) and MeCP2<sup>G273X,ΔNLS</sup>-EGFP mice (n = 9), as well as wild-type controls for each group (n = 12 and n = 10, respectively). No significant difference between survival of MeCP2<sup>G273X</sup>-EGFP and MeCP2<sup>G273X,ΔNLS</sup>-EGFP mice was observed (p = 0.78; Kolmogorov-Smirnov test). An illustrative survival curve for MeCP2-null mice (based on data from previous studies) is shown as a broken line.

(B) Average phenotypic severity score of surviving animals plotted against age for each of the groups. Data for each group are plotted up to the last point, where there are at least three surviving animals.

(C) Expression levels relative to Gapdh of genes that are upregulated (Flrt3 and Arhgap10) or downregulated (Sst, Tspan17, and Gabbr1) in brains of MeCP2 mutant mice as assessed by qPCR. Data are presented as means ± SD.

(D) Brain weights for MeCP2<sup>G273X</sup>-EGFP and MeCP2<sup>G273X,ΔNLS</sup>-EGFP mice (n = 4 for each group) as well as wild-type controls (n = 5 and n = 3). Indicated statistical significance was calculated using the t test. Data are presented as means ± SD.

(E) Cortical thickness plotted for MeCP2<sup>G273X</sup>-EGFP and MeCP2<sup>G273X,ΔNLS</sup>-EGFP mice (n = 4 each) and wild-type control animals (n = 5 and n = 3). Data are presented as means ± SD. Indicated statistical significance was calculated using the t test. The image depicts the area of the primary motor cortex (M1) measured. The scale bar represents 1,000 μm. See also Figure S2.
concentration of MeCP2 in the cytoplasm of large neurons might reduce the amount of active protein available in the nucleus. Whatever the role of MeCP2 binding to the nuclear import machinery, our work has highlighted the importance of using genetics to avoid misattributing disease relevance to evolutionarily conserved biochemical interactions.

This study has uncovered a surprising redundancy between the DNA binding domains and the NLS of MeCP2 in the nuclear localization of this factor. Given the paucity of NLS-inactivating mutations implicated in other genetic disorders (McLane and Corbett, 2009), an interesting line of future work will be to determine whether chromatin binding also contributes to nuclear sequestration of other factors. As well as providing insights into the mechanism of nuclear targeting of MeCP2, the present study should also be of interest to researchers seeking treatments for Rett syndrome. Currently, the gene therapy vectors showing the most promise in targeting the CNS have a limited capacity, leading to efforts to miniaturize MeCP2 expression constructs containing only the required functional domains (Tillotson et al., 2017). By better delineating the disease-relevant functional domains of MeCP2, our work has both improved molecular understanding of this protein and also strengthened the groundwork for one of the more promising strategies for treating Rett syndrome.

**EXPERIMENTAL PROCEDURES**

**Protein Interactions**

Co-immunoprecipitation and in vitro binding assays were performed essentially as described previously (Lyst et al., 2013), except that buffers were supplemented with 300 mM rather than 150 mM sodium chloride where indicated. GST fusion proteins were produced as described elsewhere (Lyst et al., 2016).

**Cell Imaging**

NIH 3T3 cells were maintained, transfected, harvested, fixed, and imaged as described previously (Lyst et al., 2013). LUHMES cells were maintained and transduced with lentiviruses as described previously (Shah et al., 2016). Images were quantified using the ImageJ software package with the investigator blind to the identities of the transfected MeCP2 constructs. ΔMBD and ΔMBDΔAT-hook 1 constructs lacked amino acids 111–168 and 111–191.

**Subcellular Fractionation**

For preparation of whole brain and cytoplasmic brain extracts, fresh material (not frozen) was dounce homogenized in NE1 buffer (20 mM HEPES [pH 7.5], 10 mM NaCl, 1 mM MgCl2, and 0.1% Triton X-100). This homogenate was taken as whole-brain extract, and the supernatant following centrifugation for 5 min at 500 g was taken as cytoplasmic extract.

**Animals**

All animal experiments were carried out by staff licensed by the UK Home Office in accordance with the Animal and Scientific Procedures Act 1986. Targeting vectors for generating mutant mice were based on one described elsewhere (Lyst et al., 2013). For the NLS mutation, four consecutive amino acids (RKRK) comprising the N-terminal basic cluster of this bipartite signal were mutated to alanine. Targeting vector was co-transfected into 129/Ola E14 TG2a mouse embryonic stem cells (ESCs) with Cas9 (nicking) and appropriate guide RNA expression plasmids (Ran et al., 2013). Mice were generated from these cells by standard procedures before backcrossing onto the C57BL/6 background for at least two generations. Scoring of phenotypes was performed blind to genotypes as described elsewhere (Guy et al., 2007), with wild-type and mutant male mice being weighed and monitored weekly from 4 weeks of age for one year. For biochemical analysis (co-immunoprecipitation, subcellular fractionation, and western blotting), brains were taken from male mice aged 8–18 weeks.

**Histology**

Brains from wild-type and mutant male littermates aged 10–18 weeks were dissected, removing the olfactory bulb and trimming to remove any spinal cord. Brains were weighed and immersed in fixative (3.7% paraformaldehyde in PBS). After 2 hr, brains were transferred to fresh fixative and stored overnight at 4°C. Brains were transferred to 30% sucrose solution in PBS and stored at 4°C for 48 hr, replacing the solution after 24 hr. Brains were then frozen in isopentane, chilled on dry ice, and stored at ~80°C. A Leica CM1900 cryostat set to ~26°C was used to cut 30-μm coronal sections, which were mounted on SuperFrost Plus slides and dried. Sections were photographed using an Olympus SXZ10 microscope at 2.6× magnification. The location of each section was determined with reference to “The Mouse Brain in Stereotaxic Coordinates” (Franklin and Paxinos, 2008). The primary motor cortex was defined using the atlas and the cortical thickness measured near to the center of the region. Between 22 and 33 sections were measured for each mouse, between 0.98 and ~0.46 mm from Bregma (Franklin and Paxinos, 2008).

**Gene Expression Analysis**

Total RNA was purified from whole brains of male mice aged 7–14 weeks using TRI Reagent (Sigma; T9424). The QuantiTect Reverse Transcription Kit (QiAGEN) was used for cDNA synthesis, and gene expression was quantified using the SensiMix SYBR & Fluorescein kit (Bioline) on a LightCycler 480.

**Statistical Methods**

For quantification of the nuclear to cytoplasmic ratio of EGFP-MeCP2 mutants in NIH 3T3 cells, statistical significance was calculated using the Wilcoxon test. For qPCR data as well as analysis of brain weights and cortical thicknesses, p values were calculated using the Student’s t-test. The Kolmogorov-Smirnov test was used to calculate p values for comparisons of body weights and phenotypic scores.

**Antibodies**

Antibodies used were as follows: Histone H3 (Abcam; ab1791); gamma-tubulin (Sigma; T5326); MeCP2 (Sigma; M6818 and M7443); KPNA3 (Abcam; ab6038); KPNA4 (Bethyl Laboratories; A301-627A); EGFP (CRUK and Chro-motek gta-10); and FLAG (Sigma; F3165).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes two figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.099.

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**AUTHOR CONTRIBUTIONS**

M.J.L. performed biochemical, cell imaging, and gene expression analysis. R.E. mapped KPNA3/4 binding site to the NLS of MeCP2, C.M. generated embryonic stem cell lines, J.S. generated mouse lines, J.G. performed histology. M.V.K. and D.D.S. performed phenotypic analysis of mice. M.J.L. and A.B. conceived the experiments and wrote the paper.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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