Identification and characterization of conserved noncoding cis-regulatory elements that impact MeCP2 expression and neurological functions

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While changes in MeCP2 dosage cause Rett syndrome (RTT) and MECP2 duplication syndrome (MDS), its transcriptional regulation is poorly understood. Here, we identified six putative noncoding regulatory elements of MeCP2, two of which are conserved in humans. Upon deletion in mice and human iPSC-derived neurons, these elements altered RNA and protein levels in opposite directions and resulted in a subset of RTT- and MDS-like behavioral deficits in mice. Our discovery provides insight into transcriptional regulation of MeCP2/MECP2 and highlights genomic sites that could serve as diagnostic and therapeutic targets in RTT or MDS.

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Coding variants in hundreds of genes are known to alter protein levels and lead to intellectual disability [ID] (Schanze et al. 2018). However, only a handful of disease-causing mutations in noncoding cis-regulatory elements (CREs) (Soldner et al. 2016; Oz-Levi et al. 2019) have been identified, and our understanding of how these contribute to ID is limited. MECP2 is an exemplar ID-causing, dosage-sensitive gene, with neurological dysfunction arising from both decreased [RTT] (Amir et al. 1999) and increased [MDS] (Van Esch et al. 2005) levels of MeCP2. This dosage sensitivity underscores the concept that precise control of MeCP2 levels is important for normal brain function (Chao and Zoghbi 2012, Sztainberg et al. 2015). Regulation of MECP2 occurs at the transcriptional, post-transcriptional (Gennarino et al. 2015; Rodrigues et al. 2016), and post-translational levels (Lombardi et al. 2017; Yagasaki et al. 2018), with the latter two being the most well studied. Currently, little is known about the transcriptional regulation of MECP2 beyond its core promoter region (Liu and Francke 2006; Nagarajan et al. 2006; Swanberg et al. 2009).

Results and Discussion

To identify potential CREs of MeCP2 in the brain, we profiled open chromatin in the developing and adult mouse brain using the assay for transposase-accessible chromatin with deep sequencing [ATAC-seq] (Buenrostro et al. 2013). We restricted our search to accessible regulatory elements within the 100-kb genomic region of MeCP2, flanked upstream by the Opi1nw gene and downstream by the Ilak1 gene. This genomic region, containing only MeCP2; faithfully drives expression of human MECP2 in a transgenic mouse line, rescuing all neurological defects in MeCP2-null mice (Collins et al. 2004). We identified six putative regulatory elements [Fig. 1A]: one at the promoter (Peak-4), three in intron 2 that increase in accessibility during development [Peak-1, Peak-2, and Peak-3], one upstream of MeCP2 (Peak-5) that decreases in accessibility in the adult brain compared with the postnatal day 6 brain, and one peak upstream of MeCP2 (Peak-6) that is consistently accessible throughout development. Importantly, these same peaks are present in previously published ATAC-seq data sets from multiple neuronal cell types in the adult mouse brain (Supplemental Fig. S1; Mo et al. 2015), confirming our findings.

To test whether these putative CREs regulate MeCP2 expression in the mouse brain, we deleted the genomic regions corresponding to each individual peak using CRISPR-Cas9 genome editing [Supplemental Table S1], generating five unique mouse lines. We excluded Peak-4 from our analysis because it corresponds to the MeCP2 promoter, whose deletion would likely ablate MeCP2 expression. We measured MeCP2 mRNA expression in the brains of our knockout [Peak KO] lines and found that MeCP2 expression was decreased by ~20%–30% in Peak-2KO/y, Peak-3KO/y, and Peak-5KO/y mice, while it was increased by 50% in Peak-6KO/y mice and unchanged in Peak-1KO/y mice [Fig. 1B]. Measurement of MeCP2 protein levels by Western blot showed 30% reduction (P<0.01) in Peak-2KO/y mice and 70% increase (P<0.0001) in Peak-6KO/y mice, while protein level changes in Peak-1KO/y, Peak-3KO/y, and Peak-5KO/y mice were not significant [Fig. 1C]. Interestingly, Peak-2 and Peak-6 are the only two elements with strong sequence conservation at the nucleotide level between mice and humans, suggesting that...
evolutionary pressure maintained these two gene regulatory elements [Supplemental Fig. S2].

Given that the magnitude of change in MeCP2 levels in both the Peak-2KO/y and Peak-6KO/y mice is less than that of the Mecp2flox/flox allele [Samaco et al. 2008] and MECP2 duplication mouse models [Collins et al. 2004], respectively, we wanted to identify and characterize any neurological dysfunction that may arise from more subtle changes in MeCP2 levels. Therefore, we performed a battery of behavioral tests on Peak-2KO/y and Peak-6KO/y male mice, after confirming peak knockout did not result in any gross histological abnormalities as assessed by Cresyl violet staining [Supplemental Fig. S3].

At 10 wk, Peak-2KO/y mice, which have ~30% reduction in MeCP2 expression, were hyperactive compared with their wild-type littermates [Fig. 2A]. At 24 wk, these mice had anxiety-like phenotypes [Fig. 2B] and social deficits [Fig. 2C]. At 40 wk, these mice showed social dominance deficits [Fig. 2D]. These behavioral deficits are reminiscent of those observed in Mecp2flox/flox mice and some RTT [Moretti et al. 2005] and autism mouse models [Spencer et al. 2005; Kazdoba et al. 2016]. Unlike Mecp2flox/flox mice that express 50% of the normal MeCP2 level (Samaco et al. 2008), we did not observe any sensorimotor gating deficits, motor abnormalities, or learning and memory defects in the Peak-3KO/y mice [Supplemental Fig. S4A–F].

Last, we measured the expression of several genes that are dysregulated in Mecp2-null mice [Boxer et al. 2020]. Strikingly, expression of these genes in the cortex of Peak-2KO/y mice were altered in the same direction as reported in Mecp2-null animals [Supplemental Fig. S4G,H; Chahrour et al. 2008]. These data suggest that a mild reduction in MeCP2 levels mimics a subset of behavioral and molecular changes observed in Mecp2-null mice.

In contrast, Peak-6KO/y mice, which display ~70% increase in MeCP2, show hypoactivity [Fig. 3A] and anxiety-like phenotypes [Fig. 3B,C] at 10 wk. At 24 wk, these mice showed hippocampal-dependent contextual learning deficits in the contextual fear assay [Fig. 3D]. These phenotypes resemble the behavioral profile of MECP2-Tg1 mice [which have a 100% increase of MeCP2 protein]. However, unlike the MECP2-Tg1 mice, Peak-6KO/y mice showed no deficits in sensorimotor gating, motor function, or social behavior [Supplemental Fig. S5A–E]. When we measured the expression of several known to be dysregulated in the MECP2-Tg1 mouse model [Chahrour et al. 2008; Samaco et al. 2012], we found these MeCP2 targets were altered in the same direction as reported in MECP2-Tg1 mice in the cortex of Peak-6KO/y mice [Supplemental Fig. S5F,G]. These data suggest that a moderate increase in MeCP2 protein level recapitulates a subset of MDS-like behavioral and molecular phenotypes.

We next subjected the genomic sequences of these peaks to transcription factor motif
analysis using HOMER to identify putative factors that bind within these peaks, and this analysis identified a potential CTCF binding site on Peak-6 (Supplemental Fig. S6A; Heinz et al. 2010). To validate this prediction, we conducted CTCF chromatin immunoprecipitation (ChIP) followed by qRT-PCR in the wild-type mouse frontal cortex and found CTCF enrichment on Peak-6 (Supplemental Fig. S6B). Disruption of the structural protein CTCF can result in dysregulation of genes near its binding site (Dixon et al. 2012). We measured the expression of genes upstream of Peak-6 and downstream from Mecp2 to see whether their expression is altered. We found the expression of Itcp1 (2 kb downstream) and Bgn (480 kb downstream) are increased but not Zfp185, which is 980 kb downstream from Mecp2. Similarly, the expression of Enmd (140 kb upstream) and Taz (175 kb upstream) are elevated but not Ikbkg (310 kb upstream), indicating that disruption of this regulatory element can cause dysregulation of some neighboring genes (Supplemental Fig. S6C, D). However, the expression of these genes is not affected in Peak-2 KO mice (Supplemental Fig. S6E). Mouse genetic studies have demonstrated that increasing MeCP2 protein levels alone is sufficient to cause the neurological phenotypes seen in MDS (Ramocki et al. 2010). These data together with our discovery that the phenotypes seen in Peak-6KO/y mice are a subset of those seen upon protein expression in cultured human neurons. First, we deleted the two CREs in two male, human induced pluripotent stem cell (iPSC) lines using CRISPR/Cas9 [Supplemental Table S2]. Next, we generated Vglt1-positive glutamatergic neurons (iNeurons) from these stem cells using directed differentiation via overexpression of Neurogenin2 (Ngn2) [Supplemental Fig. S8; Zhang et al. 2013]. Consistent with our mouse models, MECP2 mRNA expression is reduced in Peak-2KO/y iNeurons by ~50%, while it is increased in Peak-6KO/y iNeurons by ~30% (Fig. 4A). Protein levels of MeCP2 also showed a concomitant change (Fig. 4B,C, Supplemental Fig. S9). These data strongly suggest that Peak-2 and Peak-6 regulate MECP2 expression. Furthermore, motif analysis also identified a potential CTCF binding site on the human sequence of Peak-6 (Supplemental Fig. S7B; Heinz et al. 2010), and we validated that CTCF binds within this region using ChIP-qPCR (Supplemental Fig. S7C).

To date, several mouse models with varying levels of MeCP2 have been well characterized, notably, MeCP2flox/flox mice (50% MeCP2) and transgenic MECP2-Tg1 [200% MeCP2] show progressive behavioral deficits [Collins et al. 2004; Samaco et al. 2008]. Our two CRE KO mouse models (Peak-2 and Peak-6) perturb MeCP2 levels in similar directions (albeit milder) than the MeCP2flox/flox mice and the MECP2-Tg1 mice, respectively. Our in vivo studies show that even these subtle alterations are sufficient to produce some behavioral phenotypes observed in MeCP2flox/flox and MECP2-Tg1 mice (Fig. 5). Our CRE KO lines provide a unique allelic series demonstrating that disease severity directly corresponds with precise MeCP2 levels in the brain. We next tested whether deletion of these conserved CREs also regulates MECP2 expression in cultured human neurons. First, we deleted the two CREs in two male, human induced pluripotent stem cell (iPSC) lines using CRISPR/Cas9 [Supplemental Table S2]. Next, we generated Vglt1-positive glutamatergic neurons (iNeurons) from these stem cells using directed differentiation via overexpression of Neurogenin2 (Ngn2) [Supplemental Fig. S8; Zhang et al. 2013]. Consistent with our mouse models, MECP2 mRNA expression is reduced in Peak-2KO/y iNeurons by ~50%, while it is increased in Peak-6KO/y iNeurons by ~30% (Fig. 4A). Protein levels of MeCP2 also showed a concomitant change (Fig. 4B,C, Supplemental Fig. S9). These data strongly suggest that Peak-2 and Peak-6 regulate MECP2 expression. Furthermore, motif analysis also identified a potential CTCF binding site on the human sequence of Peak-6 (Supplemental Fig. S7B; Heinz et al. 2010), and we validated that CTCF binds within this region using ChIP-qPCR (Supplemental Fig. S7C).
demonstrate, in agreement with previous work, that normal MeCP2 dosage (100%) is required for normal brain function in mice, and either a 20%–30% decrease or a 50%–70% increase in MeCP2 may lead to some neuropsychiatric phenotypes (altered activity, anxiety, and social and learning deficits), while 50% decrease or 100% increase of MeCP2 leads to severe neurodevelopmental disorders like RTT or MDS, respectively.

MeCP2 expression has a unique spatial and temporal pattern, where it is the lowest in the liver and highest in the brain (Supplemental Fig. S10A) and increases significantly postnatally (Supplemental Fig. S10B). Accurate postnatal MeCP2 level is critical for normal brain function. Generating ATAC-seq profiles during developmental stages that coincide with the timing of MeCP2 increase identified regions with accessible chromatin structure at the MECP2 gene locus. These accessible regions may contain specific chromatin remodelers or transcription factors that regulate MECP2 expression. Further studies will be needed to identify the putative transcription factor or factors that bind to Peak-2 or Peak-6 to regulate MECP2 expression.

Understanding the phenotypic range that results from varying MeCP2 levels is critical for a number of reasons: first, assessing the type and severity of clinical manifestations of MECP2 dosage-related disorders; second, classifying and treating non-RTT patients with neuropsychiatric disorders due to mild MeCP2 mutations or noncoding mutations that affect MeCP2 levels (e.g., in CREs); and third, predicting the clinical benefits of therapeutic interventions based on the degree of modulation of MeCP2 levels. Studies on existing mouse models across a range of dosage modifications. A detailed description is in the Supplemental Material.

Figure 5. Summary of Peak KO phenotypes and comparison with MeCP2 50% loss (Mecp2lox/lox) or MDS mouse model (MECP2-Tg1).

of CREs alters MeCP2 levels in cultured human iPSC-derived neurons. Human mutations in these cis-regulatory regions could contribute to atypical RTT patients without mutations in MECP2 coding regions or patients with ID, autism, or neuropsychiatric disorders. Many genes that cause neurodevelopmental disorders and autism (Satterstrom et al. 2020) are dosage sensitive (Han et al. 2013; Rocha et al. 2016; Rice and Mclysaght 2017; Raveau et al. 2018; Schnabel et al. 2018). It is possible that unidentified mutations in regulatory elements of these genes may also affect their abundance and lead to disease phenotype. Our research highlights the importance of whole-genome sequencing (WGS) to achieve diagnoses in cases in which exome sequencing fails. Furthermore, the catalog of mutations by WGS on the X chromosome is underrepresented due to the reduced effective population size of sequenced X chromosomes, as males only carry one copy (Telenti et al. 2016). Our study also demonstrates that representation of the noncoding regions on the X-linked gene MECP2 in WGS needs to be re-examined to detect any potential variants in these critical regions.

In conclusion, we identified and functionally characterized two novel, evolutionarily conserved regulatory elements required for normal expression of Mecp2/MECP2. Deletion of either regulatory element in mice caused mild neurological dysfunction, highlighting how small changes in MeCP2 levels in either direction disrupt neurological function. More broadly, this study underscores the potential contribution of mutations in regulatory regions to various neuropsychiatric phenotypes and calls for similar studies of regulatory regions in other dosage sensitive genes involved in autism and ID.

Materials and methods

Animals

Mice were housed in an AAALAS-certified level 3 facility on a 14-h light cycle. All CRE knockout mice were generated in the Genetically Engineered Mouse Core at Baylor College of Medicine, backcrossed with wild-type C57BL/6J mice for five generations. Only male offspring were used for analysis. All procedures to maintain and use these mice were approved by the Institutional Animal Care and Use Committee for Baylor College of Medicine and Affiliates.

Generation of the knockout mice of regulatory elements

All CRE deletion mice were generated via CRISPR/Cas9-mediated gene editing. Briefly, two sgRNAs targeting the 5′ and 3′ [left (L) and right (R)] ends of the putative CREs were designed in Benchling and synthesized by IDT. sgRNAs were in vitro transcribed with the MEGASHortscript T7 transcription kit (Invitrogen). Details about injection are available in the Supplemental Material.

Behavioral assays

All data acquisition and analyses were performed by an individual blinded to the genotype. All behavioral studies were performed during the light period. At least 1 d was given between assays for the mice to recover. All the tests were performed as previously described (Chao et al. 2010) with few modifications. A detailed description is in the Supplemental Material.
ATAC-seq

Nuclear isolation was performed according to Mo et al. [2015] with two biological replicates. Nuclei were collected from fresh brain tissues for ATAC-seq. Detailed descriptions are available in the Supplemental Material.

Statistical analysis

Statistical significance was determined using GraphPad Prism software. The number of animals [n] and the specific statistical tests for each experiment are indicated in the figure legends. Sample size for behavioral studies was determined based on previous experience using mice with the same background.

Data and materials

All data needed to evaluate the conclusions in this study are present here and in the primary reference. Details of the DNA and materials are indicated in the figure legends. Sample size for behavioral studies was determined based on previous experience using mice with the same background.

Author contributions

All data needed to evaluate the conclusions in this study are presented here and/or in the primary reference. ATAC-seq data from different mouse neuronal cell lines were obtained from Mo et al. [2015] and GEO GSE63137. ATAC-seq data from human brains were obtained from GSE149268. The accession numbers for the raw and processed data files reported here are GEO GSE152719. Additional data related to this study are available on request.

Competing interest statement

The authors declare no competing interests.

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Author contributions

Y.S. conducted bioinformatic analysis. Y.S. wrote the manuscript and prepared the figures. M.C.G. and J.D.W. performed ATAC-seq experiments and conducted bioinformatic analysis. Y.S. wrote the manuscript and prepared the figures, with contributions from all authors according to their area of expertise. H.Y.Z. reviewed all of the data and edited the manuscript.

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