The Chromatin-binding Protein HMGN1 Regulates the Expression of Methyl CpG-binding Protein 2 (MECP2) and Affects the Behavior of Mice

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Background: HMGN1 is a chromatin-binding protein that modulates the cellular transcription profile. Results: Mice that either overexpress or lack HMGN1 have altered behavioral phenotypes. HMGN1 is a negative regulator of MeCP2, a protein involved in neurodevelopmental disorders. Conclusion: Misregulation of HMGN1 protein levels lead to behavioral changes in mice. Significance: HMGN1 is an epigenetic factor that contributes to the development of neurodevelopmental disorders.

High mobility group N1 protein (HMGN1), a nucleosomal-binding protein that affects the structure and function of chromatin, is encoded by a gene located on chromosome 21 and is overexpressed in Down syndrome, one of the most prevalent genomic disorders. Misexpression of HMGN1 affects the cellular transcription profile; however, the biological function of this protein is still not fully understood. We report that HMGN1 modulates the expression of methyl CpG-binding protein 2 (MeCP2), a DNA-binding protein known to affect neurological functions including autism spectrum disorders, and whose alterations in HMGN1 levels affect the behavior of mice. Quantitative PCR and Western analyses of cell lines and brain tissues from mice that either overexpress or lack HMGN1 indicate that HMGN1 is a negative regulator of MeCP2 expression. Alterations in HMGN1 levels lead to changes in chromatin structure and histone modifications in the MeCP2 promoter. Behavior analyses by open field test, elevated plus maze, Reciprocal Social Interaction, and automated sociability test link changes in HMGN1 levels to abnormalities in activity and anxiety and to social deficits in mice. Targeted analysis of the Autism Genetic Resource Exchange genotype collection reveals a non-random distribution of genotypes within 500 kbp of HMGN1 in a region affecting its expression in families predisposed to autism spectrum disorders. Our results reveal that HMGN1 affects the behavior of mice and suggest that epigenetic changes resulting from altered HMGN1 levels could play a role in the etiology of neurodevelopmental disorders.

HMGN1 is a ubiquitous vertebrate nuclear protein that binds specifically to nucleosome core particles, the building block of the chromatin fiber (5, 6). The interaction of HMGN1 with nucleosomes, which is highly dynamic (7–9), modulates the structure of chromatin (5, 6) and the levels of histone modifications (10, 11), thereby playing a role in epigenetic regulation. HMGN1 affects chromatin functions including DNA repair (12) and transcription (13, 14); however, its biological function is not fully understood.

The biological function of HMGN1 is of special interest because human HMGN1 is encoded by a gene located at 21q22.2 (15) in a region known to play a critical role in the etiology of Down syndrome (DS), one of the most common genetic disorders (16, 17). Hmgn1 is located in the syntenic region on mouse chromosome 16 and is trisomic in several mouse models for Down syndrome, including Ts65Dn, Ts1Cje, Ts1Rhr, and Tc1 (18, 19). Correspondently, the levels of HMGN1 protein are up-regulated both in Down syndrome patients and mice models for this syndrome (15). Down syndrome is characterized by a wide range of symptoms including abnormalities in the nervous system and cognitive deficits (20). It is well established that Down syndrome is caused by the presence of an extra copy of chromosome 21 or segments of it (21); however, the specific role for a triplicate gene or a combination of genes in the etiology of Down syndrome is poorly understood.

Genomic profiling of human CD4+ T cells revealed that HMGN1 preferentially localizes to chromatin regulatory sites and to promoters of transcriptionally active genes (22), a finding that is in full agreement with its widespread effects on the cellular transcription profile (14). Among the regulatory sites bound by HMGN1 is the promoter of the gene coding for the high mobility group N1 protein (HMGN1), a nucleosomal-binding protein that affects the structure and function of chromatin, is encoded by a gene located on chromosome 21 and is overexpressed in Down syndrome, one of the most prevalent genomic disorders. Misexpression of HMGN1 affects the cellular transcription profile; however, the biological function of this protein is still not fully understood. We report that HMGN1 modulates the expression of methyl CpG-binding protein 2 (MeCP2), a DNA-binding protein known to affect neurological functions including autism spectrum disorders, and whose alterations in HMGN1 levels affect the behavior of mice. Quantitative PCR and Western analyses of cell lines and brain tissues from mice that either overexpress or lack HMGN1 indicate that HMGN1 is a negative regulator of MeCP2, a protein involved in neurodevelopmental disorders. Misexpression of HMGN1 in families predisposed to Down syndrome are poorly understood.

The abbreviations used are: HMGN1, high mobility group N1 protein; DS, Down syndrome; MeCP2, methyl CpG-binding protein 2; ASD, autism spectrum disorder; AGRE, Autism Genetic Resource Exchange; FHS, Framingham Heart Study; MEF, mouse embryonic fibroblast; FAIRE, formaldehyde-assisted isolation of regulatory elements; SNP, single nucleotide polymorphism; CHOP, Children’s Hospital of Philadelphia; Dhfr, dihydrofolate reductase.
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methyl CpG-binding protein 2 (MeCP2), a nuclear protein that is expressed postnatally during mammalian brain development and is highly abundant in neurons (23). Despite its name, MeCP2 can bind both methylated and unmethylated CpG sequences (24, 25) and can either repress (26–28) or activate (29, 30) transcription. Mutations or altered levels of MeCP2 can lead to neurodevelopmental disorders such as Rett syndrome, mental retardation, learning disabilities, autism spectrum disorders (ASDs) (31, 32) repetitive behavior, hypotonia, and anxiety (33). Interestingly, MeCP2 was found to be down-regulated in Down syndrome patients (34); however, the regulation of MeCP2 expression is still not fully understood (35). The widespread effect of HMGN1 on transcription, the location of the HMGN1 gene in theDown-syndrome region of chromosome 21, the association of HMGN1 with the regulatory regions of the MeCP2 gene in the brain of Down syndrome patients and age-matched controls and used genome-wide chromatin immunoprecipitation (ChIP) sequence and targeted ChIP analyses to examine whether HMGN1 is associated with regulatory regions of the MeCP2 gene in the brain of both human and mice. In addition, we used genetically altered mice that either overexpress (36) or lack HMGN1 protein (12) to test whether HMGN1 affects the chromatin structure and expression of MeCP2 in brain. We also subjected our mice models to a battery of behavioral tests that included basic motor function, activity, anxiety, and social assessments.

In view of the effect of HMGN1 on MeCP2 expression and the strong link between MeCP2 expression and ASDs, we queried the Autism Genetic Resource Exchange (AGRE) collection of genotypes for abnormalities within the HMGN1 genomic region in families predisposed to ASDs. Our most significant finding is that changes in the levels of HMGN1 protein lead to alterations in the behavior of mice. We identify MeCP2 as one of the targets that may contribute to the effects of HMGN1 and report that the distribution of genotypes in AGRE families flanking HMGN1 is nonrandom. Taken together, our findings suggest that HMGN1 is an epigenetic factor that could contribute to the development of neurodevelopmental disorders.

EXPERIMENTAL PROCEDURES

Human Samples and Genotype Data—Brain tissue from normal individuals (#5180 and #5242) and Down syndrome patients (#M1948M, #M2135M, and #M2854M) was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The samples from Down syndrome patients were age-matched to control samples. The usage of AGRE genotype data followed AGRE protocols (see “Acknowledgments”). The usage of the Framingham Heart Study (FHS) genotype data as a control set is covered under Institutional Review Board-approved human subject protocol 10-HG-1045 at the National Institutes of Health.

Transgenic Mice—Inbred mice (HMGN1_OE) overexpressing the entire human HMGN1 gene (36) were reconstituted from cryopreservation at the Cryopreservation and Assisted Reproduction (Laboratory Animal Sciences Program) at the National Cancer Institute, Frederick. In brief, founders were made on the background of C57BL/6J mice to generate two founder lines (line 25 and line 29). F3 progeny from these crosses were crossed to produce homozygous mice for the transgene. The expression of the human HMGN1 transgene was verified using primers designed to the human specific sequence of the first intron of HMGN1 (supplemental Table S3). The Hmgn1<sup>−/−</sup> mice were described previously (12). All mice used for molecular biology analysis were 8 weeks old.

Cell Lines—Primary MEFs isolated from Hmgn1<sup>−/−</sup> mice and SV-40-transformed mouse embryonic fibroblasts (MEFs) stably transfected with a vector expressing HMGN1 were used for in vitro studies. These cell lines were previously described (14).

Antibodies and ChiP Sequence Analysis —The following antibodies were from Millipore, Temecula CA: anti-MeCP2 (elicited by a peptide spanning amino acids 465–478), rabbit polyclonal IgG, catalog #07-013; anti-H3K9me2, mouse monoclonal, catalog #17-681; anti-H3K4me2, rabbit polyclonal, catalog #07-030; anti-H3K9ac, rabbit polyclonal, catalog #17-658; anti-H3K14ac, rabbit polyclonal, catalog #07-353. Anti-β-actin mouse monoclonal, catalog #A5316 clone AC-74, anti-human HMGN1 (62, 63), and anti-mouse HMGN1 (peptide 6) (12, 64) were from Sigma. ChIP sequence analysis of CD4+ human T cells was performed as described (22). All antibodies were used for chromatin immunoprecipitation except for anti-MeCP2 and anti-β-actin, which were used for Western blot.

Real-time Quantitative RT-PCR—Total RNA was extracted from human and mouse brain tissue by Trizol reagent followed by further purification and DNase I digestion using the High Pure RNA Isolation kit (Roche Applied Science). cDNA was prepared using iScript (Bio-Rad) with 0.3–1 μg of RNA as template and diluted 1:10. Equal aliquots of cDNA were used for real-time PCR in the presence of Power SYBR Green PCR Master Mix (Applied Biosystems) and sequence-specific primers (supplemental Tables S2 and S3). Reactions were analyzed on the Applied Biosystems 7900HT Fast Real-time PCR System. Assays were carried out in triplicates. Samples lacking reverse transcriptase were used as internal negative controls. A comparative Ct method (2<sup>−ΔΔCt</sup>) was used to calculate the relative gene expression according to the formula ddCt = [dCt control gene (normal sample) – dCt control gene (genetically altered sample)] – [dCt target gene (normal sample) – dCt target gene (genetically altered sample)]. G6PD and Dhfr were used as control genes. All values are the means of 2–3 experiments.

Western Blot Analysis—Whole cell extracts from brain tissue were prepared using Laemmli sample buffer (Bio-Rad), after homogenization in PBS on a 70-μm Nylon Cell strainer (BD Biosciences), equal amounts were run on Criterion precast gels (Bio-Rad), blotted to Immobilon-P transfer membrane (Millipore), and treated with specific antibodies.
Chromatin Immunoprecipitation and HMGN1 Binding Assays—ChIP sequence analysis of human CD4+ cells was described previously (22). For all other ChIP experiments, brain tissues were homogenized on 70-μm Nylon Cell strainer (BD Biosciences) and washed twice in cold PBS. Lysates were fixed in 1% formaldehyde solution for 10 min at room temperature followed by 2.5 mM glycine and sonicated on ice on a Standard Bioruptor (Diagenode, Denville NJ) for 15 min in 60-s pulses followed by alternate 30-s cooling intervals. Immunoprecipitation was performed exactly as described previously (65). DNA was purified by phenol-chloroform extraction, and duplicates from each sample were subjected to real-time PCR in the presence of Power SYBR Green PCR Master Mix (Applied Biosystems) and sequence-specific primers (supplemental Tables S2 and S3) and analyzed on Applied Biosystems 7900HT Fast Real-time PCR System using the expression \( \frac{2^{\Delta\Delta C_{T}}} {2^{\Delta C_{T}}} \) target gene/\( 2^{\Delta C_{T}} \) control gene. APRT, Dhfr, β-actin, and α-crystallin were used as control genes.

Formaldehyde-assisted Isolation of Regulatory Elements (FAIRE)—The FAIRE procedure and buffers were adapted from Refs. 38 and 66. Mice brain tissues were homogenized on 70-μm Nylon Cell strainer (BD Biosciences) in cold PBS. Each homogenate was divided into two different samples. The first contained \( \frac{1}{3} \) of the homogenate and was left on ice for later use as a non cross-linked control. The second sample contained \( \frac{3}{4} \) of the homogenate and subjected to 5 min of cross-linking with formaldehyde in a final concentration of 1% at room temperature followed by 5 min of quenching with 2.5 mM glycine in a final concentration of 125 mM. Both the non-cross-linked and the cross-linked homogenates were spun at 700 × g at 4 °C and washed twice in cold PBS. Samples were resuspended in 1 ml of lysis buffer 1 (5% 1M HEPES KOH, pH 7.5, 2.8% 5M NaCl, 0.2% NP-40) for 10 min of shaking at 4 °C. Samples were centrifuged at 12,000 × g for 5 min.1/10 volume of 3M NaCl, 1% sodium deoxycholate 10% glycerol, 5% NP-40, 0.5 M EDTA, pH 8, 0.5 M EDTA, pH 8, 10% glycerol, 5% NP-40, 0.25% Triton X-100) for 10 min of shaking at 4 °C. Samples were centrifuged for 5 min at 1300 × g at 4 °C and resuspended in 1 ml of lysis buffer 2 (4% 5 M NaCl, 0.2% 0.5 M EDTA, pH 8, 0.1% 0.5 M EGTA, pH 8, 1% 1 M Tris, pH 8) for 10 min shaking at room temperature followed by 5 min of centrifugation at 1300 × g at 4 °C. Samples were resuspended in 3.5 ml of lysis buffer 3 (0.2% 0.5 M EDTA, pH 8, 0.1% 0.5 M EGTA, pH 8, 1% 1 M Tris, pH 8, 1% 1 M Tris, pH 8, 2% 5 M NaCl, 1% sodium deoxycholate 10%, N-lauroyl sarcosine, protease inhibitors) and sonicated on ice on a Standard Bioruptor (Diagenode) for 15 min in 30-s pulses followed by alternate 30-s cooling intervals. Cellular debris was cleared by spinning at 12,000 × g for 5 min at 4 °C. DNA was isolated by adding an equal volume of phenol/chloroform, vortexed, and spun at 12,000 × g for 5 min. The aqueous phase was transferred to a new tube, and an equal volume of phenol/chloroform was added again, vortexed, and spun at 12,000 × g for 5 min. The aqueous phase was transferred to a new tube, and an equal volume of chloroform-isooamyl alcohol (24:1) was added, vortexed, and spun at 12,000 × g for 5 min. 1/10 volume of 3 M sodium acetate (pH 5.2) and 1 μl of 20 mg/ml glycogen (Roche Applied Science) were added. Samples were mixed by gently shaking, and a 2× volume of 95% ethanol was added followed by overnight incubation at −20 °C. DNA was pelleted at 15,000 × g for 30 min at 4 °C, washed with 500 μl of 70% ethanol, spun at 15,000 × g for 5 min, and allowed to dry. Pellets were resuspended in Tris-EDTA buffer and treated with 1 μl of RNase A (10 mg/ml) for 1 h at 37 °C followed by one-step phenol/chloroform and ethanol clean-up. Samples were resuspended in 200 μl of Tris-EDTA and incubated at 65 °C overnight to avoid remaining DNA-DNA cross-links. Samples were then treated with 1 μl of proteinase K (10 mg/ml) for 1 h at 37 °C. Tris-EDTA was added to a final volume of 250 μl, and phenol/chloroform clean-up stages were repeated. Duplicates from each sample were subjected to real-time PCR in the presence of Power SYBR Green PCR Master Mix (Applied Biosystems) and analyzed on Applied Biosystems 7900HT Fast Real-time PCR System using the same Mecep2 primers that were used for the ChIP analyses as described (supplemental Table S3). The enrichment at the various sites was normalized to that received using the primer set m2, which gave the least enrichment in all samples. For any primer set, mx (x = 1, 2, 3, . . .), the relative enrichment was calculated using the expression 2^-mx(fixed − nonfixed) − m2(fixed − nonfixed). Statistical Analysis for Real-time PCR, Western Blot Quantification, ChIP, and FAIRE—Results are shown as the mean ± S.D.). p values were calculated using Student’s t test.

Mice for Behavior Analysis—Mature mice (6–7 months old) from the three different groups mentioned in this study, HMGN1 OE, Hmgn1−/−, and wild type (WT) controls, which were littermates of Hmgn1−/− or C57BL/6 age-matched retired breeders (both WT groups displayed the same behavioral phenotype and hence are considered one group), were used for behavior analysis. Each group contained eight male mice. The mice were acclimated to the test environment for more than 1 week before testing. The tests were performed on the same mice in the following order: open field test, elevated plus maze, parallel bar task, automated sociability, and novel object preference test.

Parallel Bar Task—Motor coordination, ataxia, and balance were assessed using the Parallel Bar apparatus (Cleversys, Reston, VA) adapted from Kamens and Crabbe (40). The apparatus consists of a closed transparent Plexiglas box (15 cm × 15 cm × 20 cm) with a floor made of a series of parallel stainless steel bars with a 1.6-mm diameter and an interbar spacing of 6 mm between the edges of the bars. A stainless steel base plate located underneath the bars connected with electrical clips was used to measure the number of slips between the bars. Each mouse was placed inside the apparatus and recorded for 5 min for distance and foot slips. When a mouse foot slips through the parallel metal bars and touches the base plate, it completes a circuit, and the contact is recorded as an error by LocoScan software (Cleversys). To avoid the effect of overall activity level on the total number of slips, the results were calculated as the total number of foot slips divided by total distance in cm that each individual mouse traveled.

Open Field Test—Each mouse was placed in the center of an empty bright open-field arena surrounded by walls (42 × 42 × 30 cm) to assess activity level, locomotor exploratory, and anxiety-like behaviors. Activity was recorded and quantified by TopScan, a multi-faceted tool for automated behavioral analyses (Cleversys). Each test session lasted 30 min, and the boxes were wiped clean with 70% ethanol between trials. Data were analyzed as six 5-min time intervals for total distance and num-
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umber of entries (bouts) to the center of the arena. p values were calculated using Student’s t test.

Elevated Plus Maze Test — A Plexiglas plus-shaped maze elevated 50 cm above ground (Cleversys) was used to examine anxiety related behavior. The maze consisted of two dark closed arms and two lighted open arms (30 x 5 cm each) with 18-cm high walls in the closed arms. Mice were placed in the junction of the four arms (5 x 5 cm) and allowed to move freely for 5 min. The time spent in the different parts of the maze, the total distance, and frequency of visits to the open and closed arms were recorded and scored automatically using TopScan (Cleversys). The maze was wiped clean with 70% ethanol between trials.

Automated Sociability Test and Social Memory Test (67) — Mice were placed in a polycarbonate apparatus (29 x 43 x 23 cm) divided into three equal chambers. Small, 4 x 4-cm openings were located on the dividing walls equipped with lids (dividers) that can be opened or closed. Each trial included a habituation stage of 20 min (10 min in the middle chamber with the dividers closed and 10 min with the dividers opened) followed by two stages. In the first stage (sociability test), the subject mouse was returned to the center (middle chamber), and the dividers were closed. An unfamiliar mouse (Stranger 1) was placed in one of the side chambers in a wired cup. An identical empty wired cup was placed in the opposite side chamber (alternating sides between trials). The dividers were reopened, and the subject mouse was allowed to explore the whole apparatus for 10 min. In the second stage (novel object preference test/social memory test), the subject mouse was returned to the center, and the dividers were closed. The first unfamiliar mouse (Stranger 1) was left in its wired cup at the same side chamber it was in the first stage, and a new unfamiliar control mouse (Stranger 2) was placed in the empty wired cup in the opposite side chamber. The dividers to the middle chamber were reopened, and the subject mouse was again allowed to explore the whole apparatus for 10 min. The thin, widely spaced bars of the wired cup allowed nose contact between the mice but prevented the stranger mouse from initiating any social contact, thus limiting the social contact to the tested mouse only. The amount of time the subject mouse spent in each chamber in each session was recorded and measured automatically by Any-maze video tracking system (Stoelting Co., Wood Dale, IL). All stranger (unfamiliar) mice were C57BL/6J, age- and sex-matched. These mice were subjected to two habituation sessions to placement within the wired cups of 10 min each several days before starting the trials. These mice originated from different home cages and had never been in physical contact with the subject mice or each other before the test.

Statistical Analysis for Behavior Tests — Data are shown as ± S.E. p values were calculated using Student’s t test, except for the p value of the open field test that was calculated using two-way analysis of variance, comparing each group to the WT group.

Analysis of Genotypes in the Vicinity of HMGN1 in the AGRE Collection — The AGRE consortium has genotyped a large collection of families with autistic individuals on high density single nucleotide polymorphism (SNP) genotyping platforms. AGRE provides a data set of 4327 samples from individuals (2444 unaffected and 1856 affected, 27 not phenotyped) genotyped at the Children’s Hospital of Philadelphia (CHOP) on the Illumina HapMap550 array as described in Wang et al. (43). The CHOP data have been “cleaned” by AGRE to address various types of problems (README.CHOP.clean100121.txt documentation file accompanying the CHOP data from AGRE). In addition, AGRE also provides an earlier data set of 2883 individuals (almost all of whom are included in the larger CHOP set) genotyped at the Broad Institute on an Affymetrix 500k SNP genotyping platform. Parts of the results were qualitatively confirmed on the Broad data set so as to rule out bias due to genotyping laboratory or platform. As a control data set we used a collection of 4462 (3000 unaffected and 1462 affected) genotypes done on the Affymetrix 500k chip on participants in the FHS; in FHS the “affected” individuals have cardiovascular disease (68).

HMNG1 is located on chromosome 21:39.63–39.65Mb in build 36 (hg18) coordinates. We used PLINK and additional programs to explore the genotype and haplotype distribution of the affected and unaffected individuals in the FHS and AGRE. In the FHS data set there were 60/3000 (2%) unaffected individuals with a homozygous interval spanning HMGN1, according to the homozyg metod of PLINK. In the AGRE data set, there were 19/1856 (1%) affected and 52/2444 (2.1%) unaffected individuals with a homozygous interval spanning HMGN1.

RESULTS

HMGN1 Locates to the MeCP2 Promoter — Targeted analysis of the ChIP sequence data of human CD4+ T cells (22) revealed that the enrichment levels of HMGN1 are elevated in the regulatory regions overlapping the first exon of human MeCP2 (Fig. 1A). Similar ChIP sequence analysis of chromatin extracted from human and mouse brain tissue indicated that HMGN1 is associated with the MeCP2 promoter (not shown). To verify that HMGN1 is indeed associated with the MeCP2 gene in mouse and human brain, we performed a ChIP assay with an antibody against HMGN1 with chromatin extracted from normal human brain tissue, from the brain of WT mice, and from the brains of transgenic mice overexpressing human HMGN1 (HMGN1_OE) and analyzed the precipitated DNA by real-time quantitative PCR with a set of primers spanning either residues −961 to +959 of the human MeCP2 or residues −937 to +824 of the mouse MeCp2 ( Fig. 1, C and D, and supplemental Table S1, a and b), which overlap the promoter region, the first exon, and part of the first intron. We identified a distinct HMGN1 binding site in both human and mouse brain tissue (Fig. 1, C and D). Because the antibody to mouse HMGN1 does not recognize human HMGN1, we used an antibody specific to human HMGN1 to detect this protein in the chromatin extracted from the brains of HMGN1_OE mice. Notably, the location of the human HMGN1 in the MeCp2 promoter region in the brain of the HMGN1_OE was the same as that of the endogenous mouse HMGN1. Thus, in the brains of both humans and mice, HMGN1 binds to the genomic region overlapping the promoter and first exon of MeCP2.

HMGN1 Is a Negative Regulator of MeCP2 Expression — It has been reported that the expression of MeCP2 is reduced in DS (34); however, because DS could result from the triplication of only
FIGURE 1. HMGN1 binds to the promoter of MeCP2 in both human and mouse. A, nucleosome position and HMGN1 binding in the MeCP2 promoter of human CD4+ T cells was determined by ChIP sequencing. Tag density indicates the number of adjusted reads in 10-bp windows. Data were obtained from Cuddapah et al. (22). B, shown is a schematic presentation of the MeCP2 gene. Boxes represent exons. C and D, identification of the HMGN1 binding site in human and mouse brain tissue by ChIP in the regulatory region overlapping the transcription start site present in exon 1 is shown. The boxed region represents exon #1. The genomic regions amplified, denoted as H for human and m for mouse, are listed in supplemental Table S1, a and b. Values are the averages of 3 different samples from each group. Shown is the ratio of bound/input (B/IN), normalized to APRT or Dhfr.

FIGURE 2. HMGN1 is a negative regulator of MeCP2 expression. A, shown are relative transcript levels of HMGN1 and MeCP2 in brain tissue from DS patients and age-matched controls (C) normalized to glucose-6-phosphate dehydrogenase (G6PD). The ages of the sample donors are indicated. *, p value < 0.05; **, p value < 0.02. B, shown are MeCP2 transcript levels in MEFs from WT mice, primary MEFs isolated from Hmgn1−/− mice (KO), and MEFs stably transfected with a vector overexpressing HMGN1 (OE) normalized to Dhfr. *, p value < 0.003. C, Mecp2 transcript levels in brain tissue of wild type (WT), HMGN1 overexpressing (OE), and Hmgn1−/− (KO) mice relative to β-actin. *, p values < 0.01; error bars represent S.D. D, shown is a representative Western blot depicting MeCP2 protein levels in brain extracts of wild type (WT), HMGN1 overexpressing (OE), and in Hmgn1−/− (KO) mice. E, relative levels of MeCP2 protein in mice brain extracts were quantified by Image J and adjusted to the relative intensity of β-actin for each sample. Values are the averages of three different samples from each group and two different blots. Error bars represent S.D. *, p value < 0.05; **, p value < 0.003.
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FIGURE 3. HMGN1 affects the chromatin structure of the MeCP2 promoter region. A, ChIP analysis of the effect of HMGN1 on histone modifications at its binding site on the MeCP2 promoter (m7 in Fig. 1D) in mouse brain is shown. Values are the averages of three different samples from each group. Shown is the ratio of bound/input, normalized to Dnhr. B, FAIRE analysis of HMGN1-mediated changes at the MeCP2 promoter in mouse brain. The regions amplified by the primers used are in supplemental Table S1b. Values are the averages of two experiments, normalized to m2, which gave the lowest enrichment values. Error bars represent S.D. OE, HMGN1 overexpressing; KO, Hmgn1−/− mice.

segments of chromosome 21, it is not clear whether the expression of HMGN1 was also changed in the specific patients examined. To test for a direct link between HMGN1 and MeCP2 levels in DS, we used quantitative PCR to analyze the transcript levels of HMGN1 and MeCP2 in brain tissue obtained from DS patients and age-matched controls. Our results revealed an average of 50% increase in HMGN1 and 30% decrease in MeCP2 transcript levels in brain tissue of DS patients (Fig. 2A). These results are in full agreement with previous studies.

To test whether the changes in MeCP2 expression are indeed linked to the levels of HMGN1, we used primary MEFs isolated from Hmgn1−/− mice and SV-40-transformed MEFs stably transfected with a vector overexpressing HMGN1 as described in Rochman (14). Real-time quantitative PCR analysis performed on cDNA from these cell lines revealed that overexpression of HMGN1 in MEFs resulted in a 25% reduction in MeCP2, whereas the absence of HMGN1 resulted in a 1.4-fold increase in MeCP2 expression compared with WT MEFs cells (Fig. 2B).

Next, we compared the levels of MeCP2 transcript and protein in brains obtained from wild type mice to those of brains obtained either from Hmgn1−/− mice (12) or from Hmgn1−/− mice, which contain 1.5 increased levels of HMGN1 protein (36). For this analysis we used 8-week-old mice as MeCP2-null male mice appear normal up to the age of 5 weeks but start to develop Rett-like phenotypes and die by the age of 6–12 weeks (37). Real-time quantitative PCR analysis performed on cDNA revealed that in the brain of HMGN1−/− mice, the levels of MeCP2 were reduced by 20% as compared with wild type mice. In contrast, the levels of MeCP2 transcripts in the brain of Hmgn1−/− mice were 2-fold higher than in the brain of the Hmgn1−/+ control mice (Fig. 2C). Western blot analyses confirmed that not only the transcript levels but also the MeCP2 protein levels were affected by HMGN1. Loss of HMGN1 increased the level of MeCP2 protein by 1.6-fold, whereas overexpression of HMGN1 reduced the protein levels by 40% (Fig. 2, D and E). We, therefore, conclude that HMGN1 is a negative regulator of MeCP2 expression.

HMGN1 Modifies MeCP2 Chromatin—The presence of HMGN1 in the regulatory sites of the gene coding for MeCP2 in both human and mouse tissues suggests that HMGN1 is a direct regulator of MeCP2 expression. Because HMGN1 was shown to affect chromatin structure and histone modifications, we tested whether HMGN1 affects the chromatin modifications at its binding site on the MeCP2 promoter in chromatin extracted from mouse brain tissue. ChIP analyses revealed that in the brains of Hmgn1−/− mice, the levels of H3K9ac, which is a marker of transcriptional activity, were significantly reduced, whereas those of H3K9me2, a marker of facultative heterochromatin, were significantly enriched as compared with WT and Hmgn1−/+ mice (Fig. 3A). Conversely, in brain tissue from Hmgn1−/+ mice, the levels of histone acetylation were increased as compared with Hmgn1−/− controls or to the Hmgn1−/− mice (Fig. 3A). These results agree with our findings that overexpression of HMGN1 inhibits, whereas loss of HMGN1 enhances MeCP2 expression.

As an additional test for HMGN1-induced changes at the MeCP2 promoter, we used the FAIRE assay (38) that measures the relative accessibility of regulatory regions in chromatin. The results indicated that at the MeCP2 promoter region in Hmgn1−/− mice, the DNA of the regulatory regions is less accessible, whereas in the Hmgn1−/+ mice it is somewhat more accessible than in wild type mice (Fig. 3B). In summary, all of the data are consistent with the notion that HMGN1 is a negative regulator of MeCP2 and attenuates its expression directly by affecting the chromatin structure of its promoter.

Aberrant Expression of HMGN1 Affects Activity Levels and Anxiety in Mice—Mild changes in MeCP2 levels can result in age-progressive neurological and behavioral changes in mice, and transgenic mice that have a mild overexpression of the human MeCP2 gene manifest hypoactivity and anxiety after 5 months of age (39). Therefore, we tested whether alterations in HMGN1 levels affect the behavior of mature, 6–7-month-old mice using a battery of behavioral tests. In each test we used both Hmgn1−/+ littermates of the Hmgn1−/− line and C57BL/6 wild type mice as these two control groups exhibited similar results.

We used the parallel bar task (40) to assess for motor coordination, ataxia, and balance. In this test the extent of coordination problems were quantified by the number of foot slips per distance traveled (see “Experimental Procedures” for details). In this task Hmgn1−/− mice and wild type mice showed a similar average number of slips, whereas Hmgn1−/− mice dis-
played slightly improved motor coordination and balance as judged by reduced number of foot slips (Fig. 4, A and B).

The open field and the elevated plus maze tests were used to assess locomotor activity and exploratory and anxiety-like behavior. In the open field test each mouse was placed in the center of an empty bright open-field arena surrounded by walls. Mice were recorded for 30 min, and the total distance traveled and number of entries into the center of the arena (bouts) were analyzed at six 5-min time intervals. The Hmgn1−/− mice traveled a significantly shorter distance throughout the test, at all time intervals with significantly fewer bouts to center (Fig. 4, C and D). In contrast, the HMGN1_OE mice traveled a longer distance, especially in the first 5 min, and had more bouts to center (Fig. 4, C and D). These results indicate that Hmgn1−/− mice are hypoactive and display a decreased exploratory behavior in an open field setting, whereas the HMGN1_OE mice seem to be somewhat hyperactive and tend toward increased exploration.

In the elevated plus maze we used a plus-shaped maze elevated 50 cm above ground that consisted of two dark closed arms and two lighted open arms. Mice were placed in the junction of the four arms and were allowed to move freely. The time spent in the different parts of the maze, the total distance traveled, and frequency of visits to the open and closed arms were recorded for 5 min. In this test the total distance traveled and the time spent in each part of the maze by the Hmgn1−/− mice were similar to those of the wild type mice (Fig. 4, E and F). This finding does not conflict with the results of the open field test as the two tests are different both in the length of time the mice were being observed and the environment in which they were examined. The tests were designed to probe for different aspects of activity and anxiety. Nevertheless, the HMGN1_OE mice traveled a significantly longer distance and spent significantly more time in the open arms with a concomitant decrease of time spent in the closed arms (Fig. 4, E and F). These results provide additional support for the notion that the HMGN1_OE mice are hyperactive and also show reduced anxiety.

**HMGN1 Levels Affect Sociability and Social Memory in Mice**—Because changes in MeCP2 levels have been correlated with different autism spectrum disorders (31), we assessed autistic-like behaviors in our mice models using the automated sociability test, which has been used extensively to test for autistic relevant phenotypes in mice (41) to assess for social preference and social memory in two stages. In the first stage we measured the time spent either in a chamber containing an unfamiliar wild-type mouse (Stranger 1) contained within a wired cup, in a chamber with an empty wired cup, or in a middle chamber separating these two chambers. In the second stage we placed a new wild type mouse in the previously empty cup (Stranger 2) and again measured the time that the tested mouse spent in each of the three chambers.

In the first stage the WT and the Hmgn1−/− mice spent more time in the chamber containing Stranger 1 compared with the time they spent in the chamber containing the empty cup, as expected (41). The HMGN1_OE mice, however, did not display any preference for the unfamiliar mouse, showing an equal interest in Stranger 1 and in the empty cup (Fig. 5A). Although this behavior could imply autistic-like characteristics, it could also stem from their hyperactivity or increased exploratory locomotion (42), as evident from their significantly increased total entries to both side chambers (Fig. 5, B and C).

In the second stage of the test the WT and HMGN1_OE groups displayed the expected reversal of chamber preference and spent more time with a new, unfamiliar mouse (Stranger 2) compared with the time each of the groups spent with the already familiar mouse (Stranger 1). In contrast, the Hmgn1−/− mice spent equal time with strangers 1 and 2, an indication of a lack of preference for social novelty that could be also explained by a deficit in social memory (Fig. 5D). The number of entries into the chambers did not differ significantly among the WT and Hmgn1−/− groups but was significantly higher in the HMGN1_OE group, which is a further indication of their hyperactivity and increased exploratory behavior (Fig. 5, C, E, and F). Taken together, these results indicate that aberrant HMGN1 levels lead to social deficit and autistic-like features in mice.
Biological Effects of HMGN1

Targeted Genetic Analysis of the HMGN1 Genomic Region in ASD Pedigrees Shows an Unexpected Distribution of Runs of Homozygosity—Given the HMGN1-related behavioral phenotypes and the role of HMGN1 in MeCP2 expression and in light of the link between MeCP2 levels in brain and autism spectrum disorders (29, 34, 39), we queried the AGRE consortium of genotypes from the NCBI database of Genotypes and Phenotypes (43) for SNPs in a 1-Mbp region flanking HMGN1. We used other regions of chromosome 21 in the AGRE/CHOP data and comparable FHS chromosome 21 genotypes from the NCBI database of Genotypes and Phenotypes (www.ncbi.nlm.nih.gov) as controls.

Eleven SNPs in the HMGN1 region showed a nominally significant excess of unaffected homozygous individuals for the minor allele as compared with affected individuals (Table 1). It was not surprising that these SNPs are not significantly associated in the standard tests after correcting for multiple testing, as multiple studies of these genotypes reported no association between autism and any SNPs on chromosome 21 (43, 44). Nevertheless, the excess of unaffected homozygotes prompted us to search directly for runs of homozygosity, as has been done in other Genome Wide Association Study data sets (45) but not in this AGRE data set.

The “homozyg” option of the software PLINK (46) identified 71 homozygous intervals of a size >500 kbp and flanking HMGN1. Significantly, 15/16 of the longest intervals were in unaffected family members, and the frequency of large homozygous intervals flanking HMGN1 among unaffected family members ($n = 52$) was 2.1-fold higher than among the affected members ($n = 19$) (two-sided $p < 0.005$). Furthermore, of the 52 unaffected individuals, 15 share homozygosity for a 58-marker haplotype (rs2836889 at 39,407,621 bp to rs2837022 at 39,718,621 bp) that spans HMGN1 and only four additional genes, none of which has been related to neurological abnormalities (Table 2). These 15 haplotype-sharing unaffected individuals were from different families. In contrast, similar analyses of the same region flanking HMGN1 in the FHS cohort and of additional subintervals of chromosome 21 in the AGRE cohort did not reveal any significant differences between affected and unaffected individuals.

A query of the eQTL study of Dimas et al. (47) using Genevar (48) with the term “HMGN1” revealed that four SNPs are significantly associated with HMGN1 expression, and all of them locate to the region of disproportionate homozygosity that we detected in the AGRE genotype collection (Fig. 6). It is especially interesting that the two SNPs, rs2222995 (at 39431762, $p = 4.22e-5$) and rs12482181 (at 39470722, $p = 3.11e-4$), most significantly associated with expression of HMGN1 are located closer to another gene in Table 2 than to HMGN1 itself, which would explain why the non-random distribution of genotypes is detectable by looking at a broad interval around HMGN1 but not at individual SNPs closest to HMGN1. Additional experiments are necessary to confirm the notion that sequence or expression variations in the HMGN1 gene region could be present in some families predisposed to ASDs.

FIGURE 5. HMGN1 levels affect sociability and social memory in mice. A–C, automated sociability test, stage 1, is shown. A, shown is the total time spent in each of the 3 chambers. *, $p < 0.01$; **, $p < 0.001$. B, shown is the average number of entries to each of the two side chambers. C, shown is the total sum of entries to both side chambers throughout the test. *, $p < 0.01$. D–F, shown is the automated sociability test, stage 2. D, total time spent in each of the three chambers is shown. $p < 0.01$. E, shown is the average number of entries to each of the 2 side chambers. F, total sum of entries to both side chambers throughout the test is shown. Bars represent S.E. *, $p < 0.001$. In all experiments eight age-matched male mice were tested in each group; bars represent S.E. OE, HMGN1 overexpressing; KO, Hmgn1–/– mice.

DISCUSSION

Our study demonstrates that the levels of HMGN1 protein affect the activity and social behavior of mice. Overexpression of HMGN1 leads to hyperactivity, reduced anxiety, and abnormal social interest, some of the behavioral phenotypes detected in mouse models for Down syndrome (49). Conversely, Hmgn1–/– mice display hypoactivity and abnormal social behavior and overall demonstrate features that resemble behaviors seen in models of ASDs (42).

Mouse models are used to understand the role of the numerous genes implicated in the etiology of ASDs (42). These studies
suggest that mutations or misexpression of various genes could ultimately lead to the complex phenotype of ASDs. Examples for autism-relevant behaviors in these mouse models are reduced social interactions, low sociability or reduced social approach, lack of preference for social novelty, impaired social recognition, repetitive behavior, and restricted interest.

TABLE 1
SNPs in the vicinity of HMGN1 show a nominally significant association when using the PLINK model option

| SNP     | Position (bp, hg18) | Minor allele | Major allele | PLINK p values |
|---------|---------------------|--------------|--------------|----------------|
| rs2410062 | 39,150,790          | G            | A            | 0.028/0.012    |
| rs2836716 | 39,157,929          | A            | G            | 0.041/0.027    |
| rs717871  | 39,179,325          | C            | T            | 0.014/0.012    |
| rs2142109 | 39,223,826          | G            | A            | 0.017/0.0073   |
| rs2229994 | 39,225,298          | A            | G            | 0.11/0.040     |
| rs2836788 | 39,258,761          | A            | G            | 0.11/0.042     |
| rs5615702 | 39,314,473          | G            | T            | 0.027/0.0076   |
| rs2836978 | 39,588,141          | C            | T            | 0.054/0.023    |
| rs2837029 | 39,722,241          | T            | C            | 0.059/0.022    |
| rs2837039 | 39,747,325          | T            | C            | 0.067/0.034    |
| rs2837066 | 39,816,431          | T            | C            | 0.073/0.032    |

TABLE 2
List of the genes within the 58-marker haplotype spanning from rs2836889 at 39,407,444 bp to rs2837022 at 39,718,621 bp

| Symbol | Description | Phenotype or GWAS (reference) |
|--------|-------------|-------------------------------|
| PSMG1  | Proteasome assembly chaperone 1 | Pediatric-onset inflammatory bowel disease (1), Crohn disease (2) |
| BRWD1  | Bromodomain and WD repeat domain containing 1 | Pediatric inflammatory bowel disease (3) |
| NCRNA00257 | Non protein coding RNA 257 | Unknown |
| HMGN1  | High mobility group Nucleosomal-binding protein 1 | Activity and social abnormalities in mice models (this communication) |
| WRB    | Tryptophan rich basic protein | Potential role in the pathogenesis of congenital heart disease (4) |

FIGURE 6. Scatter plot representing observed eQTLs associated with a variation in expression of HMGN1, located approximately at position 39,640,000 bp in human chromosome 21. Shown is the screen capture of Genevar results obtained by a search for SNPs (eQTLs) associated with a variation in expression of HMGN1. The top panel presents the results obtained with probe ILMN_1652123, which is associated with SNP rs9978224, whereas the bottom panel represents the results obtained with probe ILMN_2151027, which is associated with SNPs rs2222995, rs12482181, rs2026367, and rs10483056. Note that rs2222995 and rs12482181 were genotyped in the AGRE dataset and are in the region where there is an excess of homozygous runs among the unaffected individuals.

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Hmgn1−/− mice demonstrate some of these characteristics including impaired social recognition and restricted interest. Increasing evidence links behavioral abnormalities, including those seen in mental retardation and ASDs, to alteration in epigenetic regulatory mechanisms (50–52), which includes changes in chromatin structure, plasticity, and histone modifications. Hmgn1 is a prominent member of the HMGN family of nuclear proteins, which are known to specifically bind to the 147-base pair nucleosome core particles, the building block of the chromatin fiber. It has been demonstrated that the specific interaction of the protein with nucleosomes affects the local and global structure of chromatin and the levels of modification in the tails of the core histones (5, 6, 10). Thus, most likely the behavioral changes seen in mice with altered Hmgn1 expression are not solely due to its effect on MeCP2 levels but rather to the global effect due to Hmgn1 impact on gene expression. Indeed, in vitro chromatin studies indicated that Hmgn1 affects the rate of transcription (5, 13), and recent studies revealed that either up- or down-regulation of Hmgn1 levels affect the expression of numerous genes, albeit most of the changes were relatively small (14).

The pathogenic mechanism of cognitive deficits seen in Down syndrome or of the highly heterogeneous ASDs is difficult to decipher because these conditions are linked to numerous changes in gene expression and in the interaction among cellular proteins (53, 54). Likewise, given the widespread effects of Hmgn1 on gene expression and its association with regulatory sites throughout chromatin (22), it is difficult to unequivocally identify all the genes downstream from Hmgn1 that are directly responsible for the various phenotypes observed in the mice with altered Hmgn1 expression. Nevertheless by focusing on a single gene it is possible to gain insights into the possible mechanisms whereby Hmgn1 affects the global phenotype. We focused on MeCP2 as a gene regulated by Hmgn1 because it is highly expressed in brain (23) and because it is well documented that misexpression of MeCP2 or mutations in MeCP2 are strongly linked to behavioral abnormalities in human and mice (55–58). Our findings that Hmgn1 is associated with regulatory regions of MeCP2 in both human and mouse brain tissues and that the MeCP2 levels in brain tissues from mice that either overexpress or lack Hmgn1 are either reduced or elevated, respectively, suggest that Hmgn1 modulates MeCP2 levels in vivo. Likely, Hmgn1-mediated changes in MeCP2 expression contribute to some of the behavioral phenotypes seen in the mice studied here. MeCP2 is also known to have widespread effects on gene expression (29). Ultimately, the phenotypes seen are due to alterations in gene expression resulting from the combined effects caused by changes in the cellular levels of several genes, including Mecep2. Nevertheless, even though the exact pathway whereby Hmgn1 modulates behavior is still not fully clarified, it is clear that the initiating events leading to the abnormal behaviors of the mice are the changes in the levels of Hmgn1 protein. Importantly, the abnormalities detected in Hmgn1 regulatory regions in the AGRE genotype collection combined with the Hmgn1−/− mice behavior results raise a possible link between Hmgn1 and some of the phenotypes seen in ASDs.

In summary, our study provides several independent lines of evidence to support the hypothesis that either up or down-regulation of Hmgn1 expression, perhaps due to copy number variations (60) or mutations, lead to epigenetic changes that affect the cellular transcription profile, thereby playing a role in the etiology of some of the behavioral abnormalities seen in patients with Down syndrome or ASDs. These findings support the emerging notion that variations in copy number or expression of a single gene can contribute to the etiology of distinct neurodevelopmental disorders (61). Additional experiments are necessary to confirm this hypothesis.

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