nature research

Peer Review Information

Journal: Nature Structural & Molecular Biology
Manuscript Title: Differential dynamics specify MeCP2 function at nucleosomes and methylated DNA
Corresponding author name(s): Shixin Liu

Reviewer Comments & Decisions:

Decision Letter, initial version:

Message: 15th Dec 2023

Dear Professor Liu,

Thank you again for submitting your manuscript "Differential dynamics specify MeCP2 function at nucleosomes and methylated DNA". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that while all referees appreciate the results and find the potential novelconclusions timely and of wide interest, they have raised a number of important concerns. Specifically, all referees note a number technical concerns that must be addressed with more quantitative analysis, provision of raw datasets and more robust statistics. Equally important, it is also noted the the mechanism proposed needs to be supported with additional experimental data, to expand the investigation of the role of methylated DNA and exclude alternative models. Please note that we would be reluctant to go back to the referees in the absence of significant revisions.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We appreciate the requested revisions are extensive. We thus expect to see your revised manuscript within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision as long as nothing similar has been accepted for publication at NSMB or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the last revision, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: please provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon

publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage. Please find the complete NRG policies on data availability at http://www.nature.com/authors/policies/availability.html.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Carolina

Carolina Perdigoto, PhD
Chief Editor
Nature Structural & Molecular Biology
orcid.org/0000-0002-5783-7106

Referee expertise:

Referee #1: chromatin biology, biophysics and single molecule methods

Referee #2: chromatin biology, biophysics and single molecule methods

Referee #3: MeCP2, epigenetics

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:
The manuscript by Chua et al. investigates the dynamics of the Methyl-CpG-binding protein 2 (MeCP2) using correlative single-molecule fluorescence and force microscopy. This allows the authors to determine the relative diffusion and binding dynamics of MeCP2 and Rett syndrome mutants on various DNA substrates. They report that methylated DNA enhances binding and reduces diffusion with RTT mutations disrupting enhancement. On DNAs containing nucleosomes with and without methylation, they report enhanced association of MeCP2 with nucleosomes leading to stronger mechanical stability that can be modulated by linkers histones. Finally, to examine proposals that MeCP2 may act in recruitment of co-repressors, they performed further multicolor imaging experiments demonstrating that TBLR1 complexes are recruited to DNA by MeCP2 to form larger diffusion-competent assemblies.

The manuscript is well written and the main results are clearly presented and easy to follow. The use of single-molecule dynamic imaging with force control allowed the authors to uncover many new activities of MeCP2 and defects of RTT mutants that have remained inaccessible using other methods. Most compelling among these was the authors observations that MeCP2 localizes specifically at nucleosomes and the recruitment of TBLR1. Multicolor imaging provides compelling evidence for these observations.

Overall, I believe the manuscript presents many important new findings that help to clarify MeCP2 dynamics and defects of RTT variants. However, I am not sure all claims made in the discussion are supported by the experimental data without further clarification. I have provided a list of specific questions and comments below.

Comments:
1. There is a significant amount of discussion revolving around the oligomerization of MeCP2. However, the diffraction-limited resolution of the data collected and the fact that the MeCP2 is added to DNA before imaging leave many aspects of the oligomerization unclear. For example, in line 133-135: "Moreover, the MeCP2 270X trajectories contained fewer protein monomers on average compared to those for the full-length protein, supporting that the disordered TRD/CTD contribute significantly to tMeCP2 multimerization on DNA."
There isn't evidence provided that oligomerization occurs on the DNA, as opposed to in solution before loading onto DNA. It would be more convincing if an experiment looking at oligomeric states in bulk solution without DNA showed primarily the monomeric form.
2. The authors state that they heat inactivated the methyltransferase. Is that enough to be confident that it is no longer bound to DNA? How was the DNA purified after methylation? This information was not provided in the methods. If some remains bound it could account for the observed differences in diffusion between methylated and unmethylated DNAs.
3. When calculating diffusion coefficients, were both the mobile diffusing species and the immobile species included or was there a cutoff used to remove the immobile species on DNA from the calculation? The authors claim significant differences in the diffusion coefficients on methylated as compared to unmethylated DNA. Is this simply a result of inclusion of more immobile molecules? Is it possible the diffusion coefficient for scanning along DNA is the same for methylated and unmethylated when filtering for only mobile species? Or are the authors arguing that scanning diffusion is in fact slower even when MeCP2 is traveling over methylation sites. I would also imagine diffusion is more constrained as more binding occurs. The authors should clarify these points in the manuscript. Finally, in Figure 1e, error margins should be included similar to those

presented in plot 2d and mean values should be reported in the further clarification of these questions.
4. Have the authors examined the force profile of DNA bound by MeCP2 alone and compared it to the force profile with nucleosomes? This important condition was not displayed in Figure 5c or discussed. Related, in Figure 5d it appears that some H1 histones are diffusing. The authors should evaluate the dynamics of H1 and MeCP2 in the absence of nucleosomes. One explanation for the diffusion could be an interaction between the two factors, formation of a larger complex and diffusion together. Can the authors exclude this possibility. In this case, inclusion of H1 might simply start removing MeCP2 from nucleosomes to cause the force difference.
5. Depending on the results for comment 4, the authors should consider whether there are alternative hypotheses to the one stated in lines 213-217: "These results reveal that the nucleosome can simultaneously accommodate both H1 and MeCP2, but H1 attenuates the nucleosome stabilization effect of MeCP2. This indicates that the binding pose of MeCP2 in the ternary complex is distinct from that of MeCP2 bound to the nucleosome alone." If so, these statements should be adjusted.
6. In the reporting summary the authors state that "Source data are provided with this paper. Other data are available upon reasonable request." Are the raw data/kymographs deposited somewhere and accessible? Do the authors mean the displayed kymographs for source data? I do not know the reporting requirements of the journal, but I would strongly encourage the authors to deposit the raw data in a public database so others can reproduce their analysis and further benefit from their work.
7. While the authors reported n values in several places in the manuscript, they have not specified how many independent DNA tethers were used for each analysis and biological replicates. This should be reported. Are all kymographs used for analysis displayed in the supplementary materials?
8. Can the authors also display the EMSA gel used for the analysis in Figure S1b. Given the large oligomers seen in single-molecule experiments, it would be interesting to see the relative stoichiometry of binding for WT vs MeCP2C339S, C413S. The authors have displayed the EMSA gel for methylated and unmethylated binding in Figure S3b, which is very nice by the way. It is important to also include the raw gel used for analysis in Figure S1b.
9. The n value is provided for the estimate of the mean number of MeCP2 in each complex, but was not provided for the estimates of diffusion constants in Figure 1e (number of spots). This should be included.
10. In Figures 6d and 6e there are so many nucleosomes that it is hard to see if TBLR1 and MeCP2 are actually co-localizing. Maybe a zoom in would be more convincing.
11. Lines 127-128. The scanning mode is clearly changed but this does not imply discrimination between methylated and unmethylated DNA is in fact changed. See comment 3 and consider rephrasing depending on the outcome.
12. Figure 2c nicely shows differences in diffusion between wild-type and various mutants. The authors should also include estimates of the diffusion coefficients in a plot similar to figure 1e with error bars and spots for each trajectory. Same for the diffusion constant estimates Figure 3d. They should be added in a separate plot with error bars.
13. If space allows, or in the supplement, it would be useful if the authors could provide a reference for the diffusion model used.
14. The nomenclature for the bar plots should be defined somewhere. What do the *, **, ***, n.a. mean. This information should be added in the methods.

Reviewer #2:
Remarks to the Author:
In this study, Chua and colleagues monitor the dynamics and mechanics of the chromatin-binding protein Methyl-CpG-binding protein 2 (MeCP2) using cutting-edge correlative single-molecule fluorescence and force microscopy. The authors demonstrate that CpG methylation significantly attenuates the one-dimensional diffusion of MeCP2 on bare DNA, which could underlie methylation-specific activities including co-repressor recruitment. Moreover, the authors show that on chromatinized DNA, MeCP2 predominantly binds nucleosomes, which mechanically stabilizes them. Importantly, the authors examine MeCP2 variants featuring Rett syndrome-associated mutations and uncover that they compromise different aspects of the observed MeCP2-chromatin interactions.
This is well-executed, technically impressive work that sheds important new mechanistic light onto an essential chromatin-binding and disease-associated protein. In exemplifying the complexity and plasticity with which a single DNA-binding protein can engage chromatin in various contexts, this manuscript represents a major advance in the field. I am therefore happy to recommend publication, after the following points have been addressed:

(1) The authors report a range of different diffusion constants for MeCP2 on non-methylated DNA, likely due to differences in oligomeric states. Consistent with this notion, Figure ED2b displays a clearly anti-correlated behavior between the diffusion constant D and the estimated number of monomers, but only for lower numbers of monomers. Beyond a certain number of monomers, D stays constant without further decreasing. Is this due to aggregation, where the protein complex is non-physiologically stuck to the DNA/surface? What might be the physiological role of the much higher-order oligomeric species?

(2) Comparing the kymographs in Figures 1f (methylated DNA) and 3c (methylated DNA and nucleosomes), the kymographs in 3c suggest increased MeCP2 mobility. Since the observed MeCP2 diffusion does not directly involve the nucleosomes, what is the reason for this behavior? What are the experimental conditions in both cases, what is the concentration of MeCP2 in Figure 1f?

(3) It is not fully clear whether the observed changes in diffusion coefficient with methylated DNA are due to an increased oligomerization of MeCP2 when bound to methylated DNA or not. A figure analogous to ED2b for methylated DNA is not shown. Also, does the diffusive behavior depend on the applied force? It would be helpful if the authors could indicate the applied forces in the main text or in the figure legends in each case.

(4) In order to enable a direct comparison, the authors should show example MeCP2 trajectories on methylated DNA, analogous to what is depicted in Fig. 1c.

(5) The oligomeric status of MeCP2 appears to contribute to its function. When bound to naked DNA, higher-order oligomers can be formed. Are the various MeCP2 variants/mutants monomeric in the absence of DNA? Since the purification protocol involves a size exclusion step, could the authors characterize the oligomeric state of MeCP2 in the absence of DNA?

(6) The authors should discuss additional evidence from the literature in support of MeCP2

stably interacting with nucleosomes.

(7) The force-distance curves provide compelling evidence for a direct stabilizing effect that MeCP2 exerts on the nucleosome, presumably via a rather tight interaction? Can the authors estimate the Kd value for the interaction from the comparison of the F-d curves in the presence and absence of MeCP2? Is there any evidence for cooperativity in MeCP2 binding to nucleosomes?

(8) The authors could further strengthen the manuscript by discussing how the increase in transition force due to MeCP2 binding compares to the forces typically associated with biological processes such as transcription, DNA melting, overcoming nucleosome barriers etc

(9) In Figure 3d, the authors report a difference between the diffusion rate for MeCP2 on methylated DNA versus that on methylated, chromatinized DNA. Given the size of the error bars, how significant is this difference?

(10) Can histone methylation affect MeCP2?

Reviewer #3:
Remarks to the Author:
In the manuscript by Chua et al., the authors used a novel single-molecule fluorescent microscopy method to study the interactions between MeCP2 and DNA. They highlighted different binding behaviors of MeCP2 that depend on the methylation status of the DNA as well as the presence of nucleosomes. They found that MeCP2 displays a diffusion motion on bare DNA that is suppressed when the DNA is CpG methylated. They also showed that MeCP2 binds nucleosomes and enhances their stability. In addition, the authors investigated different MeCP2 mutations to identify the specific role of MeCP2 domains in DNA and nucleosome binding. While some of these findings have been reported in other studies (doi: 10.1038/ncomms11025, doi:10.1074/jbc.M704304200, doi: 10.1002/iub.386, doi: 10.1016/j.cell.2010.10.012, doi: 10.7554/eLife.51449), the method employed in this manuscript is novel and allowed the authors to show MeCP2 binding on the level of single molecules. This work helps better understand the mode of action of MeCP2 and thus contributes to the advancement of the field. I have several concerns that should be addressed prior to publication in Nature Structural & Molecular Biology.

1) The authors show that MeCP2 preferentially binds nucleosomes and enhances their stability. These experiments are informative; however, they lack one important condition: combining both nucleosomes and CpG methylation. The authors only included this condition in Figure 3 c and h, but this condition must be included and quantified in the subsequent figures as well. Including these results will benefit the manuscript as it will allow the direct comparison between the behavior of MeCP2 and its truncated versions on methylated and non-methylated chromatin. Also, a direct comparison between the affinity of MeCP2 to nucleosomes and to nucleosome-free methylated DNA is needed.

2) In Figure 4, the authors tested the binding of truncated MeCP2 proteins to nucleosomes and suggested that the TRD is required for nucleosome targeting. It would be interesting to also assess the nucleosome binding capacity of the RTT mutants shown in Figure 2a, as this would be more relevant in RTT than the binding of these mutants to DNA alone. If the

nucleosomal DNA binding of the different RTT mutants is not included in the manuscript, then in lines 70 and 248, the word "chromatin" should be replaced by "DNA".

3) Overall, the manuscript lacks a comparison between the authors' findings and previously published studies. This would be especially beneficial to the manuscript in the cases concerning the RTT-causing mutants and truncated MeCP2 proteins. There should be more comparison of the results in this study to previous findings for how these RTT mutations affect MeCP2 DNA binding, and it should also be mentioned that some of these RTT mutations significantly reduce MeCP2 protein levels (doi: 10.1016/j.cell.2013.01.038, doi: 10.1172/JCI90967, doi: 10.1093/hmg/ddy159, doi: 10.1093/hmg/ddv496).

4) The authors should comment on the limitations of their in vitro model. For instance, the number of DNA-bound nucleosomes in Figures 3,4, 5 and 6 are very different. Is this a technical issue, or is there a reason behind this difference? How does the spacing of nucleosomes on this DNA fragment compare to the in vivo spacing, and could this potentially affect MeCP2 binding? The authors mention that this assay's ability to spatially resolve molecules is at about 300 nm. How does this equate to bp length of DNA and the size of a MeCP2 molecule? Also, how were the concentrations of MeCP2, H1, TBLR1, and nucleosomes used determined? How does the ratio between the different molecules in this setup compare to the ratio in cells in physiological conditions? And is there a positive control protein with known DNA binding dynamics in cells that could be used to show that the results of this assay are consistent with DNA binding in cells?

5) Lines 126-127: The authors state that the MeCP2 P225R is unable to distinguish between methylated and unmethylated DNA based on its binding affinity and diffusivity. However, in extended data, Figure 4, the diffusivity of MeCP2 P225R is not evident, and the diffusivity on methylated DNA is not quantified. What was the basis of this conclusion? Also, the MSD plots showing diffusivity (Fig 2c and 3d) should include a split y-axis so that the data points for all conditions can be visualized.

6) In Figure 3, the authors demonstrate a high affinity of MeCP2 to nucleosomes and state that nucleosomes serve as molecular sponges for MeCP2. Would it be possible for the authors to repeat this experiment using different MeCP2 concentrations? For instance, starting from a low MeCP2 concentration, in which case all MeCP2 molecules should bind nucleosomes, then increasing the concentration until nucleosomes become saturated by MeCP2 molecules. Showing that only when all nucleosomes are saturated MeCP2 starts binding bare DNA would strengthen the authors' conclusion on this aspect. These experiments should be done with both methylated and non-methylated nucleosomes.

7) In Figure 5d, the authors find that MeCP2 and histone H1 colocalize at nucleosomes, which suggests that MeCP2 and H1 do not antagonize each other for nucleosome binding, a topic that is controversial in the literature. However, the colocalization finding would be strengthened if the authors quantify the number of MeCP2 and H1 molecules bound per nucleosome. Figure 3e shows 2 MeCP2 molecules are bound per nucleosome in the absence of H1. When H1 is added, are there 2 MeCP2 and 2 H1 molecules bound per nucleosome, or just 1 of each? And does the colocalization and number of MeCP2 and H1 molecules bound change if the concentrations of the two proteins are altered?

8) In Figure 6d, the authors showed that TBLR1 binds nucleosomal DNA even in the absence of MeCP2. It would be beneficial to include a negative control (a non-DNA binding protein) to compare its behavior in relation to DNA to the behaviors of MeCP2 and TBLR1.

Also, including MeCP2 R306C in this experiment would strengthen the authors' conclusion, as this RTT mutant is reported to disrupt the MeCP2-NCoR interaction without altering MeCP2 DNA binding (doi: 10.1038/nn.3434, doi: 10.1016/j.molcel.2019.10.032).

9) In several figures and in the text, for example Figure 2d and extended data Figure 2b, the authors quantify the "number of MeCP2 monomers". This terminology is confusing, and the intended meaning would be more clear if a term like "number of MeCP2 molecules in a multimer" were used.

10) Lines 264 to 266: The authors should explain what they mean by enzymatic digestion. The data shown in this manuscript do not lead to any conclusions regarding MeCP2 protecting methylated sites from enzymatic digestion.

11) Line 318: The word activity should be removed as the authors showed the distribution but did not explore the activity of chromatin regulators in their study.

12) Would it be technically possible to investigate MeCP2 binding on non-CpG methylated DNA using this assay? MeCP2 has been shown to bind methylated CAC sites with high affinity (doi: 10.1371/journal.pgen.1006793). Comparing MeCP2 behavior in the different methylation sequence contexts would greatly benefit this manuscript. At the very least this should be mentioned as a future direction in the discussion.

Author Rebuttal to Initial comments

Reviewers' Comments

Reviewer #1:

Remarks to the Author:

The manuscript by Chua et al. investigates the dynamics of the Methyl-CpG-binding protein 2 (MeCP2) using correlative single-molecule fluorescence and force microscopy. This allows the authors to determine the relative diffusion and binding dynamics of MeCP2 and Rett syndrome mutants on various DNA substrates. They report that methylated DNA enhances binding and reduces diffusion with RTT mutations disrupting enhancement. On DNAs containing nucleosomes with and without methylation, they report enhanced association of MeCP2 with nucleosomes leading to stronger mechanical stability that can be modulated by linkers histones. Finally, to examine proposals that MeCP2 may act in recruitment of co-repressors, they performed further multicolor imaging experiments demonstrating that TBLR1 complexes are recruited to DNA by MeCP2 to form larger diffusion-competent assemblies.

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Overall, I believe the manuscript presents many important new findings that help to clarify MeCP2 dynamics and defects of RTT variants. However, I am not sure all claims made in the discussion are supported by the experimental data without further clarification. I have provided a list of specific questions and comments below.

Response: We thank the reviewer for their thorough and positive evaluation of our study. We appreciate the questions and comments raised and have addressed them through new experiments, analyses, and textual changes to the manuscript for added clarity.

1. There is a significant amount of discussion revolving around the oligomerization of MeCP2. However, the diffraction-limited resolution of the data collected and the fact that the MeCP2 is added to DNA before imaging leave many aspects of the oligomerization unclear. For example, in line 133-135: “Moreover, the MeCP2 270X trajectories contained fewer protein monomers on average compared to those for the full-length protein, supporting that the disordered TRD/CTD contribute significantly to tMeCP2 multimerization on DNA.”
There isn’t evidence provided that oligomerization occurs on the DNA, as opposed to in solution before loading onto DNA. It would be more convincing if an experiment looking at oligomeric states in bulk solution without DNA showed primarily the monomeric form.

Response: We thank the reviewer for this excellent suggestion. To determine whether MeCP2 oligomerizes without DNA, we resorted to mass photometry (MP), a sensitive approach to quantify the mass of biomolecules in solution (Sonn-Segev et al., PMID: 32286308). The MP data clearly show that MeCP2 primarily exists in its monomeric form. This result is now included in **Extended Data Fig. 1b** of the revised manuscript. Since the molecular weight of the truncated MeCP2^{R270X} (29 kDa) is below the lower limit of the MP instrument (~40 kDa), we performed size exclusion chromatography instead, which generated a single elution peak corresponding to the monomeric form of the protein (**Fig. R1**).

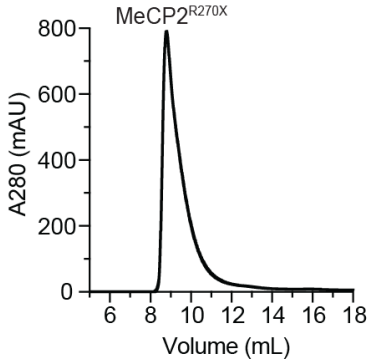

Figure R1. Superdex 200 Increase 10/30 GL chromatogram showing absorbance at 280 nm of R270X MeCP2.

2. The authors state that they heat inactivated the methyltransferase. Is that enough to be confident that it is no longer bound to DNA? How was the DNA purified after methylation? This information was not provided in the methods. If some remains bound it could account for the observed differences in diffusion between methylated and unmethylated DNAs.

Response: We appreciate this insightful question. After heat inactivation, the DNA was purified by phenol chloroform extraction followed by ethanol precipitation, which presumably removes all proteins bound to DNA. This is now described in the Methods section of the revised manuscript. To further rule out the possibility that the observed differences in MeCP2 diffusion between methylated and unmethylated DNA was due to residual methyltransferases on the DNA, we sought to use a methylation-agnostic protein that is known to diffuse on DNA. To this end, we performed new single-molecule experiments using fluorescently labeled Proliferating Cell Nuclear Antigen (PCNA), which has been shown to slide along DNA (Kochaniak et al., PMID: 19411704). Since PCNA topologically encircles DNA, leaving only a small distance (~5 angstroms) between the DNA and the protein's inner surface, any *M.SssI* methyltransferase protein (UniProt: P15840) remaining on the DNA would impede PCNA sliding. However, our results showed PCNA sliding across the entire length of the methylated λ DNA unobstructed (**Fig. R2**), indicating that DNA-bound methyltransferase after purification is negligible and that the observed changes in MeCP2 diffusion is caused by DNA methylation per se.

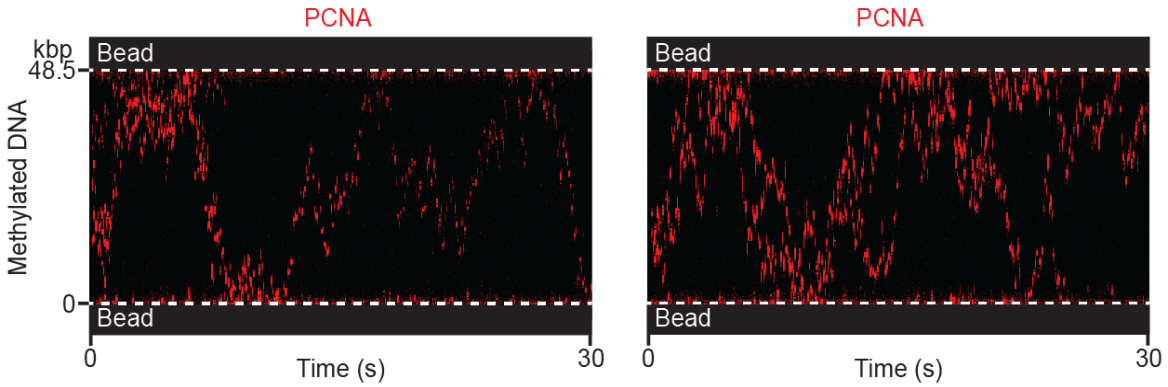

Figure R2. Two representative kymographs showing that LD647-labeled PCNA can freely slide on CpG methylated λ DNA tethers generated by our purification protocol.

3. When calculating diffusion coefficients, were both the mobile diffusing species and the immobile species included or was there a cutoff used to remove the immobile species on DNA from the calculation? The authors claim significant differences in the diffusion coefficients on methylated as compared to unmethylated DNA. Is this simply a result of inclusion of more immobile molecules? Is it possible the diffusion coefficient for scanning along DNA is the same for methylated and unmethylated when filtering for only mobile species? Or are the authors arguing that scanning diffusion is in fact slower even when MeCP2 is traveling over methylation sites. I would also imagine diffusion is more constrained as more binding occurs. The authors should clarify these points in the manuscript. Finally, in Figure 1e, error margins should be included similar to those presented in plot 2d and mean values should be reported in the further clarification of these questions.

Response: We did not categorically exclude any species from our analysis. Both mobile and immobile trajectories were included in our calculations. Because of the continuous range of the diffusion coefficients as shown in **Fig. 1f**, we felt that not applying an arbitrary cutoff is the most objective way to analyze our data. Of note, any trajectory with a *D* value smaller than ~0.01 kbp^2/s would appear "immobile" from visual inspection. We have plotted a few representative MeCP2 trajectories on methylated DNA as new **Fig. 1d** (in addition to those on unmethylated DNA in **Fig. 1c**) to exemplify the data included in our analysis.

The reviewer is correct that the more constrained MeCP2 diffusion on methylated DNA could also be caused by its increased binding. To rule out this possibility, in the original manuscript we had included example kymographs in which the methylated DNA tether was incubated with a much lower concentration of MeCP2 (**Extended Data Fig. 3c**), and low MeCP2 mobility was still observed, indicating that suppressed MeCP2 diffusion is caused by DNA methylation rather than spatial confinement. To further strengthen this conclusion, we repeated this experiment and performed MSD analysis of individual trajectories. The resulting diffusion coefficients of these sparsely bound trajectories are not significantly different than those calculated from trajectories obtained at higher MeCP2 concentration (**Fig. R3**). Finally, we have included mean values and error bars in Fig. 1e (now **Fig. 1f**). We thank the reviewer for this suggestion.

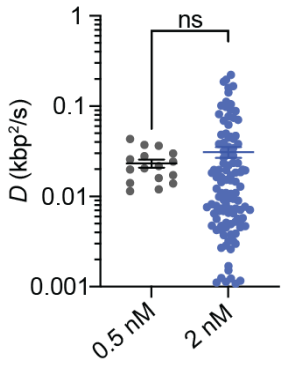

Figure R3. Diffusion coefficients (*D*) for Cy3-MeCP2 trajectories on methylated DNA tethers incubated with 0.5 nM (black) (*n* = 16 from 6 independent tethers) or 2 nM (blue) (*n* = 109 from 22 independent tethers) MeCP2. Bars represent mean and SEM.

4. Have the authors examined the force profile of DNA bound by MeCP2 alone and compared it to the force profile with nucleosomes? This important condition was not displayed in Figure 5c or discussed.
Related, in Figure 5d it appears that some H1 histones are diffusing. The authors should evaluate the dynamics of H1 and MeCP2 in the absence of nucleosomes. One explanation for the diffusion could be an interaction between the two factors, formation of a larger complex and diffusion together. Can the authors exclude this possibility. In this case, inclusion of H1 might simply start removing MeCP2 from nucleosomes to cause the force difference.

Response: We thank the reviewer for suggesting these important controls. First, we performed new pulling experiments with MeCP2-bound bare unmethylated DNA tethers, which yielded smooth force-distance curves without noticeable transitions (examples shown in **Fig. R4** below). This result indicates that the transitions observed in **Fig. 5b** came from the unwrapping of nucleosomes and/or MeCP2-nucleosome complexes. This control is now included in the revised manuscript as modified **Fig. 5b**.

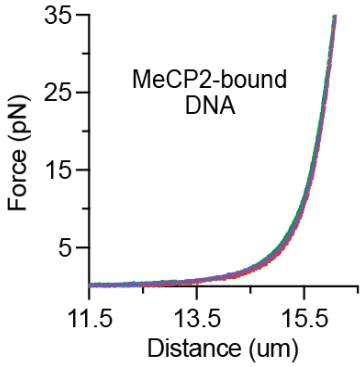

Figure R4. Representative force-distance curves of MeCP2-bound bare unmethylated DNA tethers (*n* = 5). The tethers were incubated with 2 nM MeCP2 for 30 s prior to stretching.

Secondly, we conducted new experiments to visualize the dynamics of both MeCP2 and H1 on bare DNA and observed that they rarely colocalize or diffuse together. An example kymograph is

now shown as **Extended Data Fig. 7b** in the revised manuscript. Interestingly, they often serve as barriers to confine each other's diffusion. Therefore, the interplay between H1 and MeCP2 appears to be mediated by the nucleosome, as these two factors largely act separately on bare DNA. This is now mentioned in the revised manuscript (Lines 200-202).

5. Depending on the results for comment 4, the authors should consider whether there are alternative hypotheses to the one stated in lines 213-217: "These results reveal that the nucleosome can simultaneously accommodate both H1 and MeCP2, but H1 attenuates the nucleosome stabilization effect of MeCP2. This indicates that the binding pose of MeCP2 in the ternary complex is distinct from that of MeCP2 bound to the nucleosome alone." If so, these statements should be adjusted.

Response: As described in our response to comment 4 above, the interaction between MeCP2 and H1 seems to require the nucleosome core particle. The stoichiometry and architecture of the putative nucleosome-MeCP2-H1 complex require further investigation. We have added a note to this effect in the revised manuscript (Line 212).

6. In the reporting summary the authors state that "Source data are provided with this paper. Other data are available upon reasonable request." Are the raw data/kymographs deposited somewhere and accessible? Do the authors mean the displayed kymographs for source data? I do not know the reporting requirements of the journal, but I would strongly encourage the authors to deposit the raw data in a public database so others can reproduce their analysis and further benefit from their work.

Response: As required by the journal, we will provide the source data (raw values used to generate the bar/dot plots, etc.) as well as uncropped gel images as supplementary information should the manuscript become accepted. We will also deposit all displayed kymographs and data analysis codes in a public repository such as GitHub or Zenodo. We thank the reviewer for this suggestion.

7. While the authors reported n values in several places in the manuscript, they have not specified how many independent DNA tethers were used for each analysis and biological replicates. This should be reported. Are all kymographs used for analysis displayed in the supplementary materials?

Response: We have now reported the number of independent DNA tethers analyzed for each condition, in addition to the already specified number of molecular events therein (fluorescence trajectories, force transitions, etc.) in the figure captions of the revised manuscript. We will deposit all kymographs used for analysis in a public repository should the manuscript become accepted.

8. Can the authors also display the EMSA gel used for the analysis in Figure S1b. Given the large oligomers seen in single-molecule experiments, it would be interesting to see the relative

stoichiometry of binding for WT vs MeCP2C339S, C413S. The authors have displayed the EMSA gel for methylated and unmethylated binding in Figure S3b, which is very nice by the way. It is important to also include the raw gel used for analysis in Figure S1b.

Response: We agree with the reviewer and have included representative EMSA gels for DNA binding by WT MeCP2 vs. MeCP2$^{C339S, C413S}$ in **Extended Data Fig. 1a**.

9. The n value is provided for the estimate of the mean number of MeCP2 in each complex, but was not provided for the estimates of diffusion constants in Figure 1e (number of spots). This should be included.

Response: We appreciate this suggestion and have now specified the *n* value for the number of analyzed MeCP2 trajectories on unmethylated DNA and methylated DNA in the figure caption.

10. In Figures 6d and 6e there are so many nucleosomes that it is hard to see if TBLR1 and MeCP2 are actually co-localizing. Maybe a zoom in would be more convincing.

Response: We have included a zoom-in panel in the new **Fig. 6e** with individual fluorescence signals to more clearly show the colocalization of TBLR1 and MeCP2 at the nucleosome.

11. Lines 127-128. The scanning mode is clearly changed but this does not imply discrimination between methylated and unmethylated DNA is in fact changed. See comment 3 and consider rephrasing depending on the outcome.

Response: In our response to comment 3 above, we have shown that the slowed scanning of MeCP2 on methylated DNA is not due to enhanced binding. In the case of the MeCP2^{P225R} mutant, we observed that it displays much reduced diffusion on unmethylated DNA. Again, this is unlikely to be caused by spatial constraints, as the mutant shows a similar level of binding to unmethylated DNA compared to the wild-type protein (**Fig. 2b**). We have added a note to this sentence for added clarity (Lines 125-127).

12. Figure 2c nicely shows differences in diffusion between wild-type and various mutants. The authors should also include estimates of the diffusion coefficients in a plot similar to figure 1e with error bars and spots for each trajectory. Same for the diffusion constant estimates Figure 3d. They should be added in a separate plot with error bars.

Response: We thank the reviewer for this suggestion. We have obtained the diffusion coefficient distributions for WT, T158M, P225R, and R270X MeCP2 and plotted them as new **Extended Data Fig. 4b**. We did the same for the conditions in Fig. 3d and included the plot as new **Extended Data Fig. 5a**.

13. If space allows, or in the supplement, it would be useful if the authors could provide a reference for the diffusion model used.

Response: We have added to the Methods section of the revised manuscript (Line 542) a reference (Codling et al., PMID: 18426776) for the one-dimensional diffusion model used in our data analysis.

14. The nomenclature for the bar plots should be defined somewhere. What do the *, **, ***, n.a. mean. This information should be added in the methods.

Response: This information is now stated in the Methods section of the manuscript as well as in individual figure captions.

Reviewer #2

Remarks to the Author:

In this study, Chua and colleagues monitor the dynamics and mechanics of the chromatin-binding protein Methyl-CpG-binding protein 2 (MeCP2) using cutting-edge correlative single-molecule fluorescence and force microscopy. The authors demonstrate that CpG methylation significantly attenuates the one-dimensional diffusion of MeCP2 on bare DNA, which could underlie methylation-specific activities including co-repressor recruitment. Moreover, the authors show that on chromatinized DNA, MeCP2 predominantly binds nucleosomes, which mechanically stabilizes them. Importantly, the authors examine MeCP2 variants featuring Rett syndrome-associated mutations and uncover that they compromise different aspects of the observed MeCP2-chromatin interactions.
This is well-executed, technically impressive work that sheds important new mechanistic light onto an essential chromatin-binding and disease-associated protein. In exemplifying the complexity and plasticity with which a single DNA-binding protein can engage chromatin in various contexts, this manuscript represents a major advance in the field. I am therefore happy to recommend publication, after the following points have been addressed:

Response: We thank the reviewer for their enthusiastic review of our study and their constructive feedback. We have addressed their points through new experiments, analyses, and textual changes to the manuscript for added clarity.

(1) The authors report a range of different diffusion constants for MeCP2 on non-methylated DNA, likely due to differences in oligomeric states. Consistent with this notion, Figure ED2b displays a clearly anti-correlated behavior between the diffusion constant D and the estimated number of monomers, but only for lower numbers of monomers. Beyond a certain number of monomers, D stays constant without further decreasing. Is this due to aggregation, where the protein complex is non-physiologically stuck to the DNA/surface? What might be the physiological role of the much higher-order oligomeric species?

Response: This is an excellent point. The anticorrelation between the diffusion coefficient (*D*) and the oligomer size reaches a plateau when the *D* value drops below ~0.01 kbp^2/s (**Extended Data**

Fig. 2b), which represents the detection limit of our instrument. A value on the same order of magnitude was reported for an immobile control dCas9 in a similar setup (Carcamo et al., PMID: 35876491). Therefore, with the current resolution we cannot tell whether the large oligomers were immobile or very slowly diffusing. We did observe that some of them display a modest level of diffusion (see examples below in **Fig. R5**), indicating that they were not stuck to the DNA/surface. It has been previously reported that MeCP2 can undergo higher-order oligomerization and liquid-liquid phase separation in vitro and in vivo, which is promoted by DNA and linked to heterochromatin organization (Wang et al., PMID: 32111972; Zhang et al., PMID: 35156529). Our results add to this active area of research.

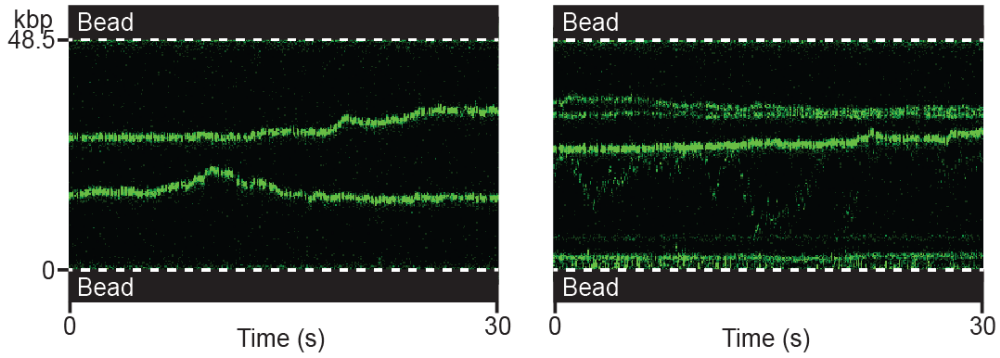

Figure R5. Two example kymographs of unmethylated bare DNA tethers where large oligomers of Cy3-labeled MeCP2 were observed to diffuse along the DNA.

(2) Comparing the kymographs in Figures 1f (methylated DNA) and 3c (methylated DNA and nucleosomes), the kymographs in 3c suggest increased MeCP2 mobility. Since the observed MeCP2 diffusion does not directly involve the nucleosomes, what is the reason for this behavior? What are the experimental conditions in both cases, what is the concentration of MeCP2 in Figure 1f?

Response: We appreciate the reviewer's sharp observation and apologize for the confusion. The experimental conditions for Fig. 1f and 3c are identical (MeCP2 concentration was 2 nM) other than 3c involving nucleosome-containing DNA tethers. As shown in Fig. 1e, individual MeCP2 trajectories exhibit a range of mobility even on methylated DNA. It happens that the previous Fig. 3c contains some MeCP2 trajectories that were visibly mobile. To avoid this confusion, we have chosen a more representative kymograph for the new **Fig. 1d**.

(3) It is not fully clear whether the observed changes in diffusion coefficient with methylated DNA are due to an increased oligomerization of MeCP2 when bound to methylated DNA or not. A figure analogous to ED2b for methylated DNA is not shown. Also, does the diffusive behavior depend on the applied force? It would be helpful if the authors could indicate the applied forces in the main text or in the figure legends in each case.

Response: These are both excellent points. First, we did not analyze the oligomeric state of MeCP2 on methylated DNA because it was difficult to distinguish multiple adjacent MeCP2 units from a single larger unit given the enhanced binding and low mobility of MeCP2 on methylated DNA. To address the reviewer's question of whether the decreased diffusion coefficient could be due to increased oligomerization of MeCP2, we performed experiments with a lower concentration of MeCP2, which reduced the chance of two separate units bound in close proximity. We then analyzed the fluorescence intensity of these units and found that MeCP2 does not seem to oligomerize into larger units on methylated DNA (see **Extended Data Fig. 3c**).

Second, all kymographs (except those during pulling experiments) were imaged at 1 pN of force applied to the DNA as mentioned in the Methods section (now also noted in the Results section of the revised manuscript). To address whether the applied force affects the diffusive behavior of MeCP2, we raised the DNA tension to 5 pN and found that MeCP2 diffusion is indeed slowed on unmethylated DNA compared to the 1 pN condition (still faster than it on methylated DNA) (**Fig. R6**). This result indicates that MeCP2's behavior on DNA is sensitive to the local mechanical state of the chromatin, which is an interesting subject for future research.

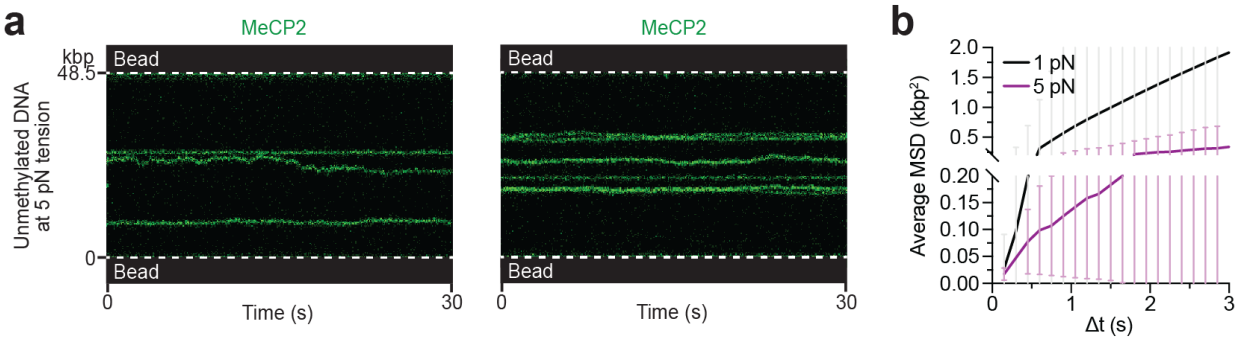

Figure R6. a, Two representative kymographs of unmethylated λ DNA tethers held at 5 pN of tension incubated with 2 nM Cy3-labeled MeCP2. **b**, Average MSD plot for MeCP2 trajectories on unmethylated DNA held at 1 pN or 5 pN of tension. Error bars represent SD.

(4) In order to enable a direct comparison, the authors should show example MeCP2 trajectories on methylated DNA, analogous to what is depicted in Fig. 1c.

Response: We have added example trajectories of MeCP2 on methylated DNA as new **Fig. 1e**, analogous to what is depicted in Fig. 1c.

(5) The oligomeric status of MeCP2 appears to contribute to its function. When bound to naked DNA, higher-order oligomers can be formed. Are the various MeCP2 variants/mutants monomeric in the absence of DNA? Since the purification protocol involves a size exclusion step, could the authors characterize the oligomeric state of MeCP2 in the absence of DNA?

Response: We appreciate this point raised by both reviewers #1 and #2. To determine the oligomeric state of MeCP2 in the absence of DNA, we performed mass photometry experiments

and found that MeCP2 primarily exists as monomers in solution (shown as **Extended Data Fig. 1b** in the revised manuscript). Similar results were obtained for other MeCP2 mutants used in this study. Since the predicted molecular weight of the truncated MeCP2^{R270X} (29 kDa) is below the lower limit of the MP instrument (~40 kDa), we performed size exclusion chromatography, which yielded a single elution peak corresponding to the monomeric form of the protein (**Fig. R1** above).

(6) The authors should discuss additional evidence from the literature in support of MeCP2 stably interacting with nucleosomes.

Response: We agree and have included more specific information about what has been previously reported regarding MeCP2's interaction with nucleosomes in the Introduction section of the revised manuscript (Lines 45-47).

(7) The force-distance curves provide compelling evidence for a direct stabilizing effect that MeCP2 exerts on the nucleosome, presumably via a rather tight interaction? Can the authors estimate the Kd value for the interaction from the comparison of the F-d curves in the presence and absence of MeCP2? Is there any evidence for cooperativity in MeCP2 binding to nucleosomes?

Response: We appreciate this comment. The pulling experiments were performed under irreversible conditions and thus the rupture force depends on the pulling rate, which was kept constant in our experiments (100 nm/s). Since it is non-trivial to derive free energy changes from non-equilibrium work, we instead used an EMSA to estimate the binding affinity of MeCP2 to a 207-bp DNA-wrapped mononucleosome and obtained a Kd value of ~3.7 nM—indeed a rather tight interaction. The nucleosomes were sparsely loaded onto the DNA tether in our single-molecule assay. To answer whether MeCP2 binds to nucleosomes in a cooperative manner would require using regularly spaced nucleosome arrays, which is an interesting direction for future studies.

(8) The authors could further strengthen the manuscript by discussing how the increase in transition force due to MeCP2 binding compares to the forces typically associated with biological processes such as transcription, DNA melting, overcoming nucleosome barriers etc.

Response: We appreciate this suggestion. The force regime in which nucleosomes unravel in the absence or presence of MeCP2 (~14 pN vs. ~29 pN) indeed encompasses the maximal forces generated by the transcription machinery and chromatin remodelers (Galburt et al., PMID: 17361130; Sirinakis et al., PMID: 21552204). The stabilizing effect (or lack thereof) of MeCP2 and its variants on chromatin is thus expected to impinge on fundamental processes such as genome organization and gene expression. This is now mentioned in the Discussion section of the revised manuscript (Lines 271-277).

(9) In Figure 3d, the authors report a difference between the diffusion rate for MeCP2 on methylated DNA versus that on methylated, chromatinized DNA. Given the size of the error bars, how significant is this difference?

Response: We thank the reviewer for raising this point. To better compare the diffusion rate between conditions, we have calculated the diffusion coefficient values for individual MeCP2 trajectories on methylated bare DNA versus at nucleosomes on unmethylated DNA and plotted them as new **Extended Data Fig. 5a**. We found the *D* values for nucleosome-associated MeCP2 were significantly lower than those for unmethylated and methylated DNA-bound MeCP2, supporting the notion that MeCP2 remains immobile when bound to nucleosomes.

(10) Can histone methylation affect MeCP2?

Response: It has been suggested by immunoprecipitation assays that MeCP2 association with chromatin is enhanced by histone methylation, particularly at H3K9 and H3K27 (e.g., Fuks et al., PMID: 12427740; Lee et al., PMID: 32561780). It is an exciting future direction to investigate how histone PTMs modulate MeCP2 dynamics on chromatin using our single-molecule platform. We have made a note about this point in the Discussion section of the revised manuscript.

Reviewer #3

Remarks to the Author:

In the manuscript by Chua et al., the authors used a novel single-molecule fluorescent microscopy method to study the interactions between MeCP2 and DNA. They highlighted different binding behaviors of MeCP2 that depend on the methylation status of the DNA as well as the presence of nucleosomes. They found that MeCP2 displays a diffusion motion on bare DNA that is suppressed when the DNA is CpG methylated. They also showed that MeCP2 binds nucleosomes and enhances their stability. In addition, the authors investigated different MeCP2 mutations to identify the specific role of MeCP2 domains in DNA and nucleosome binding. While some of these findings have been reported in other studies (doi: 10.1038/ncomms11025, doi:10.1074/jbc.M704304200, doi: 10.1002/iub.386, doi: 10.1016/j.cell.2010.10.012, doi: 10.7554/eLife.51449), the method employed in this manuscript is novel and allowed the authors to show MeCP2 binding on the level of single molecules. This work helps better understand the mode of action of MeCP2 and thus contributes to the advancement of the field. I have several concerns that should be addressed prior to publication in Nature Structural & Molecular Biology.

Response: We thank the reviewer for acknowledging the novelty of our work and its contribution to the field. We have addressed their concerns through new experiments, analyses, and textual changes to the manuscript. We have also worked to better contextualize our study within existing literature and be more explicit about the limitations of our approach.

1) The authors show that MeCP2 preferentially binds nucleosomes and enhances their stability. These experiments are informative; however, they lack one important condition: combining both nucleosomes and CpG methylation. The authors only included this condition in Figure 3 c and h, but this condition must be included and quantified in the subsequent figures as well. Including

these results will benefit the manuscript as it will allow the direct comparison between the behavior of MeCP2 and its truncated versions on methylated and non-methylated chromatin. Also, a direct comparison between the affinity of MeCP2 to nucleosomes and to nucleosome-free methylated DNA is needed.

Response: We appreciate this comment. Single-molecule experiments are very time-consuming, and it is impracticable for us to repeat all the experiments with methylated chromatin. Nonetheless, we did perform a few new experiments to reinforce some key conclusions in this study. First, in response to comment 6 below, we visualized and quantified MeCP2-nucleosome colocalization on methylated nucleosomal DNA tethers at varying MeCP2 concentrations (**Fig. R7a**). Although there existed a lot more methylated DNA sites (up to 3,113) than nucleosome sites (<10) in each tether, we found a comparable number of bare-DNA-bound MeCP2 and nucleosome-bound MeCP2, especially at low MeCP2 concentrations. Second, we pulled on MeCP2-bound methylated chromatin tethers and obtained an average transition force significantly higher than that for unbound chromatin (**Fig. R7b**). We have also performed EMSA experiments to compare the affinity of MeCP2 to nucleosomes and to methylated bare DNA. Using a 207-bp DNA sequence, we obtained an apparent Kd value of 1.0 nM for MeCP2 binding to methylated DNA (Kd for MeCP2 binding to one single DNA site is likely higher because the substrate can accommodate multiple copies of MeCP2) and 3.7 nM for MeCP2 binding to mononucleosomes wrapped with unmethylated DNA. Together, these results suggest that MeCP2 still preferentially binds nucleosomes and enhances their stability when the DNA is CpG methylated. In the future, we will characterize in more detail the behavior of MeCP2 and its RTT variants in the context of methylated chromatin.

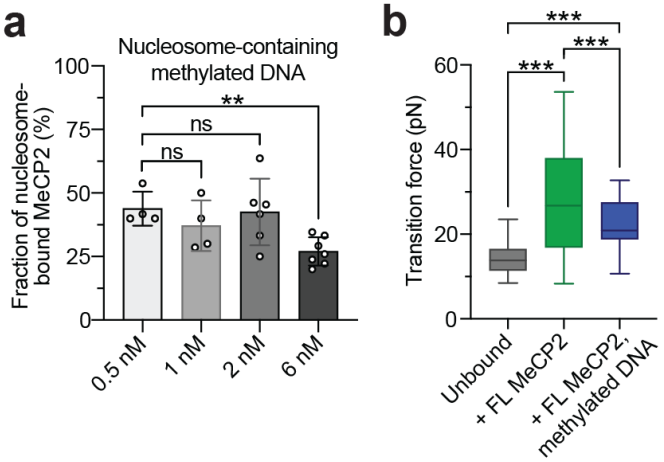

Figure R7. **a**, Fraction of Cy3-MeCP2 trajectories that were colocalized with nucleosome loci on methylated DNA in the presence of 0.5 nM (from 4 independent tethers), 1 nM (from 4 independent tethers), 2 nM (from 6 independent tethers), or 6 nM (from 7 independent tethers) MeCP2. Error bars represent SD. **b**, Distribution of transition forces recorded from force-distance curves of unmethylated nucleosomal DNA tethers with no MeCP2 (n = 84 from 5 independent tethers), bound with FL MeCP2 (n = 107 from 7 independent tethers), or of methylated nucleosomal DNA tethers bound with FL MeCP2 (n = 55 from 5 independent tethers). Box

boundaries represent 25th to 75th percentiles, middle bar represents median, and whiskers represent minimum and maximum values.

2) In Figure 4, the authors tested the binding of truncated MeCP2 proteins to nucleosomes and suggested that the TRD is required for nucleosome targeting. It would be interesting to also assess the nucleosome binding capacity of the RTT mutants shown in Figure 2a, as this would be more relevant in RTT than the binding of these mutants to DNA alone. If the nucleosomal DNA binding of the different RTT mutants is not included in the manuscript, then in lines 70 and 248, the word "chromatin" should be replaced by "DNA".

Response: We thank the reviewer for raising this point. As suggested, we performed new experiments to visualize the binding of P225R and T158M MeCP2 mutants to nucleosome-containing DNA tethers and quantified the fraction of MeCP2-bound nucleosomes under the same experimental conditions. We found that these RTT mutants retained the wild-type level of nucleosome targeting capacity. These results are now included in **Fig. 4b**.

3) Overall, the manuscript lacks a comparison between the authors' findings and previously published studies. This would be especially beneficial to the manuscript in the cases concerning the RTT-causing mutants and truncated MeCP2 proteins. There should be more comparison of the results in this study to previous findings for how these RTT mutations affect MeCP2 DNA binding, and it should also be mentioned that some of these RTT mutations significantly reduce MeCP2 protein levels (doi: 10.1016/j.cell.2013.01.038, doi: 10.1172/JCI90967, doi: 10.1093/hmg/ddy159, doi: 10.1093/hmg/ddv496).

Response: We thank the reviewer for this suggestion. We have added a more detailed discussion to compare our results on MeCP2 mutants and truncations with previous studies and mentioned reduced protein levels as another contributing factor to RTT pathology. These are included in the Discussion section of the revised manuscript (Lines 296-316).

4) The authors should comment on the limitations of their in vitro model. For instance, the number of DNA-bound nucleosomes in Figures 3,4, 5 and 6 are very different. Is this a technical issue, or is there a reason behind this difference? How does the spacing of nucleosomes on this DNA fragment compare to the in vivo spacing, and could this potentially affect MeCP2 binding? The authors mention that this assay's ability to spatially resolve molecules is at about 300 nm. How does this equate to bp length of DNA and the size of a MeCP2 molecule? Also, how were the concentrations of MeCP2, H1, TBLR1, and nucleosomes used determined? How does the ratio between the different molecules in this setup compare to the ratio in cells in physiological conditions? And is there a positive control protein with known DNA binding dynamics in cells that could be used to show that the results of this assay are consistent with DNA binding in cells?

Response: We appreciate these critical questions. We can empirically control the density of nucleosomes loaded on DNA tethers by adjusting the protein concentration and incubation time. For the experiments where we intended to visualize MeCP2's behavior on bare DNA between nucleosomes (e.g., Fig. 3, 4), we aimed to load fewer nucleosomes per tether. For the

experiments where the main purpose was to quantify MeCP2-nucleosome colocalization (e.g., Fig. 5, 6), a higher nucleosome density was aimed for. The space between nucleosomes in our experiments is on the order of kilobases. As the reviewer noted, the spatial resolution of our assay is ~300 nm, which equates to ~900 bp of dsDNA. In comparison, the DNA binding footprint of MeCP2 is ~11 bp. The protein concentrations used in our experiments were chosen such that they are high enough to allow sufficient statistics to be accumulated in a reasonable amount of time, but not too high to prevent single-molecule detection due to fluorescence background. In a practical sense, an operative concentration range is between a few to tens of nanomolar. We tried to make the relative ratio between different proteins roughly reflect the in vivo scenario. One advantage of the in vitro approach is that the ratio can be easily changed.

We acknowledge the differences between our experimental conditions and the in vivo setting where the chromatin is more densely packed (~50 bp internucleosomal spacing) and the protein concentrations are higher ([MeCP2] in neuronal nuclei is estimated to be ~10-30 μM). We have commented on the limitations of our in vitro approach in the revised manuscript (Lines 326-330). With regards to positive controls, in our earlier publications we used the same type of single-molecule assay to study other DNA-binding proteins such as the origin recognition complex and the RNA polymerase, and found a good agreement with their behaviors in cells (Li et al., PMID: 35999198; Wang et al., PMID: 36917983). The association of MeCP2 with nucleosomes, as the reviewer pointed out, has also been suggested by previous genomic and in vivo imaging studies. We have discussed in more detail in the revised manuscript how our single-molecule results help inform MeCP2's mode of action on chromatin in the context of existing literature.

5) Lines 126-127: The authors state that the MeCP2 P225R is unable to distinguish between methylated and unmethylated DNA based on its binding affinity and diffusivity. However, in extended data, Figure 4, the diffusivity of MeCP2 P225R is not evident, and the diffusivity on methylated DNA is not quantified. What was the basis of this conclusion? Also, the MSD plots showing diffusivity (Fig 2c and 3d) should include a split y-axis so that the data points for all conditions can be visualized.

Response: We apologize for the lack of clarity here. We observed that MeCP2 P225R exhibits low diffusivity on both methylated DNA and unmethylated DNA, unlike the wild-type protein that shows distinct behaviors on these substrates. We have quantified the diffusivity of MeCP2 P225R on unmethylated vs. methylated DNA and found that they are not significantly different (shown in **Fig. R8** below). This result, combined with the similar loading efficiency of MeCP2 P225R to unmethylated vs. methylated DNA (**Fig. 2b**), led us to conclude that this mutant is largely unable to distinguish between these two forms of DNA. We have clarified this point in the revised manuscript. We have also modified **Fig. 2c** and **3d** to include a split y-axis so that all data points can be visualized.

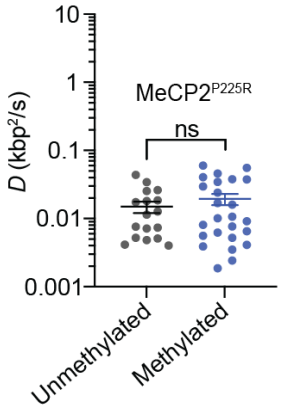

Figure R8. Diffusion coefficients (*D*) for Cy3-MeCP2 P225R trajectories on unmethylated (black) (*n* = 17 from 10 independent tethers) or CpG methylated (blue) (*n* = 25 from 10 independent tethers) DNA. Bars represent mean and SEM.

6) In Figure 3, the authors demonstrate a high affinity of MeCP2 to nucleosomes and state that nucleosomes serve as molecular sponges for MeCP2. Would it be possible for the authors to repeat this experiment using different MeCP2 concentrations? For instance, starting from a low MeCP2 concentration, in which case all MeCP2 molecules should bind nucleosomes, then increasing the concentration until nucleosomes become saturated by MeCP2 molecules. Showing that only when all nucleosomes are saturated MeCP2 starts binding bare DNA would strengthen the authors' conclusion on this aspect. These experiments should be done with both methylated and non-methylated nucleosomes.

Response: We thank the reviewer for this suggestion. We have performed new experiments in which we visualized MeCP2 binding to nucleosome-containing unmethylated DNA tethers at varying concentrations of MeCP2 and quantified the fraction of nucleosome-bound MeCP2. Consistent with the prediction made by the reviewer, we observed that at the lowest concentration (0.5 nM), almost all MeCP2 molecules were nucleosome-bound; when the concentration was increased, more MeCP2 began to bind bare unmethylated DNA. This result is now shown as new **Fig. 3f** in the revised manuscript. A similar trend was observed for nucleosome-containing methylated DNA tethers (**Fig. R7a**), although the nucleosome-bound MeCP2 fraction is lower compared to nucleosome-containing unmethylated DNA tethers across MeCP2 concentrations, because of the higher affinity of MeCP2 for methylated bare DNA.

7) In Figure 5d, the authors find that MeCP2 and histone H1 colocalize at nucleosomes, which suggests that MeCP2 and H1 do not antagonize each other for nucleosome binding, a topic that is controversial in the literature. However, the colocalization finding would be strengthened if the authors quantify the number of MeCP2 and H1 molecules bound per nucleosome. Figure 3e shows 2 MeCP2 molecules are bound per nucleosome in the absence of H1. When H1 is added, are there 2 MeCP2 and 2 H1 molecules bound per nucleosome, or just 1 of each? And does the colocalization and number of MeCP2 and H1 molecules bound change if the concentrations of the two proteins are altered?

Response: We appreciate this comment. It is possible to estimate the number of MeCP2 and H1 molecules colocalized at nucleosomes based on the intensities of their fluorescence signals in our single-molecule assay. However, the crosstalk between fluorescence channels (particularly between blue and green) complicates the data analysis. Therefore, we sought to use mass photometry—a sensitive approach to quantify the mass and interaction of biomolecules in solution (mass error within 5%)—to estimate the stoichiometry of the MeCP2-H1-nucleosome complex. When we mixed a mononucleosome sample containing 207 bp of DNA (predicted mass of 245 kDa) with MeCP2 (52 kDa) and H1 (22 kDa), we observed a major peak that is consistent with the MeCP2-H1-nucleosome complex of 1:1:1 stoichiometry. This result is now shown as new **Extended Data Fig. 7c**.

8) In Figure 6d, the authors showed that TBLR1 binds nucleosomal DNA even in the absence of MeCP2. It would be beneficial to include a negative control (a non-DNA binding protein) to compare its behavior in relation to DNA to the behaviors of MeCP2 and TBLR1. Also, including MeCP2 R306C in this experiment would strengthen the authors' conclusion, as this RTT mutant is reported to disrupt the MeCP2-NCoR interaction without altering MeCP2 DNA binding (doi: 10.1038/nn.3434, doi: 10.1016/j.molcel.2019.10.032).

Response: We thank the reviewer for this comment. As suggested, we have purified MeCP2 R306C and performed new single-molecule experiments. Satisfyingly, consistent with previous literature, we observed that this mutant fails to recruit TBLR1 to DNA regardless of the methylation status. Moreover, we observed that this mutant is deficient at recruiting TBLR1 to nucleosomes, as the fraction of TBLR1-bound nucleosomes in the presence of MeCP2 R306C was significantly lower than that in the presence of wild-type MeCP2 and similar to that in the absence of MeCP2. These results are now shown in the new **Fig. 6c and 6f**. As for the negative control, we think that the MeCP2 K210X and R162X truncations already shown in the manuscript (**Fig. 4**) are good candidates as they, unlike TBLR1 or WT MeCP2, can still bind DNA but tend not to target nucleosomes. We also would like to point out previous studies that reported TBLR1 binding to core histone proteins and reconstituted mononucleosomes (Yoon et al., PMID: 12628926; Yoon et al., PMID: 15601853). Therefore, it is not too surprising that we observed some TBLR1-nucleosome colocalization even in the absence of MeCP2. We have cited these studies in the revised manuscript.

9) In several figures and in the text, for example Figure 2d and extended data Figure 2b, the authors quantify the "number of MeCP2 monomers". This terminology is confusing, and the intended meaning would be more clear if a term like "number of MeCP2 molecules in a multimer" were used.

Response: We have replaced the terminology throughout the manuscript with the one suggested by the reviewer. For example, in Line 155 it now writes: "We then analyzed the number of MeCP2 molecules at nucleosomal loci…". We have also changed in the labels in **Fig. 2d** and **Extended Data Fig. 2b** to "Estimated # of molecules".

10) Lines 264 to 266: The authors should explain what they mean by enzymatic digestion. The data shown in this manuscript do not lead to any conclusions regarding MeCP2 protecting methylated sites from enzymatic digestion.

Response: We apologize for the confusion here. Our rationale was that since we observed that CpG methylation drastically suppresses MeCP2 diffusion on DNA, MeCP2's longer residence time or stalling at methylated sites could more effectively block DNA digestion by enzymes such as DNase I at these sites. On the other hand, when MeCP2 is rapidly scanning along unmethylated DNA—despite having only a modestly lower binding affinity compared to methylated DNA—the enzymes should still have access to their target sites. This provides a potential explanation for previous findings that MeCP2 is able to protect methyl CpG sites from enzymatic digestion but shows a lack of protection of unmodified sites. We have clarified this sentence in the revised manuscript.

11) Line 318: The word activity should be removed as the authors showed the distribution but did not explore the activity of chromatin regulators in their study.

Response: We have removed the word "activity" from this sentence.

12) Would it be technically possible to investigate MeCP2 binding on non-CpG methylated DNA using this assay? MeCP2 has been shown to bind methylated CAC sites with high affinity (doi: 10.1371/journal.pgen.1006793). Comparing MeCP2 behavior in the different methylation sequence contexts would greatly benefit this manuscript. At the very least this should be mentioned as a future direction in the discussion.

Response: We thank the reviewer for bringing up this important point. It is possible to generate DNA templates containing non-CpG methylation sites, but this is technically more challenging because there aren't known recombinant enzymes that specifically catalyze non-CpG DNA methylation. One potential strategy is to design an oligonucleotide sequence without any CpG sites, use the enzyme DNMT3A to modify cytosines in other sequence contexts such as CpA sites (Gowher and Jeltsch, PMID: 11399089), and then ligate this segment to unmethylated DNA to create a longer template (>10 kbp) for single-molecule experiments. We have added this possibility as a future direction to the Discussion section of the revised manuscript.

Decision Letter, first revision:

Message: Our ref: NSMB-A48272A

17th May 2024

Dear Professor Liu,

Thank you for submitting your revised manuscript "Differential dynamics specify MeCP2 function at nucleosomes and methylated DNA" (NSMB-A48272A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, it is important that we have a copy of the main text as a word file. If you could please send along a word version of this file as soon as possible, we would greatly appreciate it; please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology. Please do not hesitate to contact me if you have any questions.

Sincerely,

Jean Nakhle, PhD
Associate Editor
Research Cross-Journal Editorial Team for Nature Structural & Molecular Biology
ORCID: 0000-0001-9385-6577

Reviewer #1 (Remarks to the Author):

The authors have done a great job addressing my concerns. I do not have any further comments.

Reviewer #2 (Remarks to the Author):

The authors have thoroughly addressed all of my questions and comments, and I'm happy to enthusiastically recommend publication.

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed my concerns by adding new experimental data and text clarification. I think the manuscript is now suitable for publication in NSMB. My only remaining suggestion is to include the figures that were only in the rebuttal (R7 and R8) in the manuscript because I think these are important results.

Final Decision Letter:

Message: 16th Jul 2024

Dear Professor Liu,

We are now happy to accept your revised paper "Differential dynamics specify MeCP2 function at nucleosomes and methylated DNA" for publication as an Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

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Sincerely,

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