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Methyl-H3K9 Binding Protein MPP8 Mediates E-cadherin Gene Silencing and Promotes Tumor Cell Motility and Invasion

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 May 2010

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office. Three expert referee's initially agreed to assess merits and suitability of your paper for our journal. As I did receive two rather consistent reports that reveal both strength as well as current weaknesses of the study, I am in the position to make a decision at this point to prevent you from unnecessary delays with the study. As you will see, ref#1 asks for additional histone marks to be elucidated as well as clarification(s) on the microarray data. Rather more importantly, ref#2 raises crucial points on how general this model of H3K9 methyl-mark recognition by MMP8 actually is and how binding impinges on MMP8 activity and function. As the points raised by the latter referee are of crucial importance for the overall impact of your study (and thus potential suitability for our journal), I urge you to take the necessary time and care to develop these aspects of the paper much further. Conditioned on such significant additional experimentation we are happy to re-assess a future version of your work.
I do have to formerly remind you that it is EMBO_J policy to allow a single round of major revisions only and that the ultimate decision on acceptance or rejection entirely depends on the content and strength of the final version that will be assessed involving some of the original referees.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

P.S. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Kokura et al (EMBOJ-2010-74644)
In this manuscript, the authors describe novel methyl-H3K9 binding protein, MPP8. MPP8 specifically recognizes H3K9me2 and me3. Furthermore, MPP8 interacts with H3K9 methyltransferases, GLP and ESET/SETDB1 and DNA methyltransferase Dnmt3a. DNA microarray analysis showed that knockdown (KD) of MPP8 in human non-small cell lung cancer cell MDA-MB-231 induced dysregulation of multiple genes, specially involved in cell adhesion and epithelial-to-mesenchymal transition (EMT). KD of MPP8 in MDA-MB-231 cell also induced inhibition of cell growth (mild), migration and invasion. Finally, the authors focused on one of MMP8 (negatively) regulating genes, E-cadherin and they showed that MPP8 is enriched on the E-cadherin promoter and exon 2 regions. Dnmt3a is also enriched on those two regions and KD of MPP8 induced reduction of not only MPP8 but also Dnmt3a on them. MethylScreen DNA methylation analysis showed that reduction of DNA methylation was induced on the exon 2 region (but not on the promoter region) after MPP8 KD. Taken all data together, the authors concluded that MPP8 probably coordinates histone and DNA methylation for tumor suppressor gene silencing and plays an important role in EMT and metastasis.

First part (MPP8 binding to the chromatin containing H3K9me2or3) is quite clear. However, the MPP8-mediated gene silencing (regulation) mechanism part is weak. In general, promoter region of DNA methylation is crucial for gene silencing. Furthermore, which data suggests their model "MPP8 coordinates histone and DNA methylation...(Abstract)"? MPP8-mediated E-cadherin silencing is clear, but may be DNA methylation independent. The authors should clarify more about the MPP8-mediated silencing (gene regulation) mechanism. MPP8, the novel methyl-H3K9 binding protein itself is a quite interesting finding, I think. Therefore, if the authors can address my criticisms, I am supportive for this work to be published in EMBO J.

The following points are major criticisms on the current version of manuscript:

1. Figure 7. 1) should check other histone K marks, such as H3K4me, 36me, H3ac (active marks) and H4K20me3 (negative mark) and HP1, too. 2) combination KD studies with 5-aza, TSA or 5-aza+TSA (without KD also) will be useful. If such data is already available, should show.
2. Figure 4A. Microarray studies, need more explanation about horizontal-axis. Should list all the dysregulated genes by MPP8 KD in Supplemental data. Also show the number of up-regulated and down-regulated genes (MPP8 is mainly linked with gene silencing?). May be more important for gene activation like fibronectin silenced by MPP8 KD. MPP8-mediated fibronectin activation is direct effect? (MPP8 binds to the fibronectin promoter?)

Minor points
3. should apply statistics and describe P values to Figure 4E, 5D, 7B-D. Fig 5D also shown additional graph as each H3IP as 100%?
4. Figure 6A. IP efficiencies of each Flag-molecules (G9a, GLP and ESET) should be shown as another western blotting with anti-flag.

Referee #2 (Remarks to the Author):

The manuscript "Methyl-H3K9 Binding Protein MPP8 Mediates E-cadherin Gene Silencing and Promotes Tumor Cell Motility and Invasion," studies the role of the H3K9me3-binding protein in the context of cancer promotion. In a screen of H3K9 methylated peptides that bind chromodomains, the authors identify MPP8 as an H3K9 me2 and me3 recognition module (as previously demonstrated by other groups). The authors carry out an analysis beyond the previous

papers in their demonstration that MPP8 does indeed bind H3K9me2 and H3K9me3. MPP8 is shown to promote proliferation and the epithelial-to-mesenchymal transition for a breast cancer cell line. Whole genome expression analysis was used to identify E-cadherin as a target repressed by MPP8. The authors build a model that MPP8 binding to H3K9 methyl, and MPP8 interactions with two protein lysine methyltransferases and a DNA methyltransferase lead to a repressive chromatin environment that leads to inhibition of E-cadherin expression. Understanding new mechanisms by which H3K9 methylation is transduced at euchromatin is important, thus the functional characterization of MPP8-H3K9me binding is an interesting and potentially an important contribution. The main concern is that the connection between the MPP8 function (which is nicely elucidated in the manuscript) and MPP8 chromodomain binding to H3K9me3 is only tested on reporter assays. It would considerably strengthen the manuscript if this connection were to be explored in a more endogenous chromatin and biological setting.

Major Points:

1) Strengthen the connection between H3K9me3 recognition by MPP8 and MPP8 function.

Additional Points:
1) It would be helpful to show that the model is more general than one target gene. Examination of second target indentified in the expression analysis would strengthen the paper.
2) It would be interesting to make an MPP8 point mutation that can still bind to methylated H3K9 but loses the interaction with DNMT3A, which should display a loss of function. Establishing a complementation system with various MPP8 point mutants in knock-down cells would be quite powerful

Additional Editorial Correspondence 20 May 2010

I just received comments from the third referee that are very much in line with the report of particularly ref#2 asking to tighten up the functional link between H3K9- methyl-binding and functional activity of MPP8 in E-cadherin regulation. I therefore ask you to include responses to these points demanding further phenotypic characterization of the EMT-phenotype and putting the effects of MMP8 stronger into the background literature of other chromatin-modifications that govern E- cadherin expression in your revisions.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Ref#3 remarks to the authors:

The paper is overall clearly written and presents an interesting mechanism of MPP8 in the regulation of DNMT3A localization and its possible involvement in tumor progression. However, there are some concerns:

1. How general is the EMT phenotype caused by increased expression levels of MPP8 in breast cancer cells? The authors only show one cell line, can they verify this in a different invasive breast cancer cell line? It is concerning that the non-invasive MCF7 and T47D breast cancer cell lines (which express good levels of E-cadherin) show the same expression levels of MPP8 as the invasive cell lines like MDA- MB-231 cells. Is it possible to induce EMT in MCF7 cells by over-expressing MPP8?
2. The authors show that the KD of MPP8 in MDA-MB-231 cells leads to highly increased expression levels of E-cadherin, a key player in EMT processes. It would be nice to see DIC images of the KD cells to determine morphological changes of the cells, e.g. cell shape, cell-cell-contacts. It would also be necessary to show IF images of the KD cells with staining for E-cadherin to prove that E-cadherin is indeed functional, located at the cell membrane and enables the cells to establish cell-cell contacts.

3. The authors claim that the regulation of the EMT phenotype by MPP8 is via E-cadherin, can they rescue the decreased EMT phenotype caused by MPP8 KD by knocking down E-cadherin? Apparently there are global gene expression changes, is E-cadherin the lynchpin in this process?

4. Many papers, as the authors have cited in the manuscript, have shown that the E-cadherin expression can be silenced by multiple mechanisms, including histone modification pattern changes (deacetylation of H3 and H4, or methylation of H3K9, H3K27, etc. CpG island methylation at the E-cadherin locus may or may not be necessary for E-cadherin silencing. It is puzzling to see such a dramatic expresssion level changes of E-cadherin with MPP8 knockdown, but without any changes of the H3K9me2, and H3K9me3 levels and with only moderate changes of CpG methylation levels in exon2 (gene body) region? Are the authors suggesting that the transcription of E-cadherin can sometimes bypass the repressive histone modification effects as in this case, and be partially regulated by DNA methylation?

1st Revision - Authors' Response 07 August 2010

Response to the reviewers' comments

We would like to thank the reviewers again for their thoughtful comments, which have allowed us to improve our manuscript significantly. We hope that the reviewers will find that we have addressed their concerns satisfactorily.

Referee #1:

1), "Figure 7. 1 should check other histone K marks, such as H3K4me, 36me, H3ac (active marks) and H4K20me3 (negative mark) and HP1, too. 2 combination KD studies with 5-aza, TSA or 5-aza+TSA (without KD also) will be useful. If such data is already available, should show".

The reviewer has raised a very interesting question regarding whether MPP8 knockdown affects histone methylation and acetylation patterns to de-repress E-cadherin expression. To this end, we examined activation marks including H3K4me2, me3, H3K36me3 and H3ac and repression mark H4K20me3 on E-cadherin promoter in control and MPP8 knockdown MDA-MB-213 cells. However, we were not able to detect any obvious changes on these histone modifications (Fig.S7). Together, these results suggest that MPP8-mediated *E-cadherin* repression could be downstream of histone methylation and acetylation.

It has been shown that multiple epigenetic mechanisms are involved in E-cadherin gene silencing. Following the reviewer's suggestion, we treated control, MPP8 knockdown and rescue MDA-MB-231 cells with 5-Aza, TSA or both inhibitors and systematically analyzed the E-cadherin re-expression in these cells. We also analyzed H3ac and H3K9me3 pattern as well as MPP8 and DNMT3A localization in E-cadherin promoter and exon 2 regions in cells treated with different inhibitors (Fig.8). The results not only suggest that MPP8-directed DNA methylation is the major mechanism for *E-cadherin* repression, but also demonstrate the importance of MPP8 methyl-H3K9 binding in DNA methylation and *E-cadherin* repression (Fig.8).

2), "Figure 4A. Microarray studies, need more explanation about horizontal-axis. Should list all the dysregulated genes by MPP8 KD in Supplemental data. Also show the number of up-regulated and down-regulated genes (MPP8 is mainly linked with gene silencing?). May be more important for gene activation like fibronectin silenced by MPP8 KD. MPP8-mediated fibronectin activation is direct effect? (MPP8 binds to the fibronectin promoter?)

Following the reviewer's suggestion, we have included the dysregulated gene list in Supplementary Results and the raw micro-array data will be deposited in the public database. Although there are large group of genes are up-regulated and down-regulated, we still believe that MPP8 is mainly responsible for gene silencing since H3K9 methylation is one of the major repressive marks and the localization of MPP8 on promoter requires methyl-H3K9 binding. In addition, H3K9 has been associated with some activated genes as well. We speculate that MPP8 could also have a role in

gene activation through different molecular mechanisms if MPP8 targets the promoter of these genes. However, ChIP analysis indicates that it is not the case for Fibronectin (Fig.5E).

3). "should apply statistics and describe P values to Figure 4E, 5D, 7B-D. Fig 5D also shown additional graph as each H3IP as 100%?"

We have done statistic analysis for most of our results and indicated in all figures. For all histone ChIP analysis, we have provided additional graph with H3 or H4 antibodies normalized as 100%.

4). "Figure 6A. IP efficiencies of each Flag-molecules (G9a, GLP and ESET) should be shown as another western blotting with anti-flag."

We have included these IP-western results in Fig.6

Referee #2:

1). "Strengthen the connection between H3K9me3 recognition by MPP8 and MPP8 function."

The reviewer raised a great point. Dissecting the role of methyl-H3K9 binding of MPP8 in endogenous gene silencing and EMT will significantly help us to understand MPP8 function. Following the reviewer's suggestion, we have stably rescue expressed Flag-MPP8-wt or W80A mutant in MPP8 knockdown MDA-MB-231 cells and applied them for various assays. The results demonstrate that methyl-H3K9 binding of MPP8 is critical for E-cadherin repression and E-cadherin promoter localization. MPP8 methyl-H3K9 binding is also important for promoting migration and invasion in trans-well assays. In addition, ChIP analysis indicates that MPP8 methtyl-H3K9 binding is crucial for DNMT3A recruitment in E-cadherin promoter and exon 2 regions. These results together demonstrate the importance of methyl-H3K9 binding in MPP8 function.

2)." It would be helpful to show that the model is more general than one target gene. Examination of second target indentified in the expression analysis would strengthen the paper."

Following the reviewer's suggestion, we have examined more than 10 genes based on our micro-array result by RT-qPCR and ChIP assays. However, only one gene APCS/SAP (Amyloid P Component Serum) is a valid MPP8 target gene. As indicated in Fig.S9, APCS mRNA level is significantly increased in response to MPP8 knockdown and this increase is clearly MPP8-dosage dependent. ChIP analysis indicates that MPP8 targets APCS promoter in MDA-MB-231 cells while knocking-down MPP8 results in a severe reduction of MPP8 localization. Depletion APCS in mouse results in retarded induction of reactive amyloidosis (Nature Med. 3:855-859, 1997), suggesting MPP8 could regulate different biological functions by targeting different genes. Although we are very interested in these observations, the molecular dissection of these regulations is beyond the scope of the current manuscript.

3). "It would be interesting to make an MPP8 point mutation that can still bind to methylated H3K9 but loses the interaction with DNMT3A, which should display a loss of function. Establishing a complementation system with various MPP8 point mutants in knock-down cells would be quite powerful."

The reviewer raised a great point and we do appreciate this suggestion. We are actually working on characterizing MPP8-GLP and MPP8-DNMT3A interactions. We are also in process to generate DNMT3A stable knockdown/rescue system to address this question. However, it will not be possible for us to finish these experiments within the time frame given by the editor for resubmission. As an alternative approach, we treated control, MPP8 knockdown and rescue MDA-MB-231 cells with DNA methylation inhibitor 5-Aza and HDAC inhibitor TSA. As indicated in Fig.8, MPP8 knockdown induced E-cadherin de-repression is overlapped with effects caused by 5-Aza treatment, suggesting that MPP8-directed DNA methylation is critical for *E-cadherin* repression. In addition, ChIP analysis reveals that reduced MPP8 localization in E-cadherin promoter and exon 2 regions always decreases DNMT3A localization in the same region (Fig.8, S8). These observations also support that MPP8 recruits DNMT3A for DNA methylation.

Referee #3:

1). "How general is the EMT phenotype caused by increased expression levels of MPP8 in breast cancer cells? The authors only show one cell line, can they verify this in a different invasive breast cancer cell line? It is concerning that the non-invasive MCF7 and T47D breast cancer cell lines (which express good levels of E-cadherin) show the same expression levels of MPP8 as the invasive cell lines like MDA-MB-231 cells. Is it possible to induce EMT in MCF7 cells by over-expressing MPP8?"

The reviewer raised a great point. We have noticed the MPP8 expression level is not completely correlates with tumor cell behaviors. Following the reviewer's suggestion, we have tried to knockdown MPP8 in more invasive tumor cells. As indicated in Fig.S4, stably knocking-down MPP8 in non-small cell lung cancer H23 cells and cervical cancer HeLa cells results in an obvious de-repression of E-cadherin. More importantly, MPP8 knockdown in invasive H23 cells also leads to a severe reduction of migration and invasion in Boyden chamber assays, suggesting role of MPP8 in promoting migration and invasion and EMT is not limited in MDA-MB-231 breast cancer cells. We have also tried to test whether over-expression of MPP8 in MCF7 cells could induce EMT. Because of some technical issues, we did not get any colony after G-418 selection. We are now trying to use different constructs and will test this possibility again. Given that transformed cells have different background, we will also test this possibility in immortalized human mammary epithelial cells MCF10A and hMEC-hTERT-LT.

2. "The authors show that the KD of MPP8 in MDA-MB-231 cells leads to highly increased expression levels of E-cadherin, a key player in EMT processes. It would be nice to see DIC images of the KD cells to determine morphological changes of the cells, e.g. cell shape, cell- cell-contacts. It would also be necessary to show IF images of the KD cells with staining for E-cadherin to prove that E-cadherin is indeed functional, located at the cell membrane and enables the cells to establish cell-cell contacts."

Following the reviewer's suggestion, we examined cell morphological changes using DIC microscopy. As indicated in Fig.S3, MPP8 knockdown in MDA-MB-231 cells moderately changed cell shapes to a cuboidal form. Meanwhile, immunostaining results indicate that re-expressed E-cadherin indeed localizes on the cell membrane in MPP8 knockdown MDA-MB-231 cells and H23 cells (Fig.S6, S4E)

3. "The authors claim that the regulation of the EMT phenotype by MPP8 is via E-cadherin, can they rescue the decreased EMT phenotype caused by MPP8 KD by knocking down E-cadherin? Apparently there are global gene expression changes, is E-cadherin the lynchpin in this process?"

The reviewer raised a valid point. In our microarray data, we did not observe any severe changes on expression of major EMT regulators in response to MPP8 knockdown, such as SANIL, TWIST, ZEB, etc. Meanwhile, it has been demonstrated that E-cadherin controls a complex transcriptional network and loss of E-cadherin itself is sufficient to cause EMT or to afford functional traits that allow completion of the later steps of metastasis in cells and in mouse model (Cancer Res. 2008, 68:3645-54). Based on these observations and our results, we believe that MPP8 represses E-cadherin expression and in turn promotes migration, invasion and EMT. The suggested experiments are reasonable, unfortunately, it will not be possible for us to finish in the time frame given by the editor for resubmission. However, we are in process to identify more EMT-related MPP8 target genes based on our micro-array data. Hopefully we will be able to reveal a possible epigenetic EMT network regulated by MPP8.

4. "Many papers, as the authors have cited in the manuscript, have shown that the E-cadherin expression can be silenced by multiple mechanisms, including histone modification pattern changes (deacetylation of H3 and H4, or methylation of H3K9, H3K27, etc. CpG island methylation at the E-cadherin locus may or may not be necessary for E-cadherin silencing. It is puzzling to see such a dramatic expression level changes of E-cadherin with MPP8 knockdown, but without any changes of the H3K9me2, and H3K9me3 levels and with only moderate changes of CpG methylation levels in exon2 (gene body) region? Are the authors suggesting that the transcription of E-cadherin can sometimes bypass the repressive histone modification effects as in this case, and be partially regulated by DNA methylation?"

The reviewer raised a very interesting point. Generally, methyl groups on histone tail serve as docking sites to recruit methyl-binding proteins, which will in turn transmit the methyl signals to functional outcomes. As indicated in the response to another reviewer, we also tested a few more transcriptional activation and repression marks on E-cadherin promoter in addition to methyl-H3K9. However, we did not observe any obvious changes (Fig.S7). These results suggest that MPP8-mediated E-cadherin repression is downstream of histone methylation and acetylation changes. Furthermore, E-cadherin expression is up-regulated after we treated cells with HDAC inhibitor TSA and this increase is additive to MPP8 knockdown induced E-cadherin de-repression, suggesting histone acetylation also regulates E-cadherin expression in a MPP8-independent manner (Fig.8). In addition, TSA and 5-Aza treatment results in a reduction of H3K9me3 attended by a decreased localization of MPP8 and DNMT3A (Fig.8, S8). These results also suggest altered H3K9 methylation will result in MPP8 localization changes and in turn affect E-cadherin repression.

Thanks a lot

2nd Editorial Decision 30 September 2010

Upon re-review from one of the original referee's I am happy to accept your paper for publication in The EMBO Journal. Our office will soon be in touch with you related to further formalities.

Yours sincerely,

Editor
The EMBO Journal

