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DNA Methylation Status Predicts Cell Type-specific Enhancer Activity

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(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 05 November 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been evaluated by three referees and I enclose their reports below. While there is interest in the role of DNA methylation in specifying chromatin remodeling at distal regulatory elements, they referees do not currently support publication, however, there are sufficient indications that the study would be suitable for The EMBO Journal upon significant revision.

As you will see from their comments the referees are currently not completely convinced of the significance of the enrichment of CpG nucleotides at distal elements and importantly if DNA methylation plays a causative role in regulating enhancer element accessibility and GR binding. These issues clearly need to be further developed. From the comments of the referees it is important to address the following issues in order of significance. The referees request a more thorough analysis of the distribution of CpG enrichment at the different classifications of sites identified in Fig 1 and 2 and link this more directly to the bisulfite sequencing data. Importantly at this stage the current study does not provide evidence for a causative role of DNA methylation in regulating GR binding and chromatin remodeling, the in vitro GR binding data should be strengthened and the effect of depletion of Dnmt1 on DHS and GR binding should be analysed, I think based on the referees comments this is a key experiment. The referees also raise issues with the demethylation data, which also should be addressed. Other experiments such as identifying the factors that may define the origin of pre-programmed sites and the structural basis for the effect of methylation on

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GR binding to DNA while clearly would also strengthen the study are not required for current study especially given the required experiments above.

Therefore, while I appreciate that the referees request a significant amount of additional analysis and experiments the main issues they raise are central to the conclusions of the study and should provide the significant additional evidence requested to convince them of the role of DNA methylation in defining regulatory elements and regulating GR activity. If you are able to satisfactorily address these concerns we would be happy to consider a revised version of the manuscript. I would also like to point out that if additional time were required to address the core issues this would not be a problem.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Review MS EMBOJ-2010-76175 by Wiench et al

In this manuscript, Wiench et al study the connection between DNA methylation and glucocorticoid receptor (GR) binding to DNA. This work builds upon a genome-wide study of chromatin accessibility and GR binding to DNA in two distinct glucocorticoid-responsive cell lines to be published in another journal. Using this data set, the authors observe a small enrichment of CpG density in the vicinity of GR binding elements (GRBEs) preferentially in some of the sites where chromatin is nuclease sensitive irrespective of glucocorticoid treatment (pre-programmed sites). Then, they study at high resolution DNA methylation at 17 selected sites within 13 target genes. They observe that CpGs in the vicinity of cell type-specific GRBEs are generally methylated in the cell-line where the GRBEs are inactive. They also observe transient changes of small amplitude in the methylation levels of a limited number of CpGs within three genes at sites where glucocorticoids induce increase nuclease sensitivity of the chromatin (de novo sites). They conclude that their "findings provide evidence for a unique link between tissue-specific chromatin accessibility, DNA methylation and transcription factor binding at distal enhancer elements".

Although the genome-wide study of chromatin accessibility in relation to the cell type specificity of GR binding to DNA is highly interesting and complete, the present DNA methylation study is of more limited impact due to the small number of genes analyzed and to the lack of decisive demonstration of really novel properties. The study has interesting aspects that are not clearly and strongly demonstrated, and it is possible that stronger data would give higher significance to the manuscript.

1. The authors claims that they observe "a major enrichment in CpG density" (p6, line 10) when they observe a peak of CpGs density of 3.5 CpGs per 100 bp, "a significant increase" when the peak reaches 2 CpGs in 100 bp (Fig 1B) and the CpG enrichment they observe nearby GRBEs at pre-

programmed sites is only 20% above background with peaks of 1.2 CpG per 100 bp (Fig. 1 C). These average values are far from being spectacular and the authors would have to demonstrate that such an increase in CpG density exert a regulatory role in a more convincing manner than by vague correlations. Furthermore, it is misleading to use average values because they are not informative of the distribution of CpGs among the various sites considered. Are the mean peaks resulting from a global but minor increase of the CpG density distributed equally over all sites, or is there a minor subpopulation of sites with a more striking enrichment? The second option is more likely, because the authors then study in details sites with CpG densities that are all higher than 1.2 and that go up to about 6 CpG per 100 bp. To analyze the distribution of CpG density, the authors could present box-plots showing this distribution in windows of a few hundred base pairs around the sites of interest to demonstrate the different behavior of these populations. It would be important to know in particular if the de novo and pre-programmed sites have really different behavior overall or if the pre-programmed sites just contain a subpopulation of CpG enriched sites that is absent from the de novo sites (Fig. 2A). The density of CpGs found within each site analyzed by bisulfite sequencing should be clearly represented on Fig 3-4 and Supp Fig S3-5 and the effect of density on the behavior should be analyzed better. The claims and the wording should not exaggerate artificially the differences observed and to avoid unsupported claims like "dramatic contrast", "overwhelmingly due", "major enrichment".

2. The authors use throughout the text the wording "regulatory CpGs" but they bring little demonstration that these methylated CpGs play a regulatory role. The only indication is an effect on GR binding to DNA which is not well-supported by experimental data (see point 3). Demonstration that methylation directly affects glucocorticoid response of some genes in cultured cells is missing. The correlation between repressive chromatin structures and methylation is well known but it has always been difficult to distinguish whether methylation plays a causal role in most of the situations or is a secondary consequence of repression involved in its long-term stabilization. This study does little to clarify the situation and the wording should be adapted to the lack of strong demonstration.

3. The gel shift data showing that methylation of a key cytosine present in a few GRBEs interfere with GR binding to DNA is not really convincing (Fig 5). The signal intensity of the DNA-protein complex is lower, but that of the unbound DNA as well. Thus, the loss of complex is not compensated by an increase in the amount of free DNA, which would be expected if CpG methylation was lowering GR affinity for DNA. This suggests an experimental artifact (i.e., lower amount of labeled probe, complex remaining in the top of the gel, ...). The experiment shown should be more convincing. If better results can be obtained, it would be helpful to discuss the results using a figure (or a supplementary figure) showing the effect of CpG methylation on GR binding using the numerous crystallographic data available (in particular with CpG containing GRBEs (Science 2009, 324, 407).

4. The hormone-dependent demethylation shown in Fig 6 is not performed using a quantitative enough assay. The variation in the methylation levels observed are either of small intensity and/or strikingly visible at a single time point. It is not described how many clones were sequenced to determine the percentage of methylation shown. It would be necessary to sequence a sufficient number of clones to validate with a statistical test that the percentages are significantly different at different time points. Furthermore, to ensure that the differences are not due to fluctuations and carry-over contamination that often pollute bisulfite sequencing data, it would be necessary to use either barcoded hairpin bisulfite (NAR 2004, 32:e135), or to show more narrowly spaced time points showing consistent behaviors. There are previously published studies showing short-term or long-term demethylation in response to steroid hormones, including glucocorticoids, that analyzed a significantly higher number of clones and/or time points and/or genes (PNAS 2006, 103: 11112 Nature 2008, 452, 45 & 112). The present study should at least achieve similar standards.

Referee #2 (Remarks to the Author):

In this report Wiench et al investigate cell type-specific chromatin accessibility and DNA methylation of glucorticoid receptor (GR) binding sites across the genome. Based on DNAseI hypersensitivity before and after hormone stimulation the authors distinguish two types of sites: (i)"pre-programmed" or pre-existing in cells prior to stimulation with hormone and GR binding and

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(ii) "de-novo" sites which become DNAseI hypersensitive upon stimulation with hormone and GR binding. The authors show that constitutive, i.e. "pre-programmed", promoter-distal DNAseI hypersensitive sites (DHS) are mildly enriched for CpG dinucleotides (2-fold compared to flanking sequences) and are generally hypomethylated in a cell type-specific manner. In contrast, "de novo" DHS sites are depleted of CpGs and show partial DNA methylation. Limited demethylation of methylated CpGs was observed upon GR binding within the inducible ("de novo") DHS. The main message of the manuscript, as also outlined by the title, is that lack of DNA methylation at constitutive DHS defines active cell type-specific enhancers.

For the most part the data presented in manuscript is descriptive. The two novel and interesting observations are that methylated CpGs within degenerate GR response elements can partially inhibit GR binding and that upon hormone stimulation and GR binding there is rapid, but incomplete, demethylation of CpGs close to the GRE. The authors argue that such demethylation must be active as it occurs within minutes after hormone stimulation. However, it seems that the peak of hypomethylation observed at and near the Suox-GRE is at 8h after hormone treatment. Thus passive demethylation during DNA replication is also possible and could be mediated by a competition for binding between GR and Np95/UHRF1 or DNMT1 at such sites. The authors should examine DNA methylation around Mef2b and Ptprg GREs at 8h time point as well. Does demethylation take place in the presence of aphidicoline or other drugs that inhibit DNA replication? In Figure 6B, E and F, it is unclear how many sequences were investigated per time point to determine the average DNA methylation of each CpG.

I do agree that there is a good correlation between lack of DNA methylation and the presence of hormone-independent promoter-distant DHS. Given that these sites are constitutive, but tissue-specific, it is possible that they are determined by binding of other factors that destabilize nucleosomes, generate permissive chromatin and inhibit DNA methylation. Thus partial or complete lack of DNA methylation is a feature of, but not a pre-requisite for GR binding at "pre-programmed" DHS. Thus, the major question that remains unanswered is what determines the existence of "pre-programmed" tissue-specific sites. The authors could test by ChIP whether factors such as Cfp1 and MLL1, which have been implicated in maintenance of unmethylated state at CpG islands, are bound to "pre-programmed", but not "de novo" DHS.

The appearance of "de novo" sites is also poorly defined. In figure 5 the authors show that in the majority of cases DNA methylation does not inhibit GR binding. Therefore, it is unclear how "de novo" sites are chosen. The authors speculate that the appearance of "de novo" sites might be potentiated by incomplete DNA methylation. Thus they say in the discussion that "Demethylation of a limited number of CpGs could potentially destabilize a nucleosome, further exposing a response element for protein-DNA interactions." However, it seems unlikely that the presence of GRE and the amount of DNA methylation are sufficient to determine "de novo" sites as the sequence of the non-functional DHS in 3134 cells (Figure S3C) and the inducible DHS at Ptprg and Arrdc2 genes in 3134 (Figure S5) show similar levels of DNA methylation. Moreover, the two statements in the abstract (i) "De novo sites thus identify a novel class of enhancer elements that are devoid of the strong suppressive effect of methylated cytosines." and(ii) "Furthermore, treatment with glucocorticoids induces rapid changes in methylation levels at selected CpGs within de novo elements. These modifications, in turn, can impact GR binding efficiency by direct and indirect mechanisms." seem to contradict each other.

On page 5 (and the abstract) the authors say "The de novo DNAseI hypersensitive sites are surrounded by CpG-depleted sequences". This statement is not very specific. From Figure 1 and S1 it is clear that all DHS are "surrounded by" CpG-depleted sequences (-5000 to -1000 and +1000 to +5000). Probably the authors meant to say as on page 8 "de novo sites are not enriched in CpGs". The graph in Figure 2A seems to show that they are depleted of CpGs compared to flanking sequences.

In summary, I am not convinced from the data presented in the manuscript that the authors have answered satisfactory the question (formulated in the introduction) of whether DNA methylation has a cell type-specific impact on accessibility of enhancers to GR binding. Further experiments would

be necessary to demonstrate that this is the case. For example, the authors could reduce DNA methylation in 3134 cells by stable shRNA knockdown of DNMT1 and ask whether DNAseI hypersensitivity and GR binding can be observed in the presence of Dex at DHS5 and other AtT20-specific DNAseI hypersensitive sites that are normally absent in 3134 cells.

Referee #3 (Remarks to the Author):

Wiench and colleagues report a genome-wide analysis of glucocorticoid receptor (GR) binding in the context of chromatin accessibility and DNA methylation in two cancer-derived mouse cell lines.

The authors showed that most of the GR bound regions do not overlap with CpG islands nor with promoter regions.

Based on the overlap of GR bound regions with DNaseI hypersensitivity sites (DHS) detected in naive cells (before hormone treatment) they define two classes of GR bound regions:
i) "pre-programmed" DHSs: GR bound region is overlapping with DHS detected in cells before hormone treatment == constitutively open chromatin (85% of GR bound regions)
ii) "de-novo" DHSs: GR bound region is not overlapping with DHS detected in cells before hormone treatment (15% of GR bound regions)

Next, the authors studied the properties of the GR bound regions, namely the CpG content and methylation level of bound sequences. They found that "pre-programmed" class regions were enriched for CpGs, in contrast to "de-novo" class regions, which were mostly found in the CpG depleted regions. The authors speculate that the enrichment for CpGs may suggest the role of DNA methylation in enhancers.

By bisulfite sequencing they analyzed DNA methylation status of 17 regions. For the "pre-programmed" regions they found correlation between demethylation status and accessibility of DNA sequence. They also investigated the impact of methylated cytosine on GR binding. By using the EMSA assay they showed that CpG methylation could affect binding of GR but only if the methylated CpG was present directly in glucocorticoid response element.

Next they showed a detailed analysis of DNA methylation at one GR bound locus of "de-novo" class, in proximity of the Suox gene. They described rapid demethylation process initiated 10 minutes after the hormone stimulation and speculate that this observation supports the idea of active demethylation. Further they showed that prolonged hormone treatment (12 hours) resulted in remethylation of CpGs, which according to the work by John et al. 2009, corresponds to decrease of transcriptional activity of the Suox gene. Here the authors do not observe any changes in chromatin marks (acetylation of H3, H3K4me3, H3K9me1).

The observed speed of demethylation makes an active process likely, could one strengthen this point by blocking the cell cycle and thus the source of passive demethylation?

While this manuscript addresses an important problem it is unclear if it indeed shows a specific function for DNA methylation in regulating ER activity. The observation that GR sites have a higher frequency of CpG dinucleotides seems at this point insufficiently justified and even though GR seems sensitive to methylation in vitro it is unclear if this is the case in vivo.

Other questions:

Q1: The ratio of "pre-programmed" vs. "de-novo" DHS sites could depend on the sequencing depth in the DNaseI hypersensitivity assay. Lower read counts might lead to an underestimation of the number of GR bound regions overlapping with "pre-programmed" DHS sites. Was the sequencing depth sufficient to detect "all" DNaseI hypersensitive sites in the mouse genome? In this context it was noticed that the co-submitted manuscript claims that 95% of GR bound regions overlap with DHS sites detected in cells before hormone activation. This seems to disagree with the 85% claimed in the manuscript under review.

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Q2: The result of Figure 2C appears highly critical for the conclusion: the authors compared CpG density profiles for shared (between two cell lines) and unique "pre-programmed" DHSs and noticed an average 0.4 more CG within a hundred basepair. Is this subtle difference located within the GR binding site?

Importantly the authors compared two groups that are very different in size with one 50 times larger than the other. It seems likely that the differences between the shown average profiles could reflect the different sampling size as the smaller group is more noisy in general and thus the increase around the TSS could also be part of that noise. How much of the difference could be explained by the fact that the group of shared targets is much smaller and therefore more sensitive to outliers? This needs to be approached statistically by providing data that include variance (e.g. boxplots) and by performing appropriate statistical test that takes group sizes into account (e.g. some form of bootstrapping analysis).

Q3: The authors report difference in DNA methylation for the plus and minus DNA strand. However according the "methods" section they used normal bisulfite sequencing, so it seems unclear, how this would lead to strand specific methylation data

Q4: In Figure 6 (B,C,E,F): the authors show summary plots for bisulfite sequencing in three "de-novo" sites. As the differences in DNA methylation of single CpG dinucleotides are rather small, it is important to show also the error bars (C) and the methylation status of individual clones for the panels (B,E,F).

1st Revision - authors' response 25 March 2011

Response to Reviewers' Comments

REVIEWER #1:

1. Review MS EMBOJ-2010-76175 by Wiench et al In this manuscript, Wiench et al study the connection between DNA methylation and glucocorticoid receptor (GR) binding to DNA. This work builds upon a genome-wide study of chromatin accessibility and GR binding to DNA in two distinct glucocorticoid-responsive cell lines to be published in another journal. Using this data set, the authors observe a small enrichment of CpG density in the vicinity of GR binding elements (GRBEs) preferentially in some of the sites where chromatin is nuclease sensitive irrespective of glucocorticoid treatment (pre-programmed sites). Then, they study at high resolution DNA methylation at 17 selected sites within 13 target genes. They observe that CpGs in the vicinity of cell type-specific GRBEs are generally methylated in the cell-line where the GRBEs are inactive. They also observe transient changes of small amplitude in the methylation levels of a limited number of CpGs within three genes at sites where glucocorticoids induce increase nuclease sensitivity of the chromatin (de novo sites). They conclude that their "findings provide evidence for a unique link between tissue-specific chromatin accessibility, DNA methylation and transcription factor binding at distal enhancer elements". Although the genome-wide study of chromatin accessibility in relation to the cell type specificity of GR binding to DNA is highly interesting and complete, the present DNA methylation study is of more limited impact due to the small number of genes analyzed and to the lack of decisive demonstration of really novel properties. The study has interesting aspects that are not clearly and strongly demonstrated, and it is possible that stronger data would give higher significance to the manuscript.

1. The authors claims that they observe "a major enrichment in CpG density" (p6, line 10) when they observe a peak of CpGs density of 3.5 CpGs per 100 bp, "a significant increase" when the peak reaches 2 CpGs in 100 bp (Fig 1B) and the CpG enrichment they observe nearby GRBEs at pre-programmed sites is only 20% above background with peaks of 1.2 CpG per 100 bp (Fig. 1 C). These average values are far from being spectacular and the authors would have to demonstrate that such an increase in CpG density exert a regulatory role in a more convincing manner than by

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vague correlations. Furthermore, it is misleading to use average values because they are not informative of the distribution of CpGs among the various sites considered. Are the mean peaks resulting from a global but minor increase of the CpG density distributed equally over all sites, or is there a minor subpopulation of sites with a more striking enrichment? The second option is more likely, because the authors then study in details sites with CpG densities that are all higher than 1.2 and that go up to about 6 CpG per 100 bp. To analyze the distribution of CpG density, the authors could present box-plots showing this distribution in windows of a few hundred base pairs around the sites of interest to demonstrate the different behavior of these populations. It would be important to know in particular if the de novo and pre-programmed sites have really different behavior overall or if the pre-programmed sites just contain a subpopulation of CpG enriched sites that is absent from the de novo sites (Fig. 2A). The density of CpGs found within each site analyzed by bisulfite sequencing should be clearly represented on Fig 3-4 and Supp Fig S3-5 and the effect of density on the behavior should be analyzed better. The claims and the wording should not exaggerate artificially the differences observed and to avoid unsupported claims like "dramatic contrast", "overwhelmingly due", "major enrichment".

Response #1: As the reviewer suggested we have prepared box-plots of CpG distribution around DNaseI hypersensitive sites and GR-bound regions (Fig. S2B, S2E). In the latter case, we present the data for GR-bound pre-programmed DHSs since this is the only group enriched for CpGs (Fig. S2E). We would like to emphasize that the most CpG-rich sequences (CpG islands) were excluded from all our analyses. Thus high values for CpG enrichment should not be expected at sites of interest. However, we observe enrichment precisely around the center of the DHS hotspot or GR peak (± 1kb) when compared to 8 kb of the surrounding genomic background. This reflects the systematic trend in the data with statistical significance indicated in the box-plot graphs. In addition, a similar enrichment was observed in two different cell types despite the fact that the overlap between GR-bound regions is minimal. Although statistically significant, the enrichments that we describe are somewhat modest. We therefore have removed from the text all overstatements.

The reviewer also raises the possibility that the CpG enriched group might represent a sub-population within enhancers. We thank the reviewer for this insightful suggestion. To address it we sorted enhancers according to their CpG density and repeated the counting exercise in tertiles characterized by high, medium and low CpG density (see Fig 2D, E). We performed the analysis separately in pre-programmed and *de novo* sites. In both groups enhancers that are the most enriched in CpGs are also located within genomic regions characterized by high CpG density while enhancers with the lowest CpG content lie within the low CpG density regions. However, the CpG density of pre-programmed sites is always higher than the surrounding sequences and this characteristic is independent of CpG content of genomic location while *de novo* sites occur at sequences of low CpG density. Thus, *de novo* CpG content does not differ from the surrounding sequences if they are located within genomic regions of low or medium CpG density (Figure 2E). The localized decrease in CpG density within *de novo* sites is only observed when they are located within region of high CpG content. It suggests that the high CpG content at *de novo* sites could be an obstacle (especially when methylated) for rapid chromatin remodeling. We include the results in the main figure (Fig. 2D, E) and discuss them accordingly.

The density of CpGs within analyzed sites is now shown in Table S1, Fig. 3, Fig. S3 and S5 as recommended. We aimed to analyze DNA methylation at the sites within wide range of CpG density. The CpG content for analyzed pre-programmed and *de novo* sites is 0.8-4 (average 2 CpG/100 bp) and 0.7-2.3 (average 1.3 CpG/100 bp), respectively. Thus, a few sites were chosen with densities higher than indicated by the average values shown in Fig.1 and Fig. S2. The primer sets were further selected to analyze the biggest possible number of CpGs.

2. The authors use throughout the text the wording "regulatory CpGs" but they bring little demonstration that these methylated CpGs play a regulatory role. The only indication is an effect on GR binding to DNA which is not well-supported by experimental data (see point 3). Demonstration that methylation directly affects glucocorticoid response of some genes in cultured cells is missing. The correlation between repressive chromatin structures and methylation is well known but it has always been difficult to distinguish whether methylation plays a causal role in most of the situations or is a secondary consequence of repression involved in its long-term stabilization. This study does little to clarify the situation and the wording should be adapted to the lack of strong demonstration.

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Response #2: In the previous version of our manuscript we use the term "regulatory CpGs" solely to describe CpGs which are differentially methylated when an active enhancer is compared to its silent equivalent. We agree with the reviewers' concerns and now refer to them as "differentially methylated CpGs".

The reviewer raises also an important point about the primary/secondary role of DNA methylation in establishing repressive chromatin environments. Regulatory elements are complex chromatin structures where a certain equilibrium of features is established early in development by one set of factors and then maintained during differentiation by others (Xu et al, Proc Natl Acad Sci USA 104:12377-82, 2007; Xu et al, Genes Dev 23:2824-38, 2009). Given that chromatin structures are maintained by the combinatorial interactions of numerous histone modifications, transcription factors and other regulatory events, it is unlikely that elimination of a single feature such as DNA methylation would change the organization of these chromatin structures. In order to evaluate the impact of Dnmt1 on chromatin accessibility and GR binding, we have performed a series of experiments where global DNA methylation was decreased by siRNA against Dnmt1 (Fig. 4). Dnmt1 is the most abundant DNA methyltransferase in 3134 cells (data not shown) and we demonstrate that Dnmt1 knockdown indeed leads to a major decrease in methylation (Fig. S6). We also observe a moderate increase in chromatin accessibility following the decrease of DNA methylation. However, the increase in FAIRE signal is still lower than the signal detected when the site is accessible in AtT-20 cells (3-10 times). It suggests that other features besides DNA methylation contribute to the organization of active enhancers and reducing the level of DNA methylation alone is not sufficient to establish GR binding. The global changes of methylation patterns after Dnmt1 knockdown also make it challenging to infer the contribution of methylation at specific regulatory elements.

3. The gel shift data showing that methylation of a key cytosine present in a few GRBEs interfere with GR binding to DNA is not really convincing (Fig 5). The signal intensity of the DNA-protein complex is lower, but that of the unbound DNA as well. Thus, the loss of complex is not compensated by an increase in the amount of free DNA, which would be expected if CpG methylation was lowering GR affinity for DNA. This suggests an experimental artifact (i.e., lower amount of labeled probe, complex remaining in the top of the gel, ...). The experiment shown should be more convincing. If better results can be obtained, it would be helpful to discuss the results using a figure (or a supplementary figure) showing the effect of CpG methylation on GR binding using the numerous crystallographic data available (in particular with CpG containing GRBEs (Science 2009, 324, 407).

Response #3: To address the reviewer's concerns we have improved the gel shift by using radiolabeled ^{32}P-labeled Suox-GRE oligos. This method allowed for better and more reliable quantitation of free DNA and shifted complexes. We show the results in Fig. 5 and Fig. S8.

As suggested by the reviewer, Fig. S8A also includes the representation of the position of methylated cytosines based on crystallographic data (from Mejising et al, Science 342:407-410, 2009).

4. The hormone-dependent demethylation shown in Fig 6 is not performed using a quantitative enough assay. The variation in the methylation levels observed are either of small intensity and/or strikingly visible at a single time point. It is not described how many clones were sequenced to determine the percentage of methylation shown. It would be necessary to sequence a sufficient number of clones to validate with a statistical test that the percentages are significantly different at different time points. Furthermore, to ensure that the differences are not due to fluctuations and carry-over contamination that often pollute bisulfite sequencing data, it would be necessary to use either barcoded hairpin bisulfite (NAR 2004, 32:e135), or to show more narrowly spaced time points showing consistent behaviors. There are previously published studies showing short-term or long-term demethylation in response to steroid hormones, including glucocorticoids, that analyzed a significantly higher number of clones and/or time points and/or genes (PNAS 2006, 103: 11112 Nature 2008, 452, 45 & 112). The present study should at least achieve similar standards.

Response #4: We performed methylation-specific PCRs on an independent set of bisulfite-treated DNA samples. This set contains more narrowly spaced time points within the first hour of hormone treatment as well as additional time points after induction. The results are included in Fig. 6 (Suox) and Fig. S10 (Mef2b, Ptprg) and show that demethylation is initiated very early after hormone

addition, reaches a plateau and declines very slowly. These data confirm and extend our previous results obtained by bisulfite sequencing for Suox, Ptprg and Mef2b.

The number of clones per condition is now clearly indicated on Fig. 3 and Fig. S10; the separate graphs showing methylation status of individual clones are additionally presented in Fig. S9 and S11. We analyzed between 15-25 clones from each experimental condition which is consistent with previously established standards (Kangaspeska et al, Nature 452:112-115, 2008; Kim et al, Nature 461:1007-1012, 2009; Angrisano et al, Nucleic Acids Research, 2010). We thank the reviewer for spotting this omission.

REVIEWER #2:

1. In this report Wiench et al investigate cell type-specific chromatin accessibility and DNA methylation of glucorticoid receptor (GR) binding sites across the genome. Based on DNAseI hypersensitivity before and after hormone stimulation the authors distinguish two types of sites: (i)"pre-programmed" or pre-existing in cells prior to stimulation with hormone and GR binding and (ii) "de-novo" sites which become DNAseI hypersensitive upon stimulation with hormone and GR binding. The authors show that constitutive, i.e. "pre-programmed", promoter-distal DNAseI hypersensitive sites (DHS) are mildly enriched for CpG dinucleotides (2-fold compared to flanking sequences) and are generally hypomethylated in a cell type-specific manner. In contrast, "de novo" DHS sites are depleted of CpGs and show partial DNA methylation. Limited demethylation of methylated CpGs was observed upon GR binding within the inducible ("de novo") DHS. The main message of the manuscript, as also outlined by the title, is that lack of DNA methylation at constitutive DHS defines active cell type-specific enhancers.

For the most part the data presented in manuscript is descriptive. The two novel and interesting observations are that methylated CpGs within degenerate GR response elements can partially inhibit GR binding and that upon hormone stimulation and GR binding there is rapid, but incomplete, demethylation of CpGs close to the GRE. The authors argue that such demethylation must be active as it occurs within minutes after hormone stimulation. However, it seems that the peak of hypomethylation observed at and near the Suox-GRE is at 8h after hormone treatment. Thus passive demethylation during DNA replication is also possible and could be mediated by a competition for binding between GR and Np95/UHRF1 or DNMT1 at such sites. The authors should examine DNA methylation around Mef2b and Ptprg GREs at 8h time point as well. Does demethylation take place in the presence of aphidicoline or other drugs that inhibit DNA replication? In Figure 6B, E and F, it is unclear how many sequences were investigated per time point to determine the average DNA methylation of each CpG.

Response #1: We have extended the analysis of DNA methylation changes within CpGs of interest for Suox, Ptprg and Mef2b GREs using methylation-specific PCR (see also response #4 to Reviewer #1). These results show that a decrease in methylation occurs early after induction: 5 min for Suox-GRE, 10 min for Mef2b-GRE and 30 min for Ptprg-GRE (Fig. 6, Fig. S10). This is consistent with the rapid transcriptional induction of GR regulated genes. We have consequently focused our analysis on early events which are unlikely to be affected by DNA replication. Regardless, we confirm that the kinetics of DNA demethylation are unaffected by aphidicolin treatment (Fig. 6D). The number of clones sequenced per time point is now indicated in relevant figures.

I do agree that there is a good correlation between lack of DNA methylation and the presence of hormone-independent promoter-distant DHS. Given that these sites are constitutive, but tissue-specific, it is possible that they are determined by binding of other factors that destabilize nucleosomes, generate permissive chromatin and inhibit DNA methylation. Thus partial or complete lack of DNA methylation is a feature of, but not a pre-requisite for GR binding at "pre-programmed" DHS. Thus, the major question that remains unanswered is what determines the existence of "pre-programmed" tissue-specific sites. The authors could test by ChIP whether factors such as Cfp1 and MLL1, which have been implicated in maintenance of unmethylated state at CpG islands, are bound to "pre-programmed", but not "de novo" DHS.

Response #2: We agree with the reviewer that hypersensitive sites can be described and maintained by an array of factors, with DNA methylation being one feature (see response #2 to Reviewer #1). The demethylated state is reflective of hypersensitive sites and hypersensitive sites, as a chromatin

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state, is a pre-requisite for GR binding (John et. al., Nat. Genet, 2011). We have now stated this point in the manuscript.

How hypersensitive sites are formed and maintained is likely a consequence of the genome organization formed early in development mediated by a combination of factors which are unlikely to be involved in the regulation of all DHS. We would argue that this problem is beyond the scope of the current manuscript.

The appearance of "de novo" sites is also poorly defined. In figure 5 the authors show that in the majority of cases DNA methylation does not inhibit GR binding. Therefore, it is unclear how "de novo" sites are chosen. The authors speculate that the appearance of "de novo" sites might be potentiated by incomplete DNA methylation. Thus they say in the discussion that "Demethylation of a limited number of CpGs could potentially destabilize a nucleosome, further exposing a response element for protein-DNA interactions." However, it seems unlikely that the presence of GRE and the amount of DNA methylation are sufficient to determine "de novo" sites as the sequence of the non-functional DHS in 3134 cells (Figure S3C) and the inducible DHS at Ptprg and Arrdc2 genes in 3134 (Figure S5) show similar levels of DNA methylation. Moreover, the two statements in the abstract (i) "De novo sites thus identify a novel class of enhancer elements that are devoid of the strong suppressive effect of methylated cytosines." and(ii) "Furthermore, treatment with glucocorticoids induces rapid changes in methylation levels at selected CpGs within de novo elements. These modifications, in turn, can impact GR binding efficiency by direct and indirect mechanisms." seem to contradict each other.

Response #3: Chromatin accessibility varies as a continuous function and observed DNaseI sensitive regions exhibit a wide dynamic range in total tag counts between the weakest and strongest sites. We have, therefore, sequenced both hormone-naïve and dexamethasone-treated DNaseI samples to great depth of ~101 million uniquely mapping reads per condition, and computed sites of significantly elevated DHSs. Given the dense sequencing for DHS samples, *de novo* DHS are referred to as GR binding within inaccessible chromatin that triggers chromatin remodeling only after the addition of hormone(‘re programmed’ sites). Mean change in chromatin accessibility at *de novo* sites was significantly greater than at *p*re programmed sites. The results presented in Fig. 2 suggest that the *de novo* and pre-programmed sites indeed demonstrate different properties, at least in terms of CpG content.
We observe the presence of partial methylation at the *de novo* elements more frequently that at the pre-programmed sites. At this point we can only hypothesize that it might reflect the occurrence of unstable nucleosomes or the binding of unidentified proteins that function as priming factors. It might further reflect the stochastic differences in populations of responsive cells.

We have clarified the apparent contradiction between CpG content and methylation of *de novo* elements. The requirement of *de novo* sites for low CpG density is clearly shown in Fig. 2E. We describe it as a lack of critical mass of CpG dinucleotides, which, when methylated, could attract repressory complexes and prevent dynamic changes during remodeling of the site. However, even if there are fewer CpGs at these sites compared to the surrounding sequences they are still present and can be modified.

On page 5 (and the abstract) the authors say "The de novo DNAseI hypersensitive sites are surrounded by CpG-depleted sequences". This statement is not very specific. From Figure 1 and S1 it is clear that all DHS are "surrounded by" CpG-depleted sequences (-5000 to -1000 and +1000 to +5000). Probably the authors meant to say as on page 8 "de novo sites are not enriched in CpGs". The graph in Figure 2A seems to show that they are depleted of CpGs compared to flanking sequences.

Response #4: We have added new analysis where CpG distribution within and around the *de novo* and pre-programmed sites is evaluated as a function of CpG enrichment. The data are included in Fig. 2D, E and are discussed in detail in Response #1 for Reviewer #1. Briefly, the results suggest that *de novo* sites show the requirement for low CpG content and they are indeed depleted of CpGs compared to flanking sequences but only when the site is located within genomic region of high CpG density. As suggested, we have included a detailed explanation to clarify this observation.

In summary, I am not convinced from the data presented in the manuscript that the authors have answered satisfactory the question (formulated in the introduction) of whether DNA methylation has

a cell type-specific impact on accessibility of enhancers to GR binding. Further experiments would be necessary to demonstrate that this is the case. For example, the authors could reduce DNA methylation in 3134 cells by stable shRNA knockdown of DNMT1 and ask whether DNAseI hypersensitivity and GR binding can be observed in the presence of Dex at DHS5 and other AtT20-specific DNAseI hypersensitive sites that are normally absent in 3134 cells.

Response #5: As the reviewer has suggested, we have performed the Dnmt1 knockdown in 3134 cells (see Response #2 to Reviewer #1). Subsequent to the knockdown, we have analyzed the degree of demethylation, chromatin accessibility and GR binding to hypersensitive sites specific for AtT-20 and to the partially methylated *de novo* sites. The results obtained in three independent experiments are discussed in the text and shown on Fig. 4. Briefly, the data suggest that the global demethylation leads to a moderate increase in chromatin accessibility which is not observed for the control unmethylated regions (CpG island, fully demethylated pre-programmed site). However, the degree of the accessibility is below that observed at fully established hypersensitive sites and is not sufficient to facilitate GR binding.

REVIEWER #3:

Wiench and colleagues report a genome-wide analysis of glucocorticoid receptor (GR) binding in the context of chromatin accessibility and DNA methylation in two cancer-derived mouse cell lines.

The authors showed that most of the GR bound regions do not overlap with CpG islands nor with promoter regions.

Based on the overlap of GR bound regions with DNaseI hypersensitivity sites (DHS) detected in naive cells (before hormone treatment) they define two classes of GR bound regions:
i) "pre-programmed" DHSs: GR bound region is overlapping with DHS detected in cells before hormone treatment == constitutively open chromatin (85% of GR bound regions)
ii) "de-novo" DHSs: GR bound region is not overlapping with DHS detected in cells before hormone treatment (15% of GR bound regions)

Next, the authors studied the properties of the GR bound regions, namely the CpG content and methylation level of bound sequences. They found that "pre-programmed" class regions were enriched for CpGs, in contrast to "de-novo" class regions, which were mostly found in the CpG depleted regions. The authors speculate that the enrichment for CpGs may suggest the role of DNA methylation in enhancers.

By bisulfite sequencing they analyzed DNA methylation status of 17 regions. For the "pre-programmed" regions they found correlation between demethylation status and accessibility of DNA sequence. They also investigated the impact of methylated cytosine on GR binding. By using the EMSA assay they showed that CpG methylation could affect binding of GR but only if the methylated CpG was present directly in glucocorticoid response element.

Next they showed a detailed analysis of DNA methylation at one GR bound locus of "de-novo" class, in proximity of the Suox gene. They described rapid demethylation process initiated 10 minutes after the hormone stimulation and speculate that this observation supports the idea of active demethylation. Further they showed that prolonged hormone treatment (12 hours) resulted in remethylation of CpGs, which according to the work by John et al. 2009, corresponds to decrease of transcriptional activity of the Suox gene. Here the authors do not observe any changes in chromatin marks (acetylation of H3, H3K4me3, H3K9me1).

The observed speed of demethylation makes an active process likely, could one strengthen this point by blocking the cell cycle and thus the source of passive demethylation?

Response #1: Most transcription events related to GR-mediated regulation, especially chromatin remodeling take place within the first 30 minutes after stimulation with hormone and are unaffected by cell cycle. However, later events could be influenced by replication. As the reviewer has suggested, we have analyzed DNA methylation changes in the presence of the replication blocker, aphidicolin: 3134 cells were treated with 1µM aphidicolin for 24h before hormone induction. This

condition resulted in good cell viability with the cell cycle block confirmed by FACS. We observe that the kinetics of DNA demethylation remains mostly unchanged, with a decrease in methylation observed soon after hormone treatment followed by partial re-methylation 12hrs later. This result is shown in Fig. 6D.

While this manuscript addresses an important problem it is unclear if it indeed shows a specific function for DNA methylation in regulating ER activity. The observation that GR sites have a higher frequency of CpG dinucleotides seems at this point insufficiently justified and even though GR seems sensitive to methylation in vitro it is unclear if this is the case in vivo.

Response #2: We believe that the higher frequency of CpG dinucleotides at preprogrammed sites is well supported and statistically significant (See Fig. 1,2, Suppl. Fig.2 and Response #1 to Reviewer #1). Our results are also clear regarding the observation that DNA hypomethylation is a hallmark of cell type-specific regions of accessible chromatin. However, how a transcription factor binds *in vivo* is dictated by the complexity of the chromatin environment and regulatory factors that influence it. Thus, affecting one factor might not be sufficient to influence GR binding, as observed in our Dnmt1 depletion experiments. In addition, Dnmt1 depletion affects DNA methylation globally, making it challenging to study the contribution of DNA methylation at the site-specific level.

Other questions:

Q1: The ratio of "pre-programmed" vs. "de-novo" DHS sites could depend on the sequencing depth in the DNaseI hypersensitivity assay. Lower read counts might lead to an underestimation of the number of GR bound regions overlapping with "pre-programmed" DHS sites. Was the sequencing depth sufficient to detect "all" DNaseI hypersensitive sites in the mouse genome? In this context it was noticed that the co-submitted manuscript claims that 95% of GR bound regions overlap with DHS sites detected in cells before hormone activation. This seems to disagree with the 85% claimed in the manuscript under review.

Response #3: This point has been addressed in response to Reviewer#2 (Response#3). The depth of the sequencing employed in these studies (101 million uniquely mapping tags) is typically 8-10x the read depth of most published papers (compare with Boyle et al Cell 132:311-22, 2008). At a sequencing depth of over 100 million reads, we find that ~85% (actually 88.3%; this correction has been made to the Introduction) of GR peaks fall within baseline accessible chromatin. At the same read depth, in hormone-treated cells, greater than 95% of GR occupancy sites are localized within accessible chromatin. The 95% overlap between GR binding and pre-programmed DHS sites, mentioned in the abstract of the co-submitted paper refers to the AtT-20 cells, in which this component is indeed higher. We would also refer the reviewer to Fig S4 of our recently published paper (Nature Genetics, Volume 43 No 3 pp264-268) which shows the fraction of the genome in accessible chromatin as a function of sequencing depth.

Q2: The result of Figure 2C appears highly critical for the conclusion: the authors compared CpG density profiles for shared (between two cell lines) and unique "pre-programmed" DHSs and noticed an average 0.4 more CG within a hundred basepair. Is this subtle difference located within the GR binding site?

Importantly the authors compared two groups that are very different in size with one 50 times larger than the other. It seems likely that the differences between the shown average profiles could reflect the different sampling size as the smaller group is more noisy in general and thus the increase around the TSS could also be part of that noise. How much of the difference could be explained by the fact that the group of shared targets is much smaller and therefore more sensitive to outliers? This needs to be approached statistically by providing data that include variance (e.g. boxplots) and by performing appropriate statistical test that takes group sizes into account (e.g. some form of bootstrapping analysis).

Response #4: We addressed the concern of different sampling size by applying a bootstrapping analysis: 140 hotsposts from the group of unique DHSs were chosen randomly and average CpG counts were calculated repeatedly 100 times (new graph in Fig. 2C includes this analysis). It confirms the statistical significance of the subtle difference between shared and unique pre-programmed sites. Additional analysis shows that this difference is located rather outside of the

GRE element, while the sites with GRE element containing CpG show similar enrichment in both shared and unique DHSs (Fig. S2F).

Q3: The authors report difference in DNA methylation for the plus and minus DNA strand. However according the "methods" section they used normal bisulfite sequencing, so it seems unclear, how this would lead to strand specific methylation data

Response #5:
DNA treatment with sodium bisulfite leads to the conversion of unmethylated cytosines to uracils on each strand. As a result the final products for plus and minus strands are not complementary, thus the subsequent analyses are strand specific. The results presented in Fig. 6B were obtained using primers specific for either strand after bisulfite conversion.

Q4: In Figure 6 (B,C,E,F): the authors show summary plots for bisulfite sequencing in three "de-novo" sites. As the differences in DNA methylation of single CpG dinucleotides are rather small, it is important to show also the error bars (C) and the methylation status of individual clones for the panels (B,E,F).

Response #6: As suggested, we have included supplementary figures presenting the methylation status of individual clones for Suox (Supplementary Fig. S9), as well as for Mef2b and Ptprg (Supplementary Fig. S11). The summary plots are created based on the percentage of methylated cytosines calculated from sequencing of 15-25 individual clones. This is a standard method of bisulfite sequencing data presentation, for which error bars are not applicable.
We agree with the reviewer that incomplete demethylation (difference of about 20-30%) detected after hormone stimulation should be discussed more comprehensively. We would like to emphasize that small intensity changes in methylation levels are characteristic also for previously published data when active demethylation was observed after stimulation. One of the first papers describing the activity-dependent demethylation at the Bdnf gene in neurons reported a 20-40% decrease (Martinowich et al, Science 302:890-893). The change with the amplitude of ~35% was shown both at the promoter of pS2 gene (Kangaspeska et al, Nature 452:112-115, 2009) and at the CYP27B1 promoter (Kim et al, Nature 461:1007-1012, 2009). Changes of similar magnitude have been reported in additional studies (Angrisano et al, Nucleic Acids Research, 2010; Kress et al, PNAS 103:11112-11117, 2006)

2nd Editorial Decision 28 April 2011

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by the three original referees whose comments are shown below. The complicating issue here is that I have just this moment received the final report and I am on my way out of the office for the next week because we are going on vacation. As you will see two of the referees still have a number of concerns regarding the study and the decision will not be straightforward. I currently do not have the time required to go through the manuscript and reports in detail and make the correct informed decision, and therefore I will have to do so when I return on the 7th May. To make the best use of the time I propose that if possible you look through these remaining concerns and provide comments to these I can take them into consideration when I get back and make a decision on the manuscript. THis is unconventional but hopefully we can make efficient use of the time.

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Review MS EMBOJ-2010-76175R by Wiench et al

In this revised version of their manuscript, Wiench et al have made a clear effort addressing most of the criticisms and concerns raised by the reviewers. I am satisfied by most of their modifications, the complementary data they provide, and the rephrasing of the conclusions that address most but one of my concerns. I am, however, not satisfied by the DNA demethylation data that I found unconvincing now that the authors provide the numbers that were missing in the previous version. I believe that this specific analysis requires additional data to be convincing.

The complementary analyses render convincing the study of the slightly increased CpG density in the DNAse I HS and GR binding regions and the association of the methylation of these CpGs with the cell-type specific inactivity of these regulatory regions.

The revised in vitro data showing that methylation of a specific CpG within a GRBE decrease the affinity of GR for this GRBE is also convincing. This in vitro effect does not appear sufficient to interfere with GR binding in vivo as shown in Fig. 4B, thus the in vivo significance of this effect of DNA methylation on the activation of a subset of GR target genes remains to be established. These aspects were not discussed in the paper, and I believe that they should be.

The GR-triggered DNA methylation events observed on three regulatory elements are of low amplitude and because of that, they rely on too few measurements and on too low-resolution methods to be demonstrative. Furthermore, the novelty of this observation is questionable, since there is a plentiful of NR-triggered DNA demethylation events that have been described since the pioneer studies of JP Jost in 1982 (PNAS 79: 4252). The demethylation events reported here remain at the descriptive level as in 1982. In addition, the paper does not provide much novel functional and/or mechanistic insights to better understand the demethylation phenomenon.

The authors use 2 different methods to assess demethylation after bisulfite conversion of the DNA. First, they use cloning and sequencing of about 20 clones per time point and strand of regulatory regions (these values were not indicated in the previous version). They observe a lower proportion of clones corresponding to a specific methylated cytosine at one time point, and for one gene analyzed on two strands, at more than one time point on only one of the strand. The problem is that the number of clones analyzed and of clones corresponding to demethylated cytosines are most of the time too low to provide statistically significant differences. The statistical significance of the difference between the untreated and the hormone-treated samples can be evaluated using a Fisher's exact test. The p-values for the Suox -strand are 0.523 after a 10 min GC treatment, 0.111 after 20 min, and becomes significantly different from the untreated control only after 8h (0.034) and 12 h (0.041). On the + strand, the p-value is 0.022 for the 20 min GC treatment and no demethylation is visible at 8h. Thus, for Suox, there is a major difference in the kinetic of demethylation and remethylation of each strand at a single CpG when relying on only the statistically significant data. This would be an interesting observation because it is not simple to envision a mechanism achieving so different kinetics on opposing strands for a single CpG. The authors need, however, to validate this observation better, i.e., they should first demonstrate that the observation is not due to a

common artifact observed when using PCR amplification of bisulfite-converted DNA: carryover contamination by products from previous experiments. As I mentioned in my first review, the way to demonstrate with reliability the authenticity of their data is to use barcoded hairpin bisulfite (NAR 2004, 32:e135). If they perform the study on both strands with narrow time points and a sufficient number of samples sequenced they could also achieve some reliability, but not as efficiently as with the barcoded hairpin method. The Fisher's exact test can give them a feeling of how many clones they should sequence to demonstrate reliably that the demethylation is significant if the proportion of demethylated Cs is exactly as observed here: about 250 for both the control and the 10 min time point and only about 45 for the 20 min time point on the - strand. For the Mef 2b and the Ptprg CpGs that are believed to be demethylated, the p-values are 0.347, and 0.161 resp., and three times, or two times more clones for both the control and GC treated samples should be sequenced to achieve statistical significance with identical proportions. To minimize the risk of data falsification by carry-over contamination, data should be at least replicated.

The second method used in this revised version to analyze demethylation is MS-PCR using a primer which 3'most base is complementary to the T that is characteristic of bisulfite-converted demethylated C. The method is not described in the material and method section, so it is not possible to assess whether it was performed in a reliable manner. The legends to figure does not indicate which C and which strand were analyzed. Furthermore, the data presented are hardly convincing because the amplitude of the effect is low and because a large part of the effect is apparently due to normalization by a fluctuating control. To obtain reliable data with this approach it is essential to use real-time Q-PCR, primers that have a LNA at the 3'-end to ensure efficient discrimination between priming on matched and mismatched template, and to demonstrate with known varying proportions of templates corresponding to methylated and unmethylated Cs that the assay is linear in the corresponding range for every target region analyzed (the performance of the assay is highly variable from sequence to sequence). As it is now, this assay brings little value to the data and the authors would be better off sequencing more clones and using barcoded hairpin-bisulfite than optimizing this method.

Minor corrections:

1 It is not specified in the text (page 10, bottom) and figure legends how chromatin accessibility was assessed in Fig 4A. Presumably, it is by FAIRE but this should be indicated.

2 On page 14, line 4, the text between parentheses (indicated by arrows in Sup Fig 10) is presumably at the wrong place and seems to correspond to the line above: "partial demethylation of a single CpG". It is clearly not corresponding to the dex treatment.

Referee #2 (Remarks to the Author):

The manuscript by Wiench and colleagues is greatly improved by the addition of new data and changes in text and figures. My previous criticisms were addressed in full. I have no further comments.

Referee #3 (Remarks to the Author):

Since the first submission and the revised version of this MS the genome-wide analysis of GR binding sites has been published in Nature Genetics (John et al.), which has answered some of the questions regarding GR binding data.

The study under review starts from these binding data and the observation that preexisting DH and those formed upon hormone treatment show subtle differences in CpG density.

The main observation is of a slightly increased CpG content at GR binding sites while experimental evidence for a functional role of these CpGs and/or their DNA methylation states remains weak.

The revised manuscript provides additional experimental and analytical data but the main problem remains in that there appears no solid basis to conclude a general function for DNA methylation in regulating GR binding. There is only one GR binding site studied that shows DNA methylation response in vitro and it is not even attempted to connect this singular finding in vitro to the global dataset.

The observation of subtle yet fast demethylation is of interest as it supports to be an active process, which could be linked to transcription. While the kinetics are now described in more detail and more convincingly there is little follow-up experiments on the actual process. Moreover these sites are not those that show higher CpG density.

While it is in general interesting if DNA methylation could be predictive of enhancer activity or TF binding (regardless of a functional relevance) such conclusion should be based on a small and selected selected set of elements.

Points in detail :

The authors identify an increase in CpG density at GR bound regions when compared to surrounding sequences. Especially if GR peaks identified in both cell lines are considered. However the GR is not bound to typical CpG islands. They showed a similar increase for Stat3 and Oct4 using the data from Chen et al 2008.

They divided GR peaks into two classes:

a) pre-programmed (these region are sensitive to DNAse I in naïve cells)

Only for those they saw an increase in CpG density in the GR peaks compared to surrounding sequences. They split the peaks into three groups based on the CpG density in surrounding sequences and the increase in CpG density is in all of them.

b) de novo (these regions sensitive to DNAse I only after hormone treatment)

They did not see an increase in CpG density at GR bound region. When they split them into three groups they even saw the depletion of CpG for the group with highest CpG density.

Based on different CpG properties of the pre-programmed and de novo classes they speculated that DNA methylation could play a role in formation of these two different chromatin entities and that DNAseI hypersensitive sites induced by the hormone treatment could be special class of enhancers.

They measured DNA methylation levels for 17 selected regions in untreated cells.

For pre-programmed regions they measured DNA methylation over the entire bound regions. Methylation levels are bimodal (either very low <20% or very high >80%) and correlate with accessibility in a given cell type. (2 examples of shared regions and 4 cell type specific). On top of that they showed two regions, which were accessible in naïve cells and their methylation were low, however GR was not bound to them after hormone activation. They concluded that the correlation

between demethylation and accessibility seems to be a general feature of pre-programmed chromatin at enhancer elements. If validated genome-wide this would indeed be an important finding.

They also investigated the DNA methylation levels at de novo sites but in these cases they measured the DNA methylation levels only at single CpGs. Figures 3D, 3E and S5. For all of them, the methylation pattern is not that homogeneous as for the pre-programmed sites. In comparison between two cell types, they usually observed difference in methylation only for one CpG (in some cases this CpG is part of degenerated GRE motif, in other lies in the proximity of the GRE). This partial methylation varies between different sites from 40 to 80% (except Sgk1 R1 in figure 3E where there is 0% methylation for de novo site). They speculated that this partial methylation could mark these sites as closed chromatin poised for remodelling.

page 10: ... these results indicate the strong link between DNA methylation and cell type specific usage of enhancers elements. It is unclear if this statement is based on pre-programmed sites (there is solid evidence for this) or on both types of GR sites (for de novo sites the evidence is not that clear).

The knock-down experiment of Dnmt1 it does not support a regulatory role for DNA methylation.

Direct influence of DNA methylation on GR binding was only observed if a CpG locates within the GRE (which does not contain a CpG). There are ~600 degenerated GREs containing CpG. They saw a decrease of GR binding in vitro when methylated CpG was at a critical part of the motif but the binding was unaffected when the methylated CpG was in the spacer or in the proximity of GRE.

Active demethylation : The authors also performed time course experiment analysing the DNA methylation levels before and after induction of GR. Figure 6A is showing CpG overlapping the GRE in SUOX-GRE which is fully methylated in AtT-cells but only partially methylated in NH-3134 cells (50%). After induction the methylation goes down to 24% (20 mins) and later to 13% (8 hours) or 16% (12hours). However for the same region, just the other strand, they saw demethylation of another CpG from 22 to 0% and partial remethylation after 12 hours to 22% (6B) which they claimed corresponded to decrease in Suox expression after 12 hours (John 2009). However this is not true for the other strand of the same region, their the CpG stayed demethylated. Maybe this could be just explained by the different activation (Dex for minus strand and Cst fro the plus strand) but it does not make the data more understandable. For two other cases, shown in summary figure S10 (clones shown in S11), they saw decrease in methylation from 77 to 55% after 20 minutes followed by remethylation to76% after 8 hours and for last site the change is from 65 to 44 and back to 65%. No changes are observed in chromatin marks (fig 6E). It still is rather difficult to understand this data. There appears a consistent decrease ~20-30% but the absolute levels are very different and there is no clear difference between on and off state in general. Such forms of demethylation have been observed for ER before but also in cancer cell lines, which display overall disturbed patterns of DNA methylation

Additional correspondence (response to referees) 09 May 2011

We have carefully read the reviewers' comments regarding our revised manuscript "***DNA methylation status predicts cell type-specific enhancer activity***". All reviewers agree that the current version has improved considerably and that we were able to effectively answer their concerns by providing a large number of new experiments and analyses. Reviewer #2 is fully satisfied, Reviewer #1 mentions that we "***have made a clear effort addressing most of the criticisms and concerns raised***" and is "***satisfied by most of their modifications, the complementary data they provide, and the rephrasing of the conclusions that address most but***

one of my concerns" while Reviewer #3 highlights our demethylation data as detailed, convincing and of "***interest as it supports an active process which could be linked to transcription***." However, Reviewers 1 and 3 raise additional concerns regarding the study. They cite two issues: (i) the reliability and statistical significance of DNA demethylation analysis (Reviewer #1) and (ii) the lack of demonstration of a clear link between DNA methylation and regulatory process such as transcription factor binding and gene expression. We suggest that our data regarding DNA demethylation is consistent with standards established by previously published studies (see accompanying Table and attached response). Our data also establishes a strong co-relation between the demethylated state and accessible chromatin; however, obtaining the definitive result that reveals "***a general function for DNA methylation***" in regulatory processes such as transcription factor binding and gene expression is an extremely high benchmark that is beyond the current state of the field's expertise and beyond the scope of this manuscript (see attached response).

Most importantly, these two concerns do not impede with the main conclusions of our study. In this manuscript we draw attention of the research community to the status of DNA methylation at distal regulatory elements. To the best of our knowledge this issue has never been presented in a systematic and comprehensive manner. The studies published to date have focused on isolated cases and model system genes and have, therefore, ignored the common and widespread nature of methylation-related enhancer activity. DNA methylation is usually not considered when enhancers are described and our analysis fills an important gap. Significantly, we present a strong link between chromatin accessibility and DNA methylation at regulatory elements and have further confirmed the dependence of open chromatin regions on DNA methylation by depletion of the maintenance methyltransferase, Dnmt1. Notably, we compartmentalize enhancers into two groups based on the accessibility status of chromatin, designated as pre-programmed and *de novo*. The demonstration of transcription factor binding to pre-programmed and *de novo* chromatin states is a novel and developing concept. The current study shows for the first time that these two groups are distinguished a differential distribution of CpG dinucleotides and DNA methylation patterns. The reviewers do not pose any concerns regarding the main conclusions of our paper. Overall, Reviewer #1 had concerns regarding the active demethylation we observed after hormone stimulation (Figure 6). We do not present active demethylation as a novel observation (as suggested by the reviewer). Indeed, active demethylation is a previously described phenomenon. Instead, we propose dynamic changes of DNA methylation as one of the unique features of *de novo* elements. An important extension of our data is that the kinetics of changes in methylation correlates strongly with the dynamics of chromatin accessibility and the transcriptional response. Thus, we do not intend, or believe, that our study requires us to scrutinize the mechanisms of the demethylation process. Additionally, we note that some of the reviewers' 'major' objections are not entirely justified. We list bullet-points highlighting our arguments below and discuss them on a point-by-point basis in the accompanying response.

• 1. The extent of bisulfite sequencing analysis meets the standards of recently published high-profile studies
• 2. The amplitude of methylation changes is comparable to previously published examples of hormone-triggered demethylation
• 3. Strand-specificity is a known and described phenomenon
• 4. The suggestion of the requirement for barcoded hairpin bisulfite PCR is unjustified
• 5. The robustness of the demethylation experiments is validated by the good reproducibility observed in four independent experiments
• 6. The mechanistic insights requested are beyond the limits of current technology and the scope of the manuscript

We would also like to underscore that the same experiment (dynamic DNA demethylation, Figure 6) objected to by Reviewer #1 was highlighted by Reviewer #3 as an experiment of interest.

Additionally, we point out that although our conclusions are derived using the ligandactivated glucocorticoid receptor, it has become readily apparent that chromatin accessibility is a major pre-determinant of transcription factor binding. Thus, the data we present in the current manuscript is not limited to GR or nuclear hormone receptors, but can be generally applicable to

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all transcription factors. We, therefore, feel that the conclusions reached in our manuscript are of general significance and would be of interest to a wide audience.

Referee #1:

In this revised version of their manuscript, Wiench et al have made a clear effort addressing most of the criticisms and concerns raised by the reviewers. I am satisfied by most of their modifications, the complementary data they provide, and the rephrasing of the conclusions that address most but one of my concerns. I am, however, not satisfied by the DNA demethylation data that I found unconvincing now that the authors provide the numbers that were missing in the previous version. I believe that this specific analysis requires additional data to be convincing.

The complementary analyses render convincing the study of the slightly increased CpG density in the DNAse I HS and GR binding regions and the association of the methylation of these CpGs with the cell-type specific inactivity of these regulatory regions.
The revised in vitro data showing that methylation of a specific CpG within a GRBE decrease the affinity of GR for this GRBE is also convincing. This in vitro effect does not appear sufficient to interfere with GR binding in vivo as shown in Fig. 4B, thus the in vivo significance of this effect of DNA methylation on the activation of a subset of GR target genes remains to be established. These aspects were not discussed in the paper, and I believe that they should be.

Response:
The potential impact of DNA methylation on gene regulation *in vivo* has already been discussed in the Introduction (p6), Results (p11) and Discussion (p15) sections of our manuscript.

The GR-triggered DNA methylation events observed on three regulatory elements are of low amplitude and because of that, they rely on too few measurements and on too low-resolution methods to be demonstrative. Furthermore, the novelty of this observation is questionable, since there is a plentiful of NR-triggered DNA demethylation events that have been described since the pioneer studies of JP Jost in 1982 (PNAS 79: 4252). The demethylation events reported here remain at the descriptive level as in 1982. In addition, the paper does not provide much novel functional and/or mechanistic insights to better understand the demethylation phenomenon.

Response:
We suggest that the number of measurements made and the amplitude of changes observed in our demethylation experiments are similar to standards established in previously published data sets in high-profile papers. We provide a Table where we show that similar parameters were used in our studies when compared with related studies describing stimulus-triggered DNA demethylation.
We would like to reaffirm to the reviewer that the demethylation experiments were used, not to describe a novel event, but to describe a unique feature found only in *de novo* accessible chromatin sites. Furthermore, we provide evidence for an association between DNA demethylation, accessible chromatin and hormone-dependent gene expression (p13 of the manuscript). However, providing mechanistic insights into the dynamics of the demethylation process would be a digression from the primary focus of the paper.

The authors use 2 different methods to assess demethylation after bisulfite conversion of the DNA. First, they use cloning and sequencing of about 20 clones per time point and strand of regulatory regions (these values were not indicated in the previous version).

Response:
We have previously provided the average number of clones sequenced in the Material and Methods section.

They observe a lower proportion of clones corresponding to a specific methylated cytosine at one time point, and for one gene analyzed on two strands, at more than one time point on only one of the strand. The problem is that the number of clones analyzed and of clones

corresponding to demethylated cytosines are most of the time too low to provide statistically significant differences. The statistical significance of the difference between the untreated and the hormone-treated samples can be evaluated using a Fisher's exact test. The p-values for the Suox -strand are 0.523 after a 10 min GC treatment, 0.111 after 20 min, and becomes significantly different from the untreated control only after 8h (0.034) and 12 h (0. 041).

Response:

1. Standards employed in bisulfite sequencing analysis

Bisulfite sequencing is the gold standard used in studies that monitor DNA methylation (and changes in DNA methylation). These results can be further confirmed using other methods like MeDIP-PCR, MS-PCR, MS-SNuPE or restriction analysis coupled with PCR or q-PCR.

Using bisulfite sequencing we have analyzed on average 20 clones from each experimental condition. The comparison of our studies with published data is shown in Table 1. The table specifies the methodology used to detect DNA methylation, the number of clones sequenced, the amplitude of DNA demethylation, the number of time points and the number of regions analyzed. This compilation demonstrates that the parameters used in our study are entirely consistent with published reports. The estimation of statistical significance from bisulfite sequencing data is uncommon because of the cost and labour involved in sequencing an unlimited number of clones. Only Kim et al (Nature, 2009) provided statistical error for bisulfite sequencing data, based on numerous repeats of the ***same*** experiment. Given the numerous regions queried under different treatment conditions and at multiple time points, we feel that the standards used in our bisulfite analysis meet the requirements used in related studies.

2. Changes in demethylation (amplitude)

We would like to stress that small changes in methylation levels are entirely characteristic and consistent with previously published data where DNA demethylation has been observed after a stimulatory signal. One of the earliest papers describing conditional demethylation (at the Bdnf gene in neurons) reported a 20-40% decrease (Martinowich et al, Science 302:890). Similar changes were observed for both the CYP27B1 promoter (Kim et al, Nature 461:1007, 2009) and the RET gene enhancer (Angrisano et al, Nucleic Acids Research, 2010). The biggest signal-dependent change in DNA methylation has been observed at the estrogen responsive pS2 promoter (30-70% demethylation). This effect, however, was only observed after an α-amanitin block and release (Metivier et al, Nature 452:45, 2008). Thus, the changes in DNA methylation observed in our study (25-37%) seem to be typical of demonstrated changes observed in related studies.

On the + strand, the p-value is 0.022 for the 20 min GC treatment and no demethylation is visible at 8h. Thus, for Suox, there is a major difference in the kinetic of demethylation and remethylation of each strand at a single CpG when relying on only the statistically significant data. This would be an interesting observation because it is not simple to envision a mechanism achieving so different kinetics on opposing strands for a single CpG.

Response:

3. Strand-specificity of DNA methylation

Bisulfite sequencing analysis has revealed some differences in the extent and kinetics of demethylation between plus and minus strands. However, the differences are not major and what is observed is a delay in demethylation kinetics on one strand with a similar trend observed on both strands. It would be unreasonable to expect identical results from both plus and minus strands as strand-specific differences are rather common and have been previously observed: "*Periodic, strand-specific methylation/demethylation occurs during transcriptional cycling of the pS2/TFF1 gene promoter*" (Metivier et al, Nature 452:45, 2008) and "*DNA demethylation …occurs … in a distributive manner without concerted demethylation of cytosines on both strands*" (Tat-GRU element: Kress et al, PNAS 103:11112, 2006).

The authors need, however, to validate this observation better, i.e., they should first demonstrate that the observation is not due to a common artifact observed when using PCR amplification of bisulfite-converted DNA: carryover contamination by products from previous experiments. As I mentioned in my first review, the way to demonstrate with reliability the authenticity of their data is to use barcoded hairpin bisulfite (NAR 2004, 32:e135). If they

perform the study on both strands with narrow time points and a sufficient number of samples sequenced they could also achieve some reliability, but not as efficiently as with the barcoded hairpin method.

Response:
4. Barcoded hairpin bisulfite PCR
Reviewer #1 has previously suggested that "*to ensure that the differences are not due to fluctuations and carry-over contamination that often pollute bisulfite sequencing data, it would be necessary to use either barcoded hairpin bisulfite (NAR 2004, 32:e135),* ***or*** *to show more narrowly spaced time points showing consistent behaviors*". Barcoding of DNA has been used to decrease the risk of product redundancy and contamination when limited amounts of DNA template are available for amplification (eg. trace amounts ancient DNA, forsensic DNA etc). All DNA templates used in bisulfite sequencing for this study were not limited by the amount of starting material. Additionally, we have not encountered redundancy issues (ie. sequencing the same clone repeatedly) since we observe the expected random distribution of 1-2% of unconverted cytosines after bisulfite treatment.

We do, however concur with the reviewer that the study would benefit from a more detailed time course analysis in the context of hormone-dependent demethylation. This data has already been provided in a methylation specific-PCR experiment shown in Figure 6C. We feel that the request for barcoding is excessive and a thirteen time-point MS-PCR experiment more than adequately eliminates the carry-over contamination argument.

The Fisher's exact test can give them a feeling of how many clones they should sequence to demonstrate reliably that the demethylation is significant if the proportion of demethylated Cs is exactly as observed here: about 250 for both the control and the 10 min time point and only about 45 for the 20 min time point on the - strand. For the Mef 2b and the Ptprg CpGs that are believed to be demethylated, the p-values are 0.347, and 0.161 resp., and three times, or two times more clones for both the control and GC treated samples should be sequenced to achieve statistical significance with identical proportions. To minimize the risk of data falsification by carry-over contamination, data should be at least replicated.

Response:
5. Data reproducibility
Collectively, we observe hormone-triggered demethylation at the Suox gene in four independent experiments done by two different methods: bisulfite sequencing on positive and negative strands (after stimulation with dexamethasone or corticosterone) and MS-PCR across 13 time points post stimulation with dexamethasone and after treatment with dexamethasone in the presence of the cell cycle blocker aphidicolin. Thus, our results are reflective of a robust, reproducible data set, making the issue of contamination highly unlikely. Similar high levels of reproducibility were observed for the Mef2b and Ptprg genes.

The second method used in this revised version to analyze demethylation is MS-PCR using a primer which 3'most base is complementary to the T that is characteristic of bisulfite-converted demethylated C. The method is not described in the material and method section, so it is not possible to assess whether it was performed in a reliable manner. The legends to figure does not indicate which C and which strand were analyzed. Furthermore, the data presented are hardly convincing because the amplitude of the effect is low and because a large part of the effect is apparently due to normalization by a fluctuating control. To obtain reliable data with this approach it is essential to use real-time Q-PCR, primers that have a LNA at the 3'-end to ensure efficient discrimination between priming on matched and mismatched template, and to demonstrate with known varying proportions of templates corresponding to methylated and unmethylated Cs that the assay is linear in the corresponding range for every target region analyzed (the performance of the assay is highly variable from sequence to sequence). As it is now, this assay brings little value to the data and the authors would be better off sequencing more clones and using barcoded hairpin-bisulfite than optimizing this method.

Response:
MS-PCR has been described in the Material and Methods section with PCR conditions and primers listed in Supplementary Table 3. Obviously, the Cs under scrutiny here are the same Cs

where the changes in methylation have been observed before. Strand information can be added to Supplementary Table 3 or Figure 6, if required. At the Suox element demethylation detected by MS-PCR increases by 50% in the first 20 minutes of hormone treatment, stays at this level for 1 hour and then declines gradually (by ~20%). These dynamic changes in DNA methylation are shown across several time points, thereby, eliminating any concerns regarding poor temporal resolution. Similar changes in amplitude are observed for the Ptprg and Mef2b genes with slightly different kinetics. Reviewer #1 further suggests that the observed changes in DNA demethylation arise from the normalization process. We would like to stress that the changes we observe are unaffected by the normalization step.

Minor corrections:
1 It is not specified in the text (page 10, bottom) and figure legends how chromatin accessibility was assessed in Fig 4A. Presumably, it is by FAIRE but this should be indicated.

Response:
Information regarding FAIRE has already been included in the Materials and Methods section.

2 On page 14, line 4, the text between parentheses (indicated by arrows in Sup Fig 10) is presumably at the wrong place and seems to correspond to the line above: "partial demethylation of a single CpG". It is clearly not corresponding to the dex treatment.

Response:
The information in parentheses refers to the preceding sentence as spotted by the Reviewer.

Referee #2:

The manuscript by Wiench and colleagues is greatly improved by the addition of new data and changes in text and figures. My previous criticisms were addressed in full. I have no further comments.

Referee #3 (Remarks to the Author):

Wiensch et al. 1st revision
Since the first submission and the revised version of this MS the genome-wide analysis of GR binding sites has been published in Nature Genetics (John et al.), which has answered some of the questions regarding GR binding data. The study under review starts from these binding data and the observation that preexisting DH and those formed upon hormone treatment show subtle differences in CpG density.

The main observation is of a slightly increased CpG content at GR binding sites while experimental evidence for a functional role of these CpGs and/or their DNA methylation states remains weak.

Response:
6. Mechanistic insights
Mechanistic insights into the process of active demethylation are complex and have been made additionally challenging by the recent discovery of the involvement of 5-hydroxymethylation in this process. We are currently using our system to find out which pathway and components contribute to active demethylation in order to determine whether changes in methylation are required for full activation or are a by-product of the remodeling process. This approach is an extremely complex undertaking and is beyond the scope and focus of the current manuscript.

Reviewer #3 would like to see a "simple" cause and effect of DNA methylation on GR binding and hormone-dependent gene expression. Such a cause and effect has not been satisfactory resolved (in any system) and it would be extremely difficult, if possible at all, to resolve it in our system. Regulatory elements are complex chromatin structures established early in development by one set of factors and then maintained during differentiation by combinatorial interactions of numerous histone modifications, additional transcription factors and other regulatory proteins and enzymes. Given this complexity, it is highly unlikely that

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elimination of a single feature such as DNA methylation would alter the organization of these chromatin states. We have already raised and discussed this subject in our manuscript (p15). Our experiments with Dnmt1 depletion confirm and extend the association between DNA methylation and chromatin accessibility; it also demonstrates that other features besides DNA methylation contribute to the organization of active enhancers. Thus, reducing the levels of DNA methylation alone is unlikely to be sufficient to establish *de novo* GR binding. As importantly, Dnmt1 depletion has pleiotropic consequences (ie. DNA methylation is globally affected). Such a broad-spectrum effect eliminates local methylation features normally established during differentiation. The wide-ranging effects of Dnmt1 depletion could also indirectly influence GR binding by altering the expression of GR associated accessory factors. Thus, global changes in DNA methylation are somewhat crude approaches for studies at the local level. The tools to generate site-specific changes in DNA methylation (eg. through the use of anchored methyltransferases) are only in the developmental stage and are currently unavailable.

The revised manuscript provides additional experimental and analytical data but the main problem remains in that there appears no solid basis to conclude a general function for DNA methylation in regulating GR binding. There is only one GR binding site studied that shows DNA methylation response in vitro and it is not even attempted to connect this singular finding in vitro to the global dataset.

The observation of subtle yet fast demethylation is of interest as it supports to be an active process, which could be linked to transcription. While the kinetics are now described in more detail and more convincingly there is little follow-up experiments on the actual process. Moreover these sites are not those that show higher CpG density.

While it is in general interesting if DNA methylation could be predictive of enhancer activity or TF binding (regardless of a functional relevance) such conclusion should be based on a small and selected selected set of elements.

Response:
We remain unclear about what the reviewer means with this question. The reviewer says "*while it is in*
general interesting if DNA methylation could be predictive of enhancer activity or TF binding (regardless of a functional relevance) such conclusion should be based on a small and selected selected set of elements". Did the reviewer intend to say "*…conclusion should not be based on a…*". We agree with the reviewer that querying more enhancers (perhaps a genome-wide survey) would be the ideal confirmation of our hypothesis. Expensive and time-consuming genome-wide methylation studies are currently beyond the scientific aim of our manuscript.

Points in detail :

The authors identify an increase in CpG density at GR bound regions when compared to surrounding sequences. Especially if GR peaks identified in both cell lines are considered. However the GR is not bound to typical CpG islands. They showed a similar increase for Stat3 and Oct4 using the data from Chen et al 2008.

They divided GR peaks into two classes:

a) pre-programmed (these region are sensitive to DNAse I in naïve cells)
Only for those they saw an increase in CpG density in the GR peaks compared to surrounding sequences. They split the peaks into three groups based on the CpG density in surrounding sequences and the increase in CpG density is in all of them.

b) de novo (these regions sensitive to DNAse I only after hormone treatment)
They did not see an increase in CpG density at GR bound region. When they split them into three groups they even saw the depletion of CpG for the group with highest CpG density.
Based on different CpG properties of the pre-programmed and de novo classes they speculated that DNA methylation could play a role in formation of these two different chromatin entities and that DNAseI hypersensitive sites induced by the hormone treatment could be special class of

enhancers.

*They measured DNA methylation levels for 17 selected regions in untreated cells.
For pre-programmed regions they measured DNA methylation over the entire bound regions. Methylation levels are bimodal (either very low <20% or very high >80%) and correlate with accessibility in a given cell type. (2 examples of shared regions and 4 cell type specific). On top of that they showed two regions, which were accessible in naïve cells and their methylation were low, however GR was not bound to them after hormone activation. They concluded that the correlation between demethylation and accessibility seems to be a general feature of pre-programmed chromatin at enhancer elements. If validated genome-wide this would indeed be an important finding.*

Response:
As much as we agree that genome-wide validation of our hypothesis would be of great value, the time and cost of such an experiment precludes us from considering it.

*They also investigated the DNA methylation levels at de novo sites but in these cases they measured the DNA methylation levels only at single CpGs. Figures 3D, 3E and S5. For all of them, the methylation pattern is not that homogeneous as for the pre-programmed sites. In comparison between two cell types, they usually observed difference in methylation only for one CpG (in some cases this CpG is part of degenerated GRE motif, in other lies in the proximity of the GRE). This partial methylation varies between different sites from 40 to 80% (except Sgk1 R1 in figure 3E where there is 0% methylation for de novo site). They speculated that this partial methylation could mark these sites as closed chromatin poised for remodelling.
page 10: ... these results indicate the strong link between DNA methylation and cell type specific usage of enhancers elements. It is unclear if this statement is based on pre-programmed sites (there is solid evidence for this) or on both types of GR sites (for de novo sites the evidence is not that clear).*

Response:
The evidence is more evident for pre-programmed DHS elements. This sentence could be moved from p10 to p9 or removed entirely from the text as it is also mentioned in the Discussion.

The knock-down experiment of Dnmt1 it does not support a regulatory role for DNA methylation

Response:
Please see response above (point 6, Mechanistic Insights). As discussed above, the Dnmt1 knock-down experiment clearly confirms the link between DNA methylation and chromatin accessibility. However, the lack of an effect on GR binding suggests that the organization of active enhancers is maintained by complex features besides DNA methylation alone (see response above and Discussion).

Direct influence of DNA methylation on GR binding was only observed if a CpG locates within the GRE (which does not contain a CpG). There are ~600 degenerated GREs containing CpG. They saw a decrease of GR binding in vitro when methylated CpG was at a critical part of the motif but the binding was unaffected when the methylated CpG was in the spacer or in the proximity of GRE.

Active demethylation : The authors also performed time course experiment analysing the DNA methylation levels before and after induction of GR. Figure 6A is showing CpG overlapping the GRE in SUOX-GRE which is fully methylated in AtT-cells but only partially methylated in NH-3134 cells (50%). After induction the methylation goes down to 24% (20 mins) and later to 13% (8 hours) or 16% (12hours). However for the same region, just the other strand, they saw demethylation of another CpG from 22 to 0% and partial remethylation after 12 hours to 22% (6B) which they claimed corresponded to decrease in Suox expression after 12 hours (John 2009). However this is not true for the other strand of the same region, their the CpG stayed demethylated. Maybe this could be just explained by the different activation (Dex for minus strand and Cst fro the plus strand) but it does not make the data more understandable. For two other cases, shown in summary figure S10 (clones shown in S11), they saw decrease in

methylation from 77 to 55% after 20 minutes followed by remethylation to76% after 8 hours and for last site the change is from 65 to 44 and back to 65%. No changes are observed in chromatin marks (fig 6E). It still is rather difficult to understand this data. There appears a consistent decrease ~20-30% but the absolute levels are very different and there is no clear difference between on and off state in general. Such forms of demethylation have been observed for ER before but also in cancer cell lines, which display overall disturbed patterns of DNA methylation

Response:
We have commented on strand-specific differences in methylation and amplitude changes of demethylation in the response to Reviewer #1.

Table 1.

Comparison of recently published studies describing stimulus-triggered demethylation.

	Amplitude of demethylation	Bisulfite sequencing			Other methods		
		# of clones	# of time points	# of regions analyzed	Other methods	# of time points	# of regions analyzed
Martinowich et al, Science 302:890, 2003	**20-40%**	**28-35**	2	1	MS-SNuPE	2	1
Kangaspeska et al, Nature 452:112, 2008	**NA**	**NA**	NA	NA	MBD pull down; HpaII digestion followed by PCR and qPCR	19	5
Metivier et al, Nature 452:45, 2008	**30-70%**	**18-25**	16	1	HpaII digestion followed by qPCR	12	2
Kim et al, Nature 461:1007, 2009	**Average 40% across 13 CpGs**	**20 in triplicates**	Total 9; demethylation across 5 points	1	Me-DIP	9	1
Angrisano et al, NAR 2010	**20%-45%**	**20**	3	1	MassArray; pyrosequencing	3	1
Kress et al, PNAS 103:11112, 2006	**25% after 12h 60% after 24h**	**NA**	NA	1	LM-PCR; MethylQuant; Hairpin-BS-PCR	8	1
Current manuscript	**25-37% for Suox 20% for Ptprg and Mef2b**	**15-25**	3 for + strand 5 for - strand	3	MS-PCR	13	3

3rd Editorial Decision 13 May 2011

I have discussed the remaining issues with your manuscript and your subsequent responses with one of the other referees, and we have come to the conclusion that the barcoding experiments do not need to be done and the additional mechanistic expts are not required. Basically the study is ready to be accepted (although I am leaving for a conference in Vienna). Please make any text changes that are required to address the referees concerns and resubmit the final version.

Yours sincerely,

Editor
The EMBO Journal

3rd Revision - authors' response 17 May 2011

We are submitting our revised manuscript, “DNA Methylation Status Predicts Cell Type-specific Enhancer Activity.” We are pleased that reviewer 1 now accepts the sufficiency of our DNA demethylation analysis.

We have introduced the following minor changes to the manuscript:

Reviewer #1 Information r.e. the FAIRE method was added to the Fig. 4 legend, and indication of the DNA strandedness was added to the Fig. 6 legend.

Reviewer #3 The sentence from p10 has been deleted as this issue is further discussed in the Discussion section.

In addition we updated the legends for figures 5 and 6 due to the fact that these figures are intended to be B&W in the print version. We would like to confirm that figures 1-3 and 5-6 for the online version will be in color.

The reviews have greatly improved the manuscript and we would like to thank the reviewers again for insightful and constructive comments. We appreciate your help throughout the process.

4th Editorial Decision 20 May 2011

I have looked through the revised version of the manuscript and find that the remaining issues (which I previously discussed with referee #2) have been satisfactorily addressed. I am happy to accept the manuscript for publication in The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal