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Arginine methylation controls growth regulation by E2F-1

Er-Chieh Cho, Shunsheng Zheng, Shonagh Munro, Geng Lui , Simon M. Carr, Jutta Moehlenbrink, Yi-Chien Lu, Lindsay Stimson, Omar Khan, Rebecca Konietzny, Joanna McGouran, Amanda S. Coutts, Benedikt Kessler, David J. Kerr and Nicholas B. La Thangue

Corresponding author: Nicholas B. La Thangue, Oxford University

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Transaction Report:

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

1st Editorial Decision 08 June 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two of the three referees, whose comments are shown below. While the third report has been promised repeatedly, it has still not materialized and we certainly cannot justify any further delays. In fact, I must apologize for the truly excessive delay in the review process of this manuscript, which is rather atypical for this journal. We will of course forward the third report as soon as it arrives.

Unfortunately, we cannot offer to publish this dataset.

As you will see, both referees are rather critical, raising a partially overlapping set of issues related to the study. I should also note that while referee 2 is in principle more favourably disposed in terms of editorial interest of this dataset for EMBO Journals, referee1 explicitly recommends against publication.

While referee 1 finds that the biochemical data showing PRMT5 binding and the methylation of E2F1 and hence the latter's degradation is compelling, s/he argues that the evidence that E2F1 Arg methylation selectively regulates its apoptotic functions, as prominently highlighted in the title and abstract of the manuscript, is not definitive. The referee notes a previous study (JCB 2004) which described E2F1 acetylation as regulating apoptosis selectively.
Referee 2 notes the general interest of the claims made, but point 1-3 concern the lack of definitive mapping of the methylation site(s) and the PRMT5 interaction, as well as physiological evidence for

the modification. The referee finds the ubiquitination assays not definitive and requests other controls. The referee also requests analysis of other related methylases.
Both referees want clarification how the PRMT5 binding is affected by PRMT5 mediated methylation of the same motif. Both referees also comment that the clinical sample data is incomplete (also, we did not notice the colorectal data).

Given these negative opinions and in light of the fact that the EMBO Journal can only afford to invite revisions on papers which receive strong support from a majority of referees, I am afraid we can not offer to publish this dataset. Since we have a policy not to undertake extended rounds of revision, we have decided that we cannot, in this case, invite a revision, despite the editorial interest in the claims made in the manuscript.

Thank you for the opportunity to consider this manuscript and for your notable patience in awaiting this decision. I am very sorry that we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

GENERAL COMMENTS:

This study by Cho et al. establishes that the arginine methyltransferase PRMT5 binds to E2F1 and promotes its methylation at arginine 111 and 113. It further shows that this post-translational modification contributes to the regulation of E2F1 levels by inducing its ubiquitination and degradation. These findings have not been reported previously and the data supporting them are strong. However, these findings alone are insufficient to merit publication in EMBO Journal. The key hook in this paper is the authors' proposal that E2F1 arginine methylation could specifically inhibit E2F1-dependent apoptosis. This would be a significant finding, if it holds true. Unfortunately, the authors present no evidence that arginine methylation selectively inhibits E2F1's ability to activate apoptotic target genes, as opposed to cell cycle genes. Indeed, the authors' own data seem to argue against such selectively. Thus, as it stands, the paper does not merit publication in EMBO Journal.

MAJOR POINTS:

POINT 1. A primary focus of the paper is the notion that arginine methylation could selectively inhibit E2f1's ability to regulate apoptosis versus cell cycle target genes. However, the presented data provides no evidence of selective effects. Indeed, data in Fig. 4 seems to refute this hypothesis; the methylation-defective mutant E2F1 "KKK" binds and activates equally well both cell cycle (cdc6, DHFR) and proapoptotic (p73, APAF1) E2F1 target gene promoters. (Fig.4 a and 4b). Fig.4 c also shows that reduced levels of endogenous PRMT5 lead to increased E2F1 binding to the promoter of the Cdc6, p73 and E2F1 genes. Thus, E2F1 arginine methylation seems to reduce E2F1 DNA binding affinity in general.

POINT 2. The results in Fig1d and 1e show that the E2F1 mutant KK and KKK cannot bind to PRMT5, suggesting that E2F1 methylation at these residues is required in order to allow PRMT5-E2F1 binding. This ishard to reconcile with the idea that PRMT5 is the methyltrasferase responsible for E2F1 arginine methylation. (chicken and egg?).

POINT 3. Fig.3b shows identical levels of WT, KK and KKK E2F1 at time zero. However, in Fig. 3a "....both the KK and KKK mutant were expressed at increased levels compared to the wild type E2F1 Figure 3a...". How can the authors explain this discrepancy, also in view of the fact that equal

amounts of DNA were transfected in each point (1ug, see legends Fig. 3a and 3b)?

POINT 4. The ChIP experiment in Fig.4b (middle panel) shows that wild type E2F1 does not bind to the p73 promoter. However, WT E2F1 overexpression promotes p73 transcription in the luciferase assay shown in Fig.4a, middle panel. Is E2F1 indirectly mediating p73 transcription in these conditions? This would be an unexpected result. Can the authors comment on this observation?

POINT 5. A table with details of the tumor collection analyzed (how many tumors, stage, grade, time to progression correlated with E2F1 and PRMT5 levels) should be provided to allow an evaluation of the statistical relevance of the findings presented here.

POINT 6. A mechanism that selectively activate E2F1 proapoptotic potential - P/CAF-mediated E2F1 acetylation - has already been reported. This prior work should be discussed.

TYPOS.

1) page 9 second paragraph. The sentence "we reasoned that the transcriptional activity of E2F target genes might be affected by PRMT5..." should be corrected with "we reasoned that the transcriptional activity of E2F1 might be affected by PRMT5...".

2) page 4, second paragraph, third line, the sentence "... a variety of processes are know to be influenced..." should be changed with "... a variety of processes are known to be influenced..."

Referee #2

The authors show that E2F1 binds to PRMT5 and that E2F1 is methylated (me) on arginine residues (R) by PRMT5. Potential R-me targets are located in a N-terminal R/G-sequence motif close to the DNA binding/DP1 interaction domain of the E2F-1 protein. The R/G motif shares similarity to many arginine methylation sites, including p53, as previously identified as a PRMT5 target by the same lab. The authors present data to suggest that methylation within this E2F1motif by PRMT5 is involved in the regulation of apoptosis, E2F1 protein stability, transcriptional activity, and DNA binding. PRMT5 knock-down by interfering RNA correlated with E2F1 protein stabilization, increased expression of the p73 E2F1 target gene and enhanced apoptosis. The authors suggest that arginine methylation of E2F1 inhibits its apoptosis inducing function by enhanced ubiquitin mediated degradation and that DNA damage contributes to demethylation, E2F1 stabilization and enhancement of apoptosis. Analysis of PRMT5 and E2F-1 expression in tumor biopsies of colorectal cancer samples revealed negative correlation and potentially involvement in disease progression. High E2F1 expression and low PRMT5 expression suggested better prognosis than the inverse correlation.

This is an interesting manuscript that connects PRMT5, DNA damage and regulation of apoptosis with methylation of E2F1. However, it is essential that the authors thoroughly consider a number of critical points:

1. No direct evidence of methylation is provided of any of the arginine residues implied in PRMT5-E2F1 in live cells. It seems mandatory to convincingly show that methylation of the triple R (or any of these residues) occurs in cells (e.g. by mass spectrometry, as in their previous p53 paper). Data obtained with antisera and E2F1 mutations are not sufficient here (see below).

2. The E2F1 triple K mutant failed to bind PRMT5 (Figure 1e). A GST-E2F1 construct binds to PRMT5, suggesting that methylation is not a prerequisite for PRMT5 interaction. Therefore, the respective arginines (or at least R109 and R113) may not only serve as targets of PRMT5 methylation but also for binding to PRMT5. Figure 1 actually suggests that R111 is the primary PRMT5 target. Why did the authors proceed with the triple/double K mutation although the single R111K mutation would have abrogated methylation (Figure 1b)? Does R111K E2F1 bind to PRMT5? The authors need to distinguish between the effects of E2F1 R-methylation and/or lack of E2F1 PRMT5 interaction and should therefore resolve PRMT5 binding and methylation effects.

3. The experiment shown in Figure 2b does not rule out that the antibody may recognize both, un-methylated and methylated E2F1. Although one might conclude that antibody avidity distinguishes between un-/methylated peptide (Figure 2b), one cannot conclude on the endogenous E2F1 methylation state using this antibody in protein blots (Figure 2c and ff.). How would a competitive IP have looked like using this antibody plus/minus excess of competing E2F1 / RmeE2F1 peptides, followed by blotting and detection by the E2F1Rme specific antibody? One would also assume that the antibody may be applied to convincingly demonstrate R-me at aa 109/111/113 in combination with mass spectrometry and one wonders why the authors omitted direct prove of R109/111/113E2F1 (symmetric di-) methylation.

4. The interfering RNA P5 also reduces expression of the PRMT1 protein (Figure 2e). Is there a crosstalk between PRMT5 and PRMT1 on the protein level or is there a problem with the siRNA specificity? Although there is no obvious crossregulation of P1 on P5, additional controls should include other PRMTs. Along these lines, methylation deficiency seen with knock down of PRMT5 (Figure 2f) do not necessarily imply PRMT5 as the only enzyme that modifies the E2F1 motif. How would PRMT1; PRMT2, and CARM1 perform in an assay as shown in Figure 1b (positive controls for all enzymes should also be provided)? It would be important to see whether other PRMTs could be involved in methylating the E2F1 RGRGR motif (Figure 1b) and if so, how the authors confine specificity.

5. The ubiqutination data as shown in Figure 3d are not convincing. Again, a problem occurs by using the triple K mutant (see Point 2). There are also no differences seen in stability (Figure 4aii; bii), yet reporter and ChIP data indicate higher activity of the triple K mutant. Internal controls are recommended. E2F1 expression control is missing in Figure 4cii.

6. Figure 5: Fig 5b, Expression controls for E2F1 expression and concomitant E2F1 methylation levels should be shown. Fig 5c, depletion of PRMT5 marginally increased E2F-1 expression when compared to other figures, whereas apoptosis is strongly increased. The methylation status of E2F1 upon PRMT5 depletion should be demonstrated. Fig 5i, p73 levels in lane 4 are not convincing.

7. Some higher magnification immune staining microscopic data should be provided to clearly visualize nuclear/cytoplasmatic staining and E2F1/PRMT5 expression in tumor samples.

General points:

Authors should improve on labeling and arrangement of figures and clearly indicate cell lines, Ip's, lysate and mRNA controls and specific antibodies.

Rebuttal 09 June 2011

Thanks for returning the referees reports.

We have read the comments in some detail, and agree that both referees make a series of points, some of which are interpretive or reflect misunderstandings of the data. The relevant technical concerns seem, at face value, to be somewhat minimal.

Please do not regard this email as our rebuttal letter, as we will await the 3rd referee before providing a detailed more extensive response to all the referees. We do however believe that it would be useful to counter some of the comments, and make you aware of some of our responses, at this stage.

Referee 1:

1) The referee is not correct in the assertion that we claim 'methylation selectively inhibits E2F1s ability to regulate apoptosis versus cell cycle genes'. This interpretation was not made anywhere in the manuscript. What we did in fact show is that there is increased transcription of E2F target genes, some of which are connected with apoptosis (Fig 4, such as p73, APAF1 and E2F-1), when E2f1 methylation is regulated. We did not claim, anywhere, nor would we reasonably wish to, that apoptotic genes are selectively activated by methylation defective E2F-1. We did show however, that the apoptosis driven by methylation defective E2F1 requires both endogenous E2F1 and p73

activity (Fig 5 f and H), and thus both E2F1 and p73 represent E2F1 target genes which are biochemically and functionally linked to the apoptotic outcome that occurs with methylation defective E2F1.
Perhaps we were not clear enough in our explanation (although Ref 2 did not make this interpretation). But, to be clear, the point the referee makes appears on face value to be at variance with our own interpretation.

2) We don't understand the point being made here. We have used a very routine mutagenesis approach to identify E2F1 R residues methylated by PRMT5 in vitro (Fig 1). Our view is that the binding between PRMT5 and E2F1 reflects an enzyme/substrate interaction. Consequently, removing the residues targeted by PRMT5 reduces the interaction. We have no evidence to suggest that there is a separate binding domain in E2F1 for PRMT5.

3) The referee emphasised Fig 3b, commenting on the levels of the E2F1 mutants. This really is a minor point! The purpose of Fig 3b was to measure the half-life of each mutant. The absolute levels of each protein are therefore not critical; rather it is their decay rate which is the issue, and which is presented in the graph in ii). The longer half-life of the KK and KKK (meth defective) mutants explains why the steady-state level of each mutant is higher than WT E2F1 (Fig 3a, d, 5i) and why PRMT5 siRNA increases the levels of WT E2F1 (Fig 3e, f, 5c, f,g,h).

4) The referee comments on the RT ChIp in Fig 4b, specifically the low binding of E2F1 to the p73 promoter yet high transcription response in the reporter based transfection assay (Fig 4a). Rather than this being a problem, we regard this as interesting and can add more information if need be. The important point is that the referee is not correct, as indeed there is binding of E2F1 to the p73 promoter, although we agree that it is lower than the other target genes perhaps reflecting the higher back ground control (-) in this assay. However, and importantly, p73 has been extensively documented as an E2F1 target gene. The important point from our perspective is that methylation defective E2F1 has significantly enhanced binding to p73. We have an enormous amount of ChIp data and can supply other examples if need be.

5) We can provide a Table with details of the tumour collection that we screened (Fig 6 and 7). We did already cite the relevant publication describing the Phase 3 trial (Midgley et al, 2010), where the collection of CRC biopsies is appropriately annotated, but happy to supply the details again for the current manuscript.

6) We did cite the manuscript the referee comments on (Pediconi et al, 2003), and can discuss in greater detail if need be. That publication deals with acetylation of E2F1, did not map the site nor uncover a mechanism. Our paper deals with arginine methylation of E2F1, describes the biochemical and functional consequences, and relates this information to the clinical context of cancer. They are non-overlapping reports, as far as we can see. In analogous pathways, for example p53, an evolving post-translational code dictates apoptosis; it is not simply one signal. Our data support a similar scenario for E2F1.

Referee 2:

1) The referee comments that we have no evidence that the R residues are methylated in cells. This is a very confusing comment. Fig 2 shows that our antibody is firstly specific for anti-methyl R E2F1 (Fig 2a and b, which includes the peptide competition experiment requested by the referee in point 3), secondly it binds to E2F1 in cells (2c) and further its binding to E2F1 is blocked when PRMT5 is depleted (2f). We have an extensive body of additional data addressing the specificity of the antibody (eg dot blots with meth and unmeth peptides which we can show the referee). Further, the motif targeted by PRMT5 is a predicted PRMT site, and aligns nicely with the methylated site in p53. It is highly unlikely, given the above, that these R residues are not methylated in cells.

The referee requests mass spec data to support the sites of methylation. We can include this if need be. The methylated residues are clear on the ectopic protein purified from cells. However, and the reason why we did not include these data, mass spec analysis does not distinguish between asymmetric and symmetric di-methylation on arginine (both modifications are the same mass, and PRMT5 is a symmetric methyl transferase) and thus, importantly, we would still have to use an antibody which is specific for the symmetric modification. The referee perhaps is not clear on this

latter point.

2) The referee comments on residues targeted by PRMT5. He argues that R111 is the primary site. We agree, although R113K reduces the methylation to about 5% of the WT level. Thus, R111 and R113 are both critical sites. Consequently, we evaluated the KK mutant (R111K and R113K in all relevant experiments, usually in parallel with the triple KKK mutant, where no difference has ever been observed). Perhaps the image the referee viewed was not clear of Fig 1b. We can certainly provide the quantitation of the data, to assist interpretation of which residues are the targets for PRMT5.

3) The wording of this point is a little confusing, so please excuse us if we have hold of the wrong end of the stick!
Agree with the comment that Fig 2b does not prove that the antibody recognises methylated E2F1. However, the competitive IP experiment described and requested by the referee (IP plus competing peptides followed by blot with anti-meth Ab) is, as far as I can tell, shown in Fig 2b. This is one of the experiments that addresses the specificity of the antibody and the results are I believe quite clear. The referee may not have noticed (because he constantly refers to 109/111/113 R meth) that the antibody was raised against sym di-meth at R111 and R113 (ie the crucial residues implied by Fig 1b, and not meth R109).

4) The referee comments on Fig 2e, and the effect that PRMT5 siRNA has on PRMT1 levels. We have seen this effect many times (with different PRMT5 siRNAs) and cannot easily explain it. The referee questions whether other PRMTs target E2F1 in cells. In response, we do not know the answer to this question and I am not sure how relevant it is anyway to the PRMT5 regulation of E2F1. Regarding the referees request for the effect of other PRMTs on the R111/113 motif, we can certainly provide data which shows that other members of the family do not target the same sequence. As far as we can tell, R111 and R113 are specifically targeted by PRMT5.

5) The referee comments on Fig 3d, arguing that it is not convincing. It is not clear why this conclusion was made, given the quantitation underneath. Perhaps an improved exposure would help? The referee comments on the use of the KKK mutant in Fig 3d. The KK mutants behaves in exactly the same way, and we can provide these data.

The referee comments on the absence of any difference in E2F1 levels (in Fig 4aii and bii). The referee has missed some of the experimental details. We clearly state in the text (P.9 second paragraph. 4th line) that we expressed WT and KKK at equivalent levels of protein, so that we could directly compare their activities (independently of any contribution form protein levels).

6) In Fig 5b we can provide the expression level of E2F1. In Fig 5c, the difference in E2F1 level between C and P siRNA treatment is actually quite clear (induced level of E2F1 upon PRMT5 siRNA); perhaps an improved exposure would assist the referee. In Fig 5h (presumably not Fig 5i as detailed by the referee) we can supply an improved version of the p73 blot (it is a very poor antibody).

7) We can supply higher magnification and further details of the CRC biopsy immunostaining.

Thank you very much for taking the time to read these comments. I think that you might agree that some of our responses and arguments rest on solid scientific foundation, and further that several of the comments raised by the referees perhaps reflect misunderstandings relating to technical and interpretive aspects of the data.

I hope that you will give careful consideration to the above comments, and give us at least one opportunity to respond to the referees and revise the manuscript accordingly.

2nd Editorial Decision 05 July 2011

Thank you for submitting your rebuttal. I certainly agree that this reviewing process did not go as well as we had hoped (nor did it go as well as we are accustomed to at this journal): despite our repeated efforts over the last two weeks, the third referee is still not delivering a report (despite earlier repeated promises). Needless to say, we will not use this referee again in the future, but we are dismayed that we cannot obtain a third report in this case, given the fact that a) we delayed the editorial decision dramatically on account of referee 3 - apparently in vain and b) since referees 1 & 2 have opposed opinions on the basic interest of the study for The EMBO Journal.

I have gone through your point-by-point response and it is clear there is some miscommunication between you and the referees, which I will try to address in the following detailed response to your points:

ref 1:

1) you state that 'apoptosis selectivity' was never claimed and could not be claimed; misinterpretation of the referee:
The point is that referee1 WOULD have found the hypothetical selectivity interesting, but clearly s/he doesn't find scope of the present dataset compelling for EJ. Please note that the referee was likely mislead by your selective emphasis of apoptosis over the the cell cycle roles; for example, the title mentions only apoptosis and the abstract mentions cell cycle & apoptosis activity and then delves only into apoptosis, mentioning it four times!
One constructive way to address this, and indeed to enhance the scope of the work, would be to add cell cycle assays. In our view a selective role (as apparent for example for certain p53 regulatory events) would be more exciting, but is not a prerequisite for further consideration.

2) methylation/binding 'chicken & egg': you don't understand this point, although notably referee 2 raises an almost identical issue. The point is that enzyme/substrate binding appears to depend on the presence of the target site. Both referees request functional disambiguation of target binding and enzymatic target site methylation on either 111, 113 or both sites (see below).

3) I agree.

4) can be addressed with more examples - minor point.

5) minor point: data available and will be added.

6) minor point - can be addressed.

referee 2

1) In my view you misinterpret what the ref said: they said the methylation specific AB is NOT enough and wanted mass spec data; the referee did NOT say the AB did not work! We agree with the referee that additional evidence is important and clearly you followed that route in previous related studies. However, you appear to have the mass spec data and can include it - the point about symmetry is understood, but this does not render the mass spec. data meaningless.

2) ref wants disambiguation of R111 and 113 in PRMT5 binding vs. methylation target role. You did not address the binding question (see above) but comment that both 111 and 113 are critical, which is why you used the double mutant: in our view this is a legitimate point and the data should dissect the role of 111 or/and 113: are both targeted in vivo, are both implicated in enzyme binding and are both functionally relevant?
3) you state that AB controls already clearly show that the AB is specific for Me-111/113-E2F. However, the referee again requests the complementary mass spec data to support which site is actually hit (see above)!

4) while you understandably state that other PRMT family members are not relevant to this study,

you can provide data to show they do not target E2F1. While normally I'd agree with the relevance point, clearly the fact that the the loss of function also targets PRMT1 means this is a useful addition.

5) addressable: add double mutant.

6) the referee is commenting on the divergence of the change in E2F expression and the apoptotic response. The point is that this can be seen as a discrepancy.

7) You can provide extra information on clinical data. In my initial editorial assessment I had actually also noted that an absence of information for colorectal cancer.

In summary, while most of referee 1's specific issues can probably ultimately be resolved in revision, the referee does not rate the present paper highly for The EMBO Journal. In addition to the above points, we would therefore encourage you to consider how this study could be rendered more compelling for the journal. One possibility we would recommend is insight into how E2F methylation is regulated by damage.

Should you be able to address these criticisms, we could consider a revised manuscript. We would aim to return this to previous referee 2 and a new referee to ensure that we can rely on a fair and informed process. Note however, that we will certainly not disregard ref 1's clearly stated views (a world expert in the field). We would of course very much aim to demonstrate a significantly more efficient process in a further round of review. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

If you decide to thoroughly revise the manuscript for the EMBO Journal, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 23 September 2011

1. The referee commented on the connection between PRMT5 and E2F-1’s ability to influence apoptosis, suggesting that the data were inconsistent with such a binary relationship. In response, it was not our intention to claim that methylation selectively inhibits E2F-1’s ability to induce apoptosis. Rather, we believe that PRMT5 is a more general regulator of growth influenced by E2F-1. We have therefore included additional growth and cell cycle assays, as suggested by the Editor, where the role of PRMT5 and E2F-1 has been evaluated in the more general context of proliferation and growth control.

We have thus made a number of modifications in the revised manuscript. We have

incorporated new data reflecting the impact on PRMT5 on growth control and the role of E2F-1 under these conditions. The results show that PRMT5 is a positive regulator of proliferation and cell growth (by cell number and colony assays; new Figure 5), as its depletion using PRMT5 siRNA reduces both cell number and colony formation, in addition to inducing apoptosis. These effects are mediated in part through E2F-1, since co-depletion of PRMT5 with E2F-1 rescues the growth inhibitory effect of PRMT5 siRNA alone (new Figure 5). The results have been integrated into the manuscript, allowing us to conclude that PRMT5 regulates cell proliferation through the E2F pathway, and that inhibition of PRMT5 activity delays cell growth (reduced growth rate and extended doubling time) and prompts apoptosis through deregulating E2F-1 (Figure 6).

2. The referee commented that, because the KK and KKK E2F-1 mutants cannot bind to PRMT5, it is hard to reconcile that PRMT5 is the enzyme responsible for E2F-1 methylation. He/she suggested that PRMT5 bound to the methylated R residues. However, the new results presented are not consistent with this viewpoint (for example PRMT5 binds to unmethylated GST-E2F-1 and methylates the E2F-1 peptide; Figure 1b). In addition, we have provided new data that address PRMT5 binding and enzymatic modification. Revised Figure 1b shows that the integrity of both R111 and R113 is required for *in vitro* methylation by PRMT5. Revised Figure 1e shows that R111 and R113 are each required for the interaction between E2F-1 and PRMT5, and thus binding is not sufficient for methylation. The new Figure 1b (v) shows that a short peptide (20 mer) derived from E2F-1 and encompassing R111 and R113 can be methylated *in vitro*. The new Figure 2g shows that both R111 and R113 (but not R109) are methylated in cells and the principal sites of PRMT5 methylation. Since both R111 and R113 residues are required for PRMT5 binding (rather than one required for binding and the other acting as a methylation substrate), binding between PRMT5 and E2F-1 is likely to reflect an enzyme-substrate type of relationship in which R111 and R113 are functionally and biochemically equivalent.

3. The referee emphasised Figure 3b, commenting on the expression level of the E2F-1 mutants. The purpose of Figure 3b was to measure the half-life of each mutant. The absolute level of each protein is therefore not critical; rather it is the decay rate which is the issue, this being presented in the graph (Figure 3bii). The longer half-life of the KK and KKK (meth-defective) mutants explains why the steady-state level of each mutant is higher than WT E2F-1 (Figure 3a, d, 5i) and why the PRMT5 siRNA increase the level of WT E2F-1 (Figure 3e, f, 5c, f, g, h).

In addition, we have added new results which show the half-life of the single substitution mutants (R109K, R111K and R113K). As expected, R111K and R113K show an extended halflife (SI Figure 2c); R109K remains similar to wild-type E2F-1. Together, these results strongly suggest that R111 and R113 are functionally equivalent.

4. The referee commented on the RT ChIP assay in Figure 4b, specifically the low binding of ectopic E2F-1 to the p73 promoter. We believe that this interpretation reflected the higher than usual control treatment signal. As a consequence, we have replaced the RT ChIP E2F-1/p73 data with improved results (Figure 4b). However, we continue to record low DNA binding activity of ectopic E2F-1 to p73, while the methylation defective mutant (KKK) binds much more efficiently (we have provided two more examples of p73 ChIPs (Figure for the Editor and Referee 1) to emphasise the point that it is a general phenomenon). Note that the additional ChIP data (Figure 4c) shows that endogenous E2F-1 binds to the p73 promoter, which is further enhanced upon PRMT5 depletion. These results thus complement and support the enhanced DNA binding activity of KKK compared to wild-type E2F-1.

5. We have added details of the tumour collection cohort that was screened to assess PRMT5 and E2F-1 expression, detailing relevant parameters such as number of tumours, age, stage and gender (SI Table 1).

6. The referee asked us to comment on a previous report, describing the selective activation of E2F-1 dependent apoptosis by P/CAF mediated acetylation. We did indeed cite the relevant manuscript (Pediconi et al., 2003), and have re-emphasised the previous study in the revised manuscript. Pediconi *et al* document the acetylation of E2F-1 (the target residue/s were not defined) and connect this modification with apoptosis. Our study describes arginine methylation as a mechanism for suppressing E2F-1 activity, together with the biological and biochemical

consequences, and relates this information to the clinical context of cancer. The reports are therefore non-overlapping. In fact, in an analogous fashion to p53, E2F-1 may have a code (different types of modification) that dictates its biological outcome.

We would like to thank the referee for his/her valuable comments.

Response to Referee 2

1. The referee commented that we did not provide any direct evidence that the R residues are methylated in cells. In the revised manuscript, we have added data derived from mass spectrometry which documents methylation of the appropriate R residues (SI Figure 1g). We would point out, however, that the experiment the referee requested, namely peptide competition with endogenous E2F-1 in the presence of the anti-MeR E2F-1 antibody (his/her comment 3) was, in fact, shown in the original Figure 2b. This result shows that the modification specific antibody (raised against a meR111 and meR113 peptide) recognises E2F-1 in cells, and that antibody binding is competed by the methylated R peptide. Furthermore, new results in the revision show that depletion of PRMT5 eliminates anti-MeR E2F-1 binding to E2F-1(Figure 2f). Combined with other complementary data in the manuscript (Figure 1b to e, and SI Figure 1), the results support the methylation of R111 and R113 both *in vitro* and in cells.

2. The referee wanted us to assess the role of R109, R111 and R113, and thereby provide further justification for the use of the KKK and KK mutants in the study. We have provided an extensive body of new data which address this point. Thus, both R111 and R113 are required for E2F-1 methylation *in vitro* (new Figure 1b), and R111 and R113 are each sufficient for PRMT5 binding (Figure 1e); R109 impacts minimally on methylation by PRMT5 and binding to PRMT5 (Figure 1b and e). Both KKK and KK mutants lack R methylation and PRMT5 binding, which we believe justifies their use as arginine methylation and PRMT5 binding defective mutants. We have, as requested, added a large body of new data on the properties of the single substitution derivatives (R109K, R111K and R113K). Both R111K and R113K sites are methylated in cells (new Figure 2g), both sites are required to bind PRMT5 (new Figure 1e), both sites regulate the stability of E2F-1 (SI Figure 2c), and both sites affect the transcription properties of E2F-1 (SI Figure 2e). R109K has similar properties to wild-type E2F-1 (Figure 1b and e, 2g, SI 2c and 2e). We believe therefore that both R111 and R113 are biochemically and functionally equivalent, at least within the confines of our experimental systems.

3. The experiment that the referee requested, namely the competitive IP plus and minus competing peptides, was shown in Figure 2b. These data have been complemented by the additional mass spec data (SI Figure 1), together with the effect of PRMT5 depletion on E2F-1 methylation (Figure 2f).

4. The referee commented on the impact of PRMT5 siRNA on PRMT1 levels (previous Figure 2e). We believe that the blot provided in the original submission was not typical of what we have routinely seen. We have therefore re-blotted the samples, and have incorporated the new data into the revised manuscript (revised Figure 2e).

5. The referee remarked on the ubiquitination data (Figure 3d), indicating that they were not convincing. The quantification of the data is shown underneath the primary blot, where a 50% reduction in ubiquitination was apparent with the mutant. We have tried to improve the exposure of the primary blot in the revised manuscript, to assist in interpreting the data. We have, in addition, provided new data on the ubiquitination of the KK mutant, which behaves in a similar fashion to the KKK mutant (SI Figure 2c).

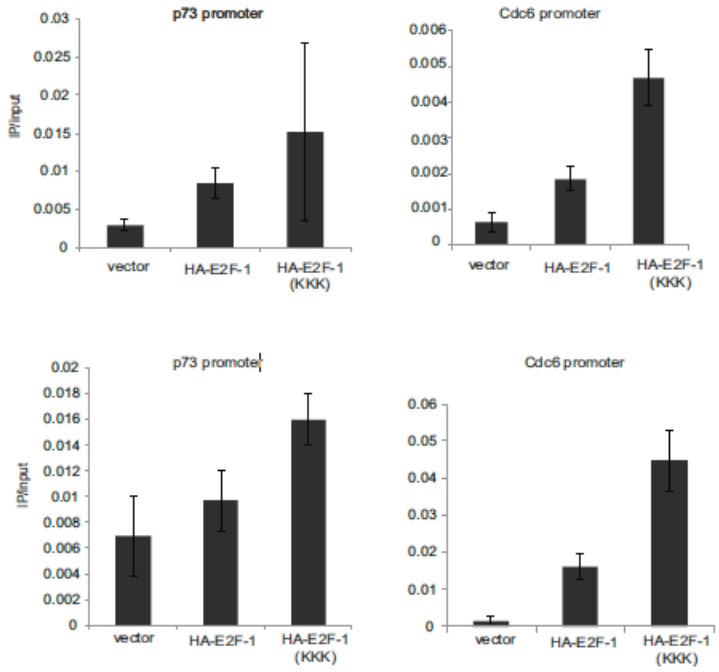
The referee also mentioned the level of the mutants in Figure 4a. It is important to note that we deliberately expressed equal levels of the ectopic proteins, so that we could directly compare their transcriptional activity (this was stated in the text; P.9 2nd paragraph).

6. The referee commented on the expression level of E2F-1 in Figure 5c (revised Figure 6c), which now shows a clearer increase in E2F-1 levels upon PRMT5 depletion. An improved image of p73 levels upon PRMT5 depletion has been included in the revised SI Figure 1f.

7. We have provided better resolution images of the immunohistochemistry on tumour biopsies (40x and 200x), as requested by the referee (Figure 7a and b).

We would like to thank the referee for his/her valuable comments.

Figure for the Editor and Referee 1



3rd Editorial Decision 06 November 2011

Thank you for submitting your revised manuscript. As discussed, we returned it to original referee 2 and replaced referees 1 and 3 with a new arbitrating referee (referee 4) to ensure that we could rely on a constructive and informed editorial process; the comments of both referees are enclosed. As you will see, both referees express interest in your manuscript and are broadly in favour of publication, pending satisfactory further revision.

The key point raised by referee 2 is that the mass spec data to support physiologically relevant di-methylation on R111 and R113 is restricted to recombinant protein. We agree with the referee that this is not made clear in the brief discussion on p. 8 (first paragraph), which should be revised. Moreover, we also concur with the referee that mass spectrometric data to provide a second and a more direct level of evidence for the key conclusion of di-methylation of E2F-1 in tumour cells is to be strongly encouraged. This level of experimentation is standard in high level publications - and indeed such data has been provided by your own lab in similar studies previously.
We hope that by now such data is available in your laboratory and that it can be included in revision. We would not undertake formal re-review of such data, but would validate it briefly with an expert advisor.

Referee 4's main point is that the tissue staining data has to be presented in a more statistically robust manner (point 4).

Referee 4 also requests a number of citation changes and concludes that the note on R109 methylation is overstated. The referee revisits the discussion initiated by referee 1 on a general role in E2F function vs. an apoptosis specific role, which had not been your intended claim. The referee takes issue with implying a role of E2F in cell growth (bottom of p. 8 and discussion). We

recommend that this is referenced in more detail; it may be appropriate to revise the text to imply a more general cell cycle role.

Finally, we agree with referee 2 that new fig 6c is merely a longer exposure of previous fig 5c. We would recommend that a quantification of the Western blot is included in revision.

Please note that we encourage presentation of uncropped source data of key blots as 'Source Data' files associated with the figures. We would be please if you could provide such data for inclusion in the published paper.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the reviewers.

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

We would hope to receive a revision within one month, but if cancer cell mass spec data has to be generated, this can be extended as necessary. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The authors have dealt with most of the points that were raised by this referee and provide modified figures, text and some additional data. As stated before, this is an interesting manuscript that suggests a novel regulatory level of E2F1. There are two points that require clarification:

Major point:

- This referee requested proof of arginine methylation on R109, 111, 113 in live cells. "...It seems mandatory to convincingly show that methylation of the triple R (or any of these residues) occurs in cells (e.g. by mass spectrometry, as in their previous p53 paper). Data obtained with antisera and E2F1 mutations are not sufficient here (see below)..." (of previous review). The authors now show by mass spectrometry that a GST-construct expressed in bacteria can be methylated in vitro (residues 91-113). The authors do not mention in the main text or in the Figure legend that mass spectrometric evidence, as presented in SI Figure 1G, is based on an in vitro experiment. This becomes only evident after reading the Material and Methods section (page 24 bottom; there is also a typo in this paragraph: LDS, should be SDS). The authors thus skirt around the very important issue of providing direct evidence for in vivo methylation of E2F1. What one would like to see is the occurrence of methylated E2F1 R109, 111,103 (or any of these residues) in tumor cells that express high PRMT5 level, as suggested by the authors.

Minor point:

- Figure 5C was previously criticized by this reviewer and the authors claim in their rebuttal letter that in the revised version a clearer increase of E2F1 levels upon PRMT5 depletion is now shown in

Figure 6C. However, in my copy previous Figures 5C and revised Figure 6C do look identical.

Referee #4

Review of EMBOJ-2011-77754R1

This manuscript describes the identification and characterization of arginine methylation of the transcription factor E2F1. This is a novel and timely discovery by these researchers and they demonstrate a clear link between the modification and the functional properties of E2F1. In this regard, the work is novel and important. The response to previous reviewers seems relatively thorough and my assessment is that only minor revisions are needed for publication. They are listed below:

Items to revise:

1) On page 3 the authors state, 'E2F1-/- mice suffer from an increased incidence of tumors, ...' they should cite Yamasaki et al. Cell 1996, not the parallel Field et al. report, because Field et al. didn't follow the mice long enough to see tumors.

2) Also on page 3 they state, 'In DNA damaged cells, E2F-1 is induced...' The appropriate references for this are Blattner et al. MCB 1999, and Hofferer et al. NAR 1999.

3) Near the top of page 6 they state that mutation of R109K causes a modest reduction in methylation, but there is little data to support it in Figure 1 and the authors themselves investigate effects of mutation and modification at the other two sites almost exclusively. They should conclude that mutating R109K has no detectable effect.

4) Quantification of tissue staining is suspect because of the lack of statistical analysis of the data. Given that staining is categorized into discontinuous variables (eg. high vs. low) it is better to express the data as proportions (5 out of 10 vs. 1 out of 10) whose differences can be compared with a chi-squared test and whose significance can be assigned a P value. As it is, it is not clear if the different percentages shown for each compared category are meaningful or merely observed by chance.

5) In the discussion on page 17 the authors suggest that increased E2F1 expression can have a negative growth effect. I am unaware of any study that demonstrates a requirement for E2F1 in growth arrest, the authors also don't offer a reference for this statement. There is considerable data to indicate that increased E2F1 levels contribute to apoptosis, so the authors should edit this portion of the discussion to remove any mention of growth inhibition and just refer to previous work on apoptosis.

2nd Revision - authors' response 02 December 2011

Referee 1:

The referee indicated that we had dealt with most of his/her points within the existing revisions. However, the referee commented that it was not sufficient to present mass spectrometry data derived from *in vitro* methylation of GST-E2F-1. Consequently, we have put considerable effort into mapping arginine methylation on E2F-1 expressed in MCF7 cells, and present the new data in the revised Figure 2h. The tandem mass spectrometry analysis shown here establishes that E2F-1 purified from tumour cells is methylated at R111 and R113; similar mass spectrometry data were obtained from E2F-1 in U2OS cells (data not shown).

We have noted the comment made by the referee about the previous Figure 5c and, as advised by yourself, provided in the revision quantitation of the data (detailed in SI Figure 1f legend).

Referee 4:

We have added the references and cited the literature suggested by the referee (see page 3). Further, as recommended, the text has been modified to accommodate the minimal methylation that we observed of R109 (first paragraph, P.6). The referee's point relating to tissue immunostaining is well taken, and we have revised the data, as recommended, and performed statistical analysis on relevant data (see revised legend for Figure 7d and e). The basis of the scoring system for the tumour biopsies is described in SI Materials and Methods.

In his/her final comment, the referee asked us to modify the discussion on negative growth control by E2F-1. In response, we have revised the discussion to accommodate the referee's concerns, and also cited previous literature which describes a role for E2F-1 in negative growth control (p.16; Lee and Farnham, 2000; Wang et al, 2007).

Finally, we have provided uncropped data for the key blots in the manuscript (in SI Figure 3 of the revised manuscript).

