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E2F1 mediates DNA damage and apoptosis via HCF-1 and the MLL family of histone methyltransferases

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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

10 March 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I am sorry that it has taken us longer than usual to get back to you with a decision, which was due to delayed referee reports as well as the fact that somewhat conflicting opinions of the three referees necessitated additional consultations with the experts before eventually reaching a conclusion. As you will see, two of the referees find your results interesting and potentially suited for publication pending satisfactory revision of a number of important issues, while referee 1 raises more serious concerns especially with regard to the novelty and advance over your earlier study on this topic.

Following additional consultations, we have come to the conclusion that we should, in light of the positive overall evaluation of referees 2 and 3, be happy to consider a revised version of the manuscript for publication should you be able to address the most salient concerns of the referees. However, given the current novelty issues, I have to make clear that successful revision would in this case entail a significant extension of the study along the lines suggested by the referees, including new experiments. I am aware from your correspondence that this may not be easy for you to achieve given your co-author's leaving the lab - I would thus also understand it if you were to rather decide to publish the manuscript rapidly and without any significant changes elsewhere. In light of these special circumstances, it might be a good idea if you would get back to me after having carefully gone through the referees' comments, and suggest which issues you might be able to address (and how) within the limited amount of time left to your co-author. I would then be happy to discuss whether or not it would be sensible to try and revise the study for publication in The

EMBO Journal.

In any case, thank you for the opportunity to consider your work for publication. I look forward to hearing from you regarding your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the current study, Tyagi et al. investigate the functional role of the interaction between E2F1 and HCF-1 and the MLL-family of H3K4 methyltransferases. The authors show that apoptosis induced by E2F1 over-expression is in part dependent on the HCF-1 binding sequence. Tyagi et al. also provide evidence that DNA damage and S-phase entry stimulated by E2F1 expression are dependent on the interaction. The authors further show that HCF-1, MLL, WDR5 are recruited along with E2F1 to the promoters of pro-apoptotic genes after adriamycin treatment correlating with increased levels of H3K4me3, and that HCF-1 is required for full expression of these genes.

The same group has previously mapped the interaction between E2F1 and HCF-1 and shown that HCF-1 along with MLL/SET1 is recruited to E2F1 responsive promoters at the G1/S transition (Tyagi et al. 2007). The current study demonstrates the functional importance of the E2F1 and HCF-1 interaction. However based on the previously published data, the finding that the HCF-1 binding motif, and HCF-1 is required for E2F1-induced replication, DNA damage and apoptosis could be expected, and the novelty of the study can therefore be questioned.

The authors further show that HCF-1, MLL and WDR5 co-localize with E2F1 on pro-apoptotic promoters after Adriamycin treatment. They argue that "HCF-1 appears to be selectively involved in the transcriptional activation of pro-apoptotic genes in a DDR" as HCF-1 is not recruited to the p21 promoter after adriamycin treatment. As the level of E2F1 is also not increased on this promoter after treatment, it is unclear why the authors expect HCF-1 to be so.

Other comments:

1. Throughout the study the authors only use the transformed U2OS cell line. It would be interesting to know whether HCF-1 binding to E2F1 is required for the ability of the protein to transactivate promoters, induce S phase and apoptosis in other cell types. In fact, the authors show in this study that E2F1 is required for S phase entry in U2OS cells, whereas this is not the case in many other cell types, including mouse embryonic fibroblasts where E2F3 appears to have a more prominent role.
2. The authors have previously shown that E2F3a and E2F4 also bind to HCF-1, and it would be appropriate to analyze how the depletion of HCF-1 affect the activity of these 2 proteins, e.g. the ability of E2F3a to induce S phase and DNA damage response.
3. On several occasions the authors state that "deregulation of E2F1 leads to oncogenic DNA damage and subsequent anti-oncogenic apoptosis" (e.g. abstract, introduction), i.e. a strict order of (causal) events. The authors refer to Bartkova et al, 2005 for these statements. However, Bartkova and colleagues did not show this. They showed that overexpression of E2F1 can induce DNA damage and apoptosis. However, they did not provide a causal role between the two events. Moreover, they did not analyze the requirement for E2F1 induced DNA replication for DNA damage and apoptosis (p. 7). Bartkova and colleagues speculated that the connection could be the one, the authors state as a fact, and I recommend that the authors rephrase the manuscript accordingly.
4. Several laboratories have shown that E2F1 can induce apoptosis without its transactivation domain. The authors do not analyze transactivation mutants in their study, nor do they comment on this role of E2F1, which is hard to reconcile with this manuscript. The authors should at least discuss this in the manuscript.
5. IP experiments validating the decreased/increased interaction between the E2F1 mutants and

HCF-1 (fig. 1) should be shown.

6. ChIP experiments should be presented as "% of input" instead of "Relative Units". What is "relative units"

7. It should be mentioned in the text referring to Fig. 2F that it has been published previously that HCF-1, MLL, WDR5 co-localize with E2F1 at these promoters (Tyagi et al. 2007).

8. It should also be mentioned in the text to Fig. 1A that the comparison of E2F1 sequences between species was described in their previous paper.

Referee #2 (Remarks to the Author):

This work by Tyagi and Herr sheds some light on the ability of E2F1 to induce DNA damage response and share the properties of both an oncogene and a tumor suppressor. The experiments are quite straightforward and the results generally support the conclusions. Given that the HCF-1 and MLL methyltransferases have already been implicated in the role of E2F1 as a transcription factor, and the main conclusion here is that it is the transcription activity of E2F1 that is instrumental for causing the DNA damage and also favouring apoptosis, this raises a couple of issues that need to be clarified (see specific points below. In general, I believe this is a solid study and novel, exciting data that deserve to be considered for publication in EMBO J., provided the few critical points can be addressed.

Specific points

1. The whole paper emphasizes that there are only two options for cells to respond to DNA damage, either arrest the cell cycle to facilitate DNA repair, or trigger apoptosis. What is ignored completely, is the large evidence for cell senescence as a possible outcome, particularly for non-lymphoid cells. The senescence fate as a possible outcome after DNA damage should be mentioned (e.g. DiMicco et al., Nature 2006; Bartkova et al., Nature 2006) along with the other two options, and the intriguing possibility that senescence might be favoured in cells lacking HCF-1, for example, could be addressed directly. This could further support the authors conclusion that HCF1 is critical for the cells to take the pro-apoptotic route.

2. The authors' data are interesting but avoid the major issue of what is it about the E2F1 that leads to DNA damage under some conditions, while not under normal proliferation conditions. I agree this is a tough question, but the authors could at least examine whether the timing or quantitative aspects such as altered ratios of the factors on the selected promoters are aberrant under conditions that result in the E2F1-induced DNA damage, as compared to E2F1-induced S phase progression without DNA damage. Any results that could shed some light here would be very valuable and the authors have all the tools to address this point.

Referee #3 (Remarks to the Author):

Tyagi et al, make significant progress on the vexing question of how E2F1 promotes both DNA damage accumulation and apoptosis. They show that a region of E2F1 between the cyclin A binding and DNA binding domains interacts with HCF, a factor originally found associated with HSV VP-16. HCF, together with MLL, colocalize with E2F1 on promoters and interactions among these factors are required for E2F1-mediated DNA damage signaling and apoptosis. Tyagi et al also show preferential interactions of E2F1, HCF, and MLL with promoter regions associated with apoptosis signaling rather than a cell cycle checkpoint (p21) following drug-induced DNA damage. These results have the potential to provide significant mechanistic insight into earlier observations that E2F1 contributes to DNA damage signaling mediated by DNA damaging agents.

On whole, the experiments are well designed and the results clear. The one overall concern is that E2F1 overexpression is used to demonstrate the contribution of the HCF interacting domain and HCF binding is responsible for E2F1-mediated DNA damage signaling. Others have shown that even modest changes in E2F1 levels (2-3X increases) can activate DNA damage signaling in cells. Some evidence that HCF and MLL contribute to DNA damage signaling mediated by endogenous E2F1 is needed to address this issue.

Other concerns:

The authors conclude that the HCF-1 binding site on E2F1 is necessary for S phase induction and the interaction of E2F1 on S-phase promoter regions. However, it seems that the interaction between HCF-1 and E2F1 has a more complicated phenotype because relatively more G2/M cells (4N) are observed (Figure 1C), and more BrdU positive cell were observed (Figure 2 E) in E2F1HBMmut compared to vector alone.

The results in Figure 4 show that HCF-1 and MLL preferentially interact with promoters associated with DNA damage signaling following adriamycin treatment. However, they do not link these observations to E2F1 biochemically or phenotypically. Indeed, MLL and WDR5 interactions with p14ARF and p21 promoter regions differ from the results observed for E2F1 (Fig 4A). Some evidence linking E2F1 with HCF and MLL in contributing to DNA damage signaling following DNA damage induction is needed to tie these data to the theme of the manuscript (ie, its title).

Given that HCF-1 and MLL interact with other E2Fs besides E2F1, it unclear why these E2Fs do not also promote DNA damage accumulation or apoptosis. Some further clarification and/or model would helpful in understanding the specificity of biology associated with HCF-1/MLL/E2F1.

Additional correspondence

11 March 2009

First, thank you very much for handling our manuscript and for your thoughtful decision.

Dr. Shweta Tyagi and I have studied the reviews and considered what we might be able to accomplish in the short term. Reviewer 1 had the most concrete suggestions and so we have decided to work on addressing his or her comments.

I appreciate the opportunity to discuss with you what additional experimentation might be sufficient to consider a revised manuscript. Since, however, we are not making a decision between one experiment or another but rather doing all that we can, I suggest that Shweta go ahead and see what she can get done. Then, when we have results in hand, I can get back to you with a response to the reviewers and description of how the manuscript can be revised. My thought is to save both of us time and allow for you to make a decision with more concrete information.

Please do let me know whether this suits you or not.

Again, thank you.

Best regards,

Winship Herr

Additional correspondence

13 March 2009

Thank you for your response to my decision letter. I agree that your plan of trying to address the referees' criticisms as completely as possible, and to then see how far this goes, is a reasonable one. Since you indicated you would start with referee 1's concerns (it was not clear to me whether this meant his/her more specific 'other comments' or also included his/her more general conceptual concerns), I would just like to provide some further input and information here. As I alluded to in my original letter, my decision to consider a revised manuscript despite the major conceptual (novelty) issues of referee 1 was also based on additional consultations and advice, which in this case had come from referee 2. This referee agreed to some extent with the novelty/advance concerns but also indicated that the suggestions raised by him/herself would go a long way towards boosting

this novelty aspect if addressed successfully, and thus be rather important. In this light, may I therefore kindly suggest you also attempt to resolve the two specific points of referee 2 as completely as possible within the time constraints.

Hope this extra information is of help, and best of luck for your revision work!

Best regards,

Editor
The EMBO Journal

Additional correspondence

05 June 2009

We are preparing our revision of the above mentioned manuscript. I am happy to report that Dr. Shweta Tyagi was able to solidify our results in response to the reviews. We thank the reviewers for the suggestions. In preparing our response, however, and re-examining your e-mail below, it has become clear to me that before submitting a revision it is essential to clarify your sense as to what is essential for acceptance in the EMBO Journal.

The bottom line is that I feel we can address Reviewer 1's concerns but are not able to extend things along the lines proposed by Reviewer 2. Let me explain...

Reviewer 1 makes two important points. The first is along the lines of whether our study has sufficient general interest for publication in the EMBO Journal and the second point is some specific experimental concerns. I feel that these are very appropriate concerns. The other two reviews are generally positive although I can imagine that more enthusiasm could have helped! But the important point is that they generally don't raise critical experimental concerns but rather aspects of how the study is integrated into the current literature and the breadth of analysis.

My understanding from your e-mail below is that to allay your well-founded concerns about Reviewer 1's comments, you spoke to Reviewer 2 who then focused on his/her interests: senescence and how E2F1 induces DNA damage under only some conditions (i.e., not during normal passage from G1-to-S). These are valid interests but are simply beyond our capabilities and/or direct interests. Concerning the first point, senescence, it certainly would be of interest to know how HCF-1 might be involved in such a response in other cell types but I do not see how this raises new principles related to inappropriate expression of E2F1. I feel it is like adding a fourth leg to a three-legged stool.

It would certainly add to the story (albeit with probably up to a year's work when all is said and done) but, whatever the result, it would not change principles already established in our manuscript (e.g., that in association with E2F1, HCF-1 and MLL play both oncogenic (induce DNA damage) and tumor suppressive (Induce apoptosis) roles). Our research program focuses on the roles of HCF-1 as an integrator of promoter-specific DNA-binding proteins (e.g., E2F1 and E2F4) and chromatin modifiers (e.g., MLL histone methyltransferases and Sin3 histone deacetylases) and we simply do not have the resources to study a multitude of different ways in which different cell-types will respond to E2F1 overexpression.

Concerning the second point of how E2F1 induces DNA damage under only some conditions, I think it is very interesting and is indeed in line with our research program but even as the reviewer points out this is a tough question (we hope to answer it by CHIP-seq analyses of RNA polymerase to identify genes that are activated by E2F1 in early G1 phase and the G1-to-S transition) and is simply a whole project onto itself.

Thus, we are not in a position to address these two points of Reviewer 2 with additional experimentation and if this is a sine qua non of consideration of a revised manuscript it would indeed be best to begin again elsewhere.

We wonder, however, whether with your agreement we might see whether, by better explanation of the broad interest of the study and the additional experimentation (Reviewer 1's points 1 and 5) that we have done, we can obtain the support of the reviewers for acceptance of a revised manuscript. To give you a sense of the argumentation that we would use and how the revised manuscript will look, I attach a draft response to reviewers for your examination.

If you could let me know how you feel about our arguments, I will be most appreciative.

Best regards,

Winship

Additional correspondence

05 June 2009

Thanks for your message concerning the resubmission of your revised manuscript - it catches me while finishing up a number of things before leaving the office for two weeks from tomorrow on. I have therefore only briefly looked at your draft response and the explanations presented in your letter. In any case, it would be difficult for me to predict how the referees would respond in light of your argumentation and of the new data you have obtained. While it is true that referee 2's positive opinion had tipped the balance towards me inviting a revision of the manuscript, that does not necessarily mean that failure to decisively address his/her comments might make them turn more negative; likewise, I am however also not able to predict whether addressing referee 1's more specific concerns will be able to swing his/her overall more negative opinion regarding the conceptual advance of the study. In any case, I would certainly not object to seeking their opinion on your manuscript (and on the diligent responses in the spirit of the draft you provided me with) before taking a final decision. At this stage, I feel there is nothing to be lost by sampling the reviewers' enthusiasm for a revised version, and should such an attempt fail, you could still try from the start elsewhere.

In case you agree with this course of action, I would like to suggest you submit your revised version and response letter (using the URL in my original decision letter), and upon my return I will try to get the original referees to look at it again, and we'd take it from there.

Hope that this helps - if you would need further discussion/clarification I am afraid this would have to wait until my return as well.

With best regards,

Editor
The EMBO Journal

1st Revision - authors' response

03 July 2009

Response to reviewers evaluations:

We thank the three reviewers for their very helpful comments. We feel that, in responding to the reviewers' comments, the manuscript has greatly improved. To aid the reviewers in evaluating the revision, we first give a brief global description of the changes made to the manuscript and then describe point by point how we have addressed each of the reviewers' comments.

Overall description of modifications made to the manuscript in response to the reviewers'

comments:

Except for some specific points addressed below, the reviewers were generally pleased with the quality of the work and yet made some specific recommendations, which led to further experimentation. In particular, we have made the study more definitive by analyzing the ability of the recombinant wild-type and mutant E2F1 proteins to associate with endogenous HCF-1 (new Figure 2B) and by analyzing the ability of the E2F1 mutants to induce apoptosis in two additional cell lines: a second p53 and pRb positive cancer cell line (MCF7) and a primary embryonic cell line (IMR90tert) (new Figure 3). The association studies showed that HCF-1 interacts well with all the HCF-1 binding motif (HBM)-containing E2F1 proteins but not with the HBM mutant. And the apoptosis induction assays showed that the second cancer-cell line MCF7 reacted to the E2F1 mutants as do the U2OS cells initially described whereas the non-transformed IMR90tert cells also displayed HBM-dependent apoptosis but not increased apoptosis with the E2F1 VP16HBM mutant.

To accommodate the new data and to make the study more accessible to the general reader, we have reorganized the figures as follows:

Old	New
Figure 1A	Figure 1
Figure 1B	Figure 2A
-----	Figure 2B
Figure 1C-E	Figure 2C-E
-----	Figure 3
Figure 2A-D	Figure 4A-D
Figure 2E + F	Figure 5A + B
Figure 3	Figure 6
Figure 4	Figure 7

We feel that the most serious reviewer comments pertained to the general interest of the study. To emphasize better the general interest we have extensively revised the manuscript to bring out points that we feel were lost in the original version as well as, along the lines suggested by the reviewers, to better place the study in the context of published studies.

Author response to specific referee remarks. (Original review remarks are reproduced in quotes.)

Referee #1 :

"In the current study, Tyagi et al. investigate the functional role of the interaction between E2F1 and HCF-1 and the MLL-family of H3K4 methyltransferases. The authors show that apoptosis induced by E2F1 over-expression is in part dependent on the HCF-1 binding sequence. Tyagi et al. also provide evidence that DNA damage and S-phase entry stimulated by E2F1 expression are dependent on the interaction. The authors further show that HCF-1, MLL, WDR5 are recruited along with E2F1 to the promoters of pro-apoptotic genes after adriamycin treatment correlating with increased levels of H3K4me3, and that HCF-1 is required for full expression of these genes.

"The same group has previously mapped the interaction between E2F1 and HCF-1 and shown that HCF-1 along with MLL/SET1 is recruited to E2F1 responsive promoters at the G1/S transition (Tyagi et al. 2007). The current study demonstrates the functional importance of the E2F1 and HCF-1 interaction. However based on the previously published data, the finding that the HCF-1 binding motif, and HCF-1 is required for E2F1-induced replication, DNA damage and apoptosis could be expected, and the novelty of the study can therefore be questioned."

We appreciate the concern of the reviewer for the expected nature of the study. We argue below, however, that while certainly coherent, the results are not necessarily expected and that furthermore they represent a molecular understanding and clarity that well surpasses the norm of the field of E2F factor activation of cellular processes. In relation to expectation, our previous results showed that HCF-1 association with E2F proteins is cell-cycle regulated: thus HCF-1 associates with E2F4 in S phase and early G1 but not in late G1 and the G1-S phase transition, and HCF-1 associates with E2F1 predominantly in the G1-S phase transition and S phase and not during the early G1 phase and

little in late G1. Given the cell cycle-specific HCF-1-E2F factor associations, we do not feel that, except in retrospect, one could argue that E2F1 activities when expressed at inappropriate times in the cell cycle would necessarily depend on HCF-1 and the MLL family of histone methyl transferases for activity.

We also feel that the finding that changing the HCF-1 binding site in E2F1 to the herpes simplex virus VP16 sequence improves E2F1's ability to promote apoptosis is exceptional. We feel that it is an important result because it suggests that the E2F1-HCF-1 interaction is limiting for apoptosis and thus an attractive target for modulation of E2F1 function. Lastly, we note that our results indicate that E2F1 with HCF-1 selectively regulates the apoptotic response vs cell-cycle arrest. As a result of the reviewer's comments, these novel elements are clarified and emphasized better in the revised manuscript.

We also feel that it is important to point out that, compared to mechanisms of E2F factor repression with for example pRb, at a molecular level relatively little concrete has been known about the function and precise interaction of critical cofactors essential for E2F1 activation of processes such as cell-cycle progression, DNA damage, and apoptosis, which are key aspects of cancer progression.

"Review 1 (cont.): The authors further show that HCF-1, MLL and WDR5 co-localize with E2F1 on pro-apoptotic promoters after Adriamycin treatment. They argue that "HCF-1 appears to be selectively involved in the transcriptional activation of pro-apoptotic genes in a DDR" as HCF-1 is not recruited to the p21 promoter after adriamycin treatment. As the level of E2F1 is also not increased on this promoter after treatment, it is unclear why the authors expect HCF-1 to be so."

We thank the reviewer for pointing out this discrepancy. We should have stated that E2F1 and HCF-1 appear to be selectively involved together. The point remains that, by apparently other means, MLL and WDR5 are recruited to the p21 promoter. These results underline a tighter link between E2F1 and HCF-1 as opposed to with the MLL methyltransferases. This is now clarified in the revised manuscript.

Other comments:

"1. Throughout the study the authors only use the transformed U2OS cell line. It would be interesting to know whether HCF-1 binding to E2F1 is required for the ability of the protein to transactivate promoters, induce S phase and apoptosis in other cell types."

In our Tyagi et al. 2007 Molecular Cell paper, we showed that HCF-1 is important for activation of E2F1 promoters at G1/S and our laboratory has previously shown that loss of HCF-1 leads to a G1 arrest in HeLa cells (Julien and Herr, 2003). Encouraged by this reviewer, we now show that, as in U2OS cells, the E2F1 HCF-1 binding site is critical for induction of apoptosis in the transformed MCF7 cell line and nontransformed IMR90(tert) cell line. As in U2OS cells, in MCF7 cells we observe the enhanced ability of the E2F1VP16HBM mutant to promote apoptosis (new Figure 3).

"In fact, the authors show in this study that E2F1 is required for S phase entry in U2OS cells, whereas this is not the case in many other cell types, including mouse embryonic fibroblasts where E2F3 appears to have a more prominent role."

Pathways for transcriptional regulation are often cell-type-specific. We cannot and do not argue that E2F1 and HCF-1 are critical for S-phase entry in all cell types. In the MEF example given here, we note with interest that E2F3a also associates with HCF-1 (see Tyagi et al. 2007 and reviewer comment below) and thus in some cell types HCF-1 could be involved in promoting cell-cycle progression through different E2F partners. Such a finding, however, is outside the scope of this study.

"2. The authors have previously shown that E2F3a and E2F4 also bind to HCF-1, and it would be appropriate to analyze how the depletion of HCF-1 affect the activity of these 2 proteins, e.g. the ability of E2F3a to induce S phase and DNA damage response."

The strength of the current study is that E2F1 has a well-characterized dominant effect when deregulated (it induces DNA damage and apoptosis), which can be analyzed. E2F4 is involved in

repression of transcription and in very different aspects of cell cycle control than E2F1. The E2F3 gene produces two E2F proteins (E2F3a and E2F3b), which appear to play opposing roles in transcriptional regulation and cell cycle control. To analyze these proteins would require separate studies.

"3. On several occasions the authors state that "deregulation of E2F1 leads to oncogenic DNA damage and subsequent anti-oncogenic apoptosis" (e.g. abstract, introduction), i.e. a strict order of (causal) events. The authors refer to Bartkova et al, 2005 for these statements. However, Bartkova and colleagues did not show this. They showed that overexpression of E2F1 can induce DNA damage and apoptosis. However, they did not provide a causal role between the two events. Moreover, they did not analyze the requirement for E2F1 induced DNA replication for DNA damage and apoptosis (p. 7). Bartkova and colleagues speculated that the connection could be the one, the authors state as a fact, and I recommend that the authors rephrase the manuscript accordingly."

We thank the reviewer for pointing out this error, which we have corrected.

"4. Several laboratories have shown that E2F1 can induce apoptosis without its transactivation domain. The authors do not analyze transactivation mutants in their study, nor do they comment on this role of E2F1, which is hard to reconcile with this manuscript. The authors should at least discuss this in the manuscript."

We thank the reviewer for this helpful comment. We feel, however, that given that we show that the HCF-1 binding site, which lies outside the transactivation domain, promotes apoptosis that it is reconcilable that E2F1 transactivation domain mutants retain proapoptotic activity. Indeed, we suggest that our results clarify why the transactivation domain mutants can still activate apoptosis since they can still associate with HCF-1. We now incorporate this point into the manuscript.

"5. IP experiments validating the decreased/increased interaction between the E2F1 mutants and HCF-1 (fig. 1) should be shown."

We thank the reviewer for suggesting this experiment. The data are now presented in Figure 2B. These results clearly show that HCF-1 association with E2F1 is HBM dependent. The co-immunoprecipitation does not necessarily show (nor does it negate) increased association of HCF-1 with the E2F1VP16HBM mutant. The significance of the E2F1VP16HBM mutant came from studies with yeast two-hybrid analyses, in which we showed that the E2F1VP16HBM mutant has a relaxed interaction with E2F1 (Tyagi et al., 2007). In revising the manuscript, we have now taken care not to translate this relaxed interaction into higher affinity interaction. The point remains that we have been able to change 8 out of 10 positions within and surrounding the HBM by replacing the wild-type E2F1 sequence with the viral VP16 sequence and in so doing increase the ability of E2F1 to activate apoptosis.

"6. ChIP experiments should be presented as "% of input" instead of "Relative Units". What is "relative units?" "

We thank the reviewer for pointing this out. We have now defined the term "Relative Units" for both ChIP experiments in Figure 5B and 7A in the manuscript.

In Figure 5B, real-time PCR of E2F1, HCF-1, MLL, WDR5, and H3K4 trimethylation (TriMe) ChIP analyses of U2OS cells transfected with plasmids encoding empty vector, E2F1, or E2F1HBMmut, was done in triplicate. Real-time PCR quantification of ChIP DNAs was done using Delta Relative CT Quantification, where the values are calculated relative to input as follows: $\Delta CT = CT(\text{input}) - CT(\text{sample})$. (0.3% of total ChIP input DNA was used in the input amplification.) To determine the relative quantity of HCF-1, MLL, and WDR5 recruited with the empty vector, or E2F1 or E2F1HBMmut expression vectors, the signals in cells immunoprecipitated using the anti-E2F1 antibody are set to 1 for each transfected sample with all other signals (i.e. HCF-1, MLL, WDR5, and H3K4 trimethylation) in that transfected sample indicated as relative-fold differences. Hence "Relative Units" denote the enrichment of that protein relative to E2F1.

In Figure 7A, real-time PCR quantification of ChIP DNAs was done as in Figure 5A. Relative Units = $2^{\Delta CT}$.

"7. It should be mentioned in the text referring to Fig. 2F that it has been published previously that HCF-1, MLL, WDR5 co-localize with E2F1 at these promoters (Tyagi et al. 2007)."

Certainly and we were not the first to describe E2F1 binding to these promoters as made clear now in the text. Here, we use these characterized promoters to study E2F1-HCF-1 interaction in the cell.

"8. It should also be mentioned in the text to Fig. 1A that the comparison of E2F1 sequences between species was described in their previous paper."

Agreed. This figure is to aid the reader by way of introduction to the mutants and position of elements described in the text.

Referee #2:

"This work by Tyagi and Herr sheds some light on the ability of E2F1 to induce DNA damage response and share the properties of both an oncogene and a tumor suppressor. The experiments are quite straightforward and the results generally support the conclusions. Given that the HCF-1 and MLL methyltransferases have already been implicated in the role of E2F1 as a transcription factor, and the main conclusion here is that it is the transcription activity of E2F1 that is instrumental for causing the DNA damage and also favouring apoptosis, this raises a couple of issues that need to be clarified (see specific points below). In general, I believe this is a solid study and novel, exciting data that deserve to be considered for publication in EMBO J., provided the few critical points can be addressed.

"Specific points

"1. The whole paper emphasizes that there are only two options for cells to respond to DNA damage, either arrest the cell cycle to facilitate DNA repair, or trigger apoptosis. What is ignored completely, is the large evidence for cell senescence as a possible outcome, particularly for non-lymphoid cells. The senescence fate as a possible outcome after DNA damage should be mentioned (e.g. DiMicco et al., Nature 2006; Bartkova et al., Nature 2006) along with the other two options, and the intriguing possibility that senescence might be favoured in cells lacking HCF-1, for example, could be addressed directly. This could further support the author's conclusion that HCF1 is critical for the cells to take the pro-apoptotic route."

We thank the reviewer for this comment from which we have now better integrated our study into the current state of the field. We have thus revised the manuscript to incorporate the issue of senescence as a response to oncogene activity and DNA damage, an outcome that we do not address in our study. A study of the role of HCF-1 in senescence would be interesting but it is not one that we are able to address in the context of this manuscript. To our knowledge most of the studies so far linking E2F factors to senescence focus on repression of E2F function with for example pRb, not activation as the case of our study with HCF-1.

Concerning HCF-1, we note with interest that loss of HCF-1 does not lead to senescence in the temperature sensitive cell line tsBN67. tsBN67 cells harbor a point mutation in the HCF-1 gene that causes the cells to arrest at the nonpermissive temperature but does not cause senescence as the cells are able to re-enter the cell cycle once they are returned to the permissive temperature (Goto et al., Genes Dev. 1997). Thus, it is indeed possible that HCF-1 also plays a role in oncogene-induced senescence, thus playing a role in both oncogenic defense pathways, apoptosis and senescence. Elucidating such a possibility is beyond the scope of this manuscript.

"2. The authors' data are interesting but avoid the major issue of what is it about the E2F1 that leads to DNA damage under some conditions, while not under normal proliferation conditions. I agree this is a tough question, but the authors could at least examine whether the timing or quantitative aspects such as altered ratios of the factors on the selected promoters are aberrant under conditions that result in the E2F1-induced DNA damage, as compared to E2F1-induced S phase progression without DNA damage. Any results that could shed some light here would be very valuable and the authors have all the tools to address this point."

We agree that this is a very interesting point. In future studies, we plan to look at the genome-wide program of gene activation (by ultra-high-throughput ChIP seq analyses) during G1-S phase progression and during inappropriate E2F1 expression to identify what might be different. We also feel, however, as the reviewer rightfully points out, that these questions are not likely to be easily addressed, and that they are simply beyond the scope of this study.

Referee #3 :

"Tyagi et al, make significant progress on the vexing question of how E2F1 promotes both DNA damage accumulation and apoptosis. They show that a region of E2F1 between the cyclin A binding and DNA binding domains interacts with HCF, a factor originally found associated with HSV VP-16. HCF, together with MLL, colocalize with E2F1 on promoters and interactions among these factors are required for E2F1-mediated DNA damage signaling and apoptosis. Tyagi et al also show preferential interactions of E2F1, HCF, and MLL with promoter regions associated with apoptosis signaling rather than a cell cycle checkpoint (p21) following drug-induced DNA damage. These results have the potential to provide significant mechanistic insight into earlier observations that E2F1 contributes to DNA damage signaling mediated by DNA damaging agents.

"On whole, the experiments are well designed and the results clear. The one overall concern is that E2F1 overexpression is used to demonstrate the contribution of the HCF interacting domain and HCF binding is responsible for E2F1-mediated DNA damage signaling. Others have shown that even modest changes in E2F1 levels (2-3X increases) can activate DNA damage signaling in cells. Some evidence that HCF and MLL contribute to DNA damage signaling mediated by endogenous E2F1 is needed to address this issue."

There may be a misunderstanding of how we did our E2F1-mediated DNA damage experiments. We created stable cell lines that express the E2F1-estrogen receptor fusion proteins at levels equal to or twofold the level of the native protein and induced E2F1 activity by addition of the innocuous drug OHT. Thus, we are exactly within the range desired by the reviewer.

"Other concerns:

"1/ The authors conclude that the HCF-1 binding site on E2F1 is necessary for S phase induction and the interaction of E2F1 on S-phase promoter regions. However, it seems that the interaction between HCF-1 and E2F1 has a more complicated phenotype because relatively more G2/M cells (4N) are observed (Figure 1C), and more BrdU positive cell were observed (Figure 2 E) in E2F1HBMmut compared to vector alone."

Although our data indicate that HCF-1 association with E2F1 is important for cell cycle progression, induction of DNA damage, and an apoptotic response to DNA damage, we do not argue that HCF-1 may not be involved in other activities of E2F1 as well. We note that E2F factors have also been shown to have functions in the G2 phase and we are intrigued by the idea that these functions could be involved in the roles observed for HCF-1 in mitosis and cytokinesis.

"2/ The results in Figure 4 show that HCF-1 and MLL preferentially interact with promoters associated with DNA damage signaling following adriamycin treatment. However, they do not link these observations to E2F1 biochemically or phenotypically. Indeed, MLL and WDR5 interactions with p14ARF and p21 promoter regions differ from the results observed for E2F1 (Fig 4A). Some evidence linking E2F1 with HCF and MLL in contributing to DNA damage signaling following DNA damage induction is needed to tie these data to the theme of the manuscript (ie, its title)."

To our eye, MLL and WDR5 interactions with the p14ARF promoter are the same as observed for E2F1 in response to adriamycin (new Figure 7A); in each case the interactions increase with administration of adriamycin. And as stated above, the association of E2F1 and HCF-1 with the p21 promoter goes in parallel but interestingly differs from MLL and WDR5. We link E2F1 with HCF-1 and MLL in contributing to DNA damage signaling by showing that loss of HCF-1 and WDR5 lead to a reduction in apoptosis in response to adriamycin (new Figure 7C).

"3/ Given that HCF-1 and MLL interact with other E2Fs besides E2F1, it unclear why these E2Fs do not also promote DNA damage accumulation or apoptosis. Some further clarification and/or model would helpful in understanding the specificity of biology associated with HCF-1/MLL/E2F1."

We do not at present have a definitive answer to this point. We do note, however, that when HCF-1 associates with E2F4 the repressive Sin3 histone deacetylase is present; we suggest that the E2F4-HCF-1 complex is involved in other processes than activation of apoptosis. We additionally note that the E2F3a and E2F3b proteins possess a non-canonical HCF-1 binding motif, which may direct different E2F factor functions. Encouraged by the reviewer, we have included these points in the revised discussion.

In conclusion, we agree completely that there are still many interesting issues to be addressed in how E2F1 promotes cell-cycle progression, DNA damage, and apoptosis. We sincerely hope, however, that the reviewers and editor will agree that our current study makes an important contribution to this issue.

Decision letter

22 July 2009

Thank you for submitting your revised manuscript. It has now been seen once more by the original referee 1, and I am happy to inform you that s/he considers the main concerns adequately addressed and has no further objections to its publication in The EMBO Journal. The only two minor points remaining pertain to interpretation/discussion of the findings in light of the literature. The simplest way to deal with these issues (see below) would be for you to effect these minor changes in the manuscript text, and return the modified text document by email as soon as possible. We would then replace the manuscript file in the system and after that should be able to swiftly proceed with the acceptance and production of your study!

With best regards,

Editor
The EMBO Journal

Referee 1:

The authors have nicely addressed the comments by the reviewers, and I can recommend publication.

I have two comments however:

1. As noted in my previous review, the Bartkova paper from 2005 only addresses what can happen, when E2F1, CDC25A and Cyclin E are overexpressed, they do not show that pRb inactivation leads to DNA double-strand breaks. This is actually an easy experiment with the availability of the Rb loxP/loxP MEFs, however, to my knowledge this finding has not been published. Unless the authors know of an appropriate reference for this experiment, I suggest they do not over-interpret the Bartkova paper.

2. In response to reviewer #2 the authors state: "To our knowledge most of the studies so far linking E2F factors to senescence focus on repression of E2F function with for example pRb, not activation as the case of our study with HCF-1". This is correct, but the concept that E2F1 will induce senescence by activating ARF expression is quite straightforward, since E2F1 has been shown to activate ARF, and ectopic expression of ARF induces senescence. This has both been shown in vitro and in vivo in the following papers:

Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor. Dimri GP, Itahana K, Acosta M, Campisi J. Mol Cell Biol. 2000 Jan;20(1):273-85.

Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. Lazzerini Denchi E, Attwooll C, Pasini D, Helin K. Mol Cell Biol. 2005

Additional correspondence

05 August 2009

I thank you again for the rapid handling of our revised manuscript. I am now back from my holidays and have been able to revise the manuscript. As you have suggested, I am attaching the revised manuscript to this e-mail.

Concerning point 1 of Reviewer 1, we now have added an additional reference:

Pickering MT, Kowalik TF. (2006) Rb inactivation leads to E2F1-mediated DNA double-strand break accumulation. *Oncogene* 25: 746-755

This paper directly shows that disruption of Rb leads to E2F1-mediated DNA double strand breaks. We thank the reviewer for alerting us to the fact that the Bartkova paper did not address this specific aspect. (See modification to the end of the first paragraph of the Introduction.)

Concerning point 2, this pertains to our argumentation concerning Reviewer 2's comments and does not concern the paper directly. We thank the reviewer for this information but since it does not pertain to the paper we have not modified the paper concerning this point.

Please do let me know if there is anything additional that we should do.