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The SNF2-like helicase HELLS mediates E2F3-dependent transcription and cellular transformation

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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 04 July 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports from three expert reviewers, which are copied below. The referees on the whole all appreciate the potential importance of implicating HELLS as a new E2F3 cofactor and acknowledge the comprehensive nature of your work. Nevertheless, especially referees 2 and 3 raise a number of substantive concerns regarding both the experimental analysis and its presentation/interpretation in the current state. Furthermore, the referees are also concerned that the large amount of data currently included not only fosters possible internal discrepancies, but also potentially distract from the main message that HELLS contributes to E2F3-mediated transcription and proliferation.

In light of the referees comments, we shall be open to consider a substantially revised and restructured manuscript further for publication. In this respect, although we realize that the study is already very data-heavy, additional experimental data will nevertheless be required to provide a sufficiently definitive basis for the main conclusions; at the same time, we however also feel that not all points of the referees would need exhaustive addressing within the scope of the current submission, and that some data could potentially be taken out of the present study.

Key points that would need to be addressed for a successful revision would be:
- to clarify whether E2F3 and HELLS interact directly or only indirectly/via DNA, by following the experimental suggestions of referees 2 and 3
- to clarify the current discrepancies between the ChIP results and the ChIP-seq datasets, with the latter also requiring improved presentation

- to clarify the impact of HELLS expression on S-phase entry/progression (along the lines asked by referees 2 pt 4 and ref 3 pt c). A related request by referee 1 is to discuss how HELLS knockdown cells could accomplish a (delayed) S-phase entry in the absence of E2F3 target gene expression
- with regard to the somewhat counterintuitive identification of HELLS - previously assigned mostly co-repressive function - as a new transcriptional 'co-activator', we strongly feel that at least some thoughtful discussion on the possible mechanistic explanations and implications will need to be added to the manuscript

On the other hand, points that may not need to be directly addressed (but will nevertheless have to be diligently answered and discussed) are:
- In-depth ChIP analysis to dissect respective E2F3A/B roles - however, it will be important to clarify the referees' more specific questions regarding the current indiscriminate use/discussion of the two functionally distinct isoforms!
- adding genome-wide mRNA expression data and histone modification analysis
- extensive follow-up on the HELLS/E2F3 connection with MLL and in prostate cancer cells (e.g. ref 2 pt 5, pt 8, ref 3 pt 3). However, we also feel that at least the prostate cancer overexpression data criticized by referee 3 should not be completely removed from the manuscript, as we do think they add important supporting physiological evidence.

When preparing a revised manuscript, please also carefully proofread and edit the manuscript both regarding presentation/accessibility and spelling/grammar, trying to avoid use of informal narrative as much as possible.

Regarding the more specific presentational and technical points, please also try to adequately answer/clarify all of them in a revised manuscript and response letter. I should point out that it is our policy to allow only a single round of major revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Therefore, should you have any concerns regarding the comments and requests by the reviewers, I would encourage you to get back to me for further consultations. Should you have good suggestions for removing certain non-essential data to streamline the manuscript, I would also appreciate if you could briefly discuss such proposals with us before resubmission.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised 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.

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

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript by von Eyss et al. presents novel roles for HELLS in co-regulation of E2F3-

dependent transcription and establishes a new connection between these two transcription factors in tumorigenesis. After identification of HELLS as a potential E2F3 binding partner by mass spectrometry, the authors go on to thoroughly and compellingly demonstrate via multiple approaches the physical binding of HELLS with E2F3 and that numerous E2F3-dependent transcriptional targets are co-dependent on HELLS and E2F3. For example, they show that overexpression of E2F3 is unable to activate target genes or transform cells in the absence of HELLS. Importantly, the manuscript supports a previously unreported role for cooperation between HELLS and E2F3 in prostate cancer, and a potential mechanism through MLL1 for HELLS/E2F3 driven tumorigenesis. Overall, experiments are comprehensive, rigorous and physiological, and conclusions drawn are well supported. However, there are minor issues to be addressed:
1. The Title of the manuscript undersells the discoveries made: how about "The SNF2-like HELLs mediates E2F3-dependent transcription and cellular transformation".
2. On page 3 in the Intro, they state that "loss of Rb and amplification of E2F3 are obligate events for tumorigenesis", but this cannot be concluded from these associations.
3. Using chimeras between E2F1 and E2F3, they show that the 331333 chimera (with only the coiled-coil domain of E2F3 replaced by that of E2F1) fails to bind HELLS. Ideally, testing the 113111 chimera would determine whether the E2F3 coiled-coil domain is sufficient for HELLS binding. But in lieu of this, the authors need to recognize that the 331333 chimera could have structural defects. If they showed that the 331333 chimera could still bind Rb or DNA, this concern would be alleviated.
4. Page 8, top paragraph: they refer to Figure 1D in last line, but this should be to Figure 1H.
5. In showing HELLS enrichment at promoters of E2F3-dependent target genes, Bmyb is shown in Figure 2E, but should also be referred to as a E2F3-dependent target gene in the body of text.
6. On page 13, referring to Suppl Fig S4A,B, they state that there "is a general trend for E2F3 and HELLS co-expression" in other cancers, but the data presented do not indicate which tumors show co-expression.
7. The ChIP-seq data are important and appear quite supportive, but the Results section describing these results is hard to follow. It is clear that E2F3 and HELLS "share a largely overlapping target set", which is important, but some of the other relationships are difficult to understand (such as with H3K27me3).
8. Another discrepancy that the authors should address is that HELLS knockdown cells do enter S-phase, but with substantial delay, and yet E2F3 target genes are not induced at timepoints that are after this delayed S-phase entry (Fig 3). So unless the authors want to argue that the cells make it into S phase without E2F targets like CDC6, they need to discuss how the cells are doing it (i.e. is a little CDC6 enough?).
9. There are multiple grammar/punctuation errors throughout.

Referee #2 (Remarks to the Author):

In the proposed manuscript ÑThe SNF2-like HELLS interacts with E2F3 and is critical to allow S-phase entry" the authors identify HELLS as a novel binding partn er of E2F3 and fine map the interaction domains of the two proteins. In the following experiments both HELLS and E2F3 were found to co-localize to the promoters of known E2F3 target genes. The expression of the E2F3 target genes was obstructed by the down-regulation of HELLS, which also blocked the re-entry in the S-phase of the cell cycle. Since E2F3 up-regulation is known to be associated with various tumors, the examination of biopsies of prostate cancer patients revealed a positive correlation in the expression of HELLS and E2F3. HELLS appears to be a permissive factor for the E2F3 tumorigenicity and down-regulation of HELLS in an aggressively growing prostate cancer cell line transfected with E2F3 leads to a lesser number of colonies in the soft agar assay. A genome-wide ChIP study showed that E2F3 and HELLS co-occupy a significant number of promoters of active genes, among which the histone methyltransferase MLL1.

Major points

1. Despite a detailed study of the HELLS and E2F3B interaction domains, it does not become clear whether the interaction is direct or indirect. To clear that point, the authors will have to show a binding between recombinantly produced E2F3B and HELLS.

2. The interaction domain in E2F3B is common to both E2F3A and E2F3B, and HA-tagged versions

of both proteins were able to co-immunoprecipitate HELLS. Although both proteins have a highly related protein structure, the function overlaps only partially. Generally E2F3A is known to be expressed in a cell cycle dependent manner and to activate its target genes, whereas E2F3B is expressed during the whole cell cycle and appears to have a repressive function at specific loci (e.g. Arf). Since the authors use in all following experiments a "pan-E2F3" antibody that recognizes both E2F3A and E2F3B, there is no way to understand which one of these two proteins was ChIP-ed on which promoter. It is essential to know, which isoform - E2F3A or E2F3B - is cooperating with HELLS function and is critical for tumor proliferation.

3. For the understanding of E2F3 - HELLS cooperation and its impact on the expression of E2F target genes it is important to connect promoter occupancy, mRNA expression and protein levels in wild type cells and in HELLS knockdown experiments. The authors present in Figures 2, 3 and 4 different experiments in asynchronous and synchronized cell populations but for none of them the complete data set is shown and discussed. Additionally some results are inconsistent - results in western blots of Figure 3A and 4A for CDC6, E2F1, p107 are not the same; in Figure 3 the protein and mRNA expression of E2F1 and E2F3B do not correlate and it is impossible to compare the experiments shown in Figure 3A and 4A, since different time points after serum addition are shown.

4. When HELLS is downregulated by shRNA, T98G cells show a delayed S-phase entry compared to controls. Since the absence of HELLS leads also to a downregulation of the E2F3 protein expression, it is not clear if the S-phase entry delay is a secondary effect. In Figure 4 the authors try to address this problem with an overexpression of HA-ER-E2F3A (but not E2F3B), but they show no analysis of the cell cycle progression by BrdU labeling in presence of the ectopically expressed E2F3A. It would be also important to rescue the phenotype with an ectopic HELLS expression and to show that the interaction with E2F3 is essential for the effect, e.g. use a mutant form of the HELLS protein, which is not able to interact with E2F3.

5. E2F3 and HELLS expression levels are correlated in prostate cancer. The downregulation of HELLS decreases the transformation capacity of overexpressed E2F3. This is interesting, but in order to epistatically order E2F3 and Hells in this assay additional experiments are needed, e.g. overexpression of HELLS alone and HELLS-E2F3 combination, overexpression of HELLS mutant, which is not able to interact with E2F3.

6. In Figure 6 the authors show ChIP-seq data for HELLS, E2F3 and H3K27me3 from wild type MEFs and E2F3-/- MEFs. 22% of the HELLS occupied transcriptional start sites (TSS) are also enriched for H3K27me3. 93% of these promoters are also bound by E2F3. Contradictory to the data presented in Figure 2E, where it is shown that HELLS occupancy on p107 (i.e. rbl1) and Bmyb promotors is decreased in E2F3-/- MEFs, there is no HELLS binding to neither to the p107 promoter nor to the Bmyb promotor in the ChIP-seq data set done in the same cell lines and HELLS binding does not appear to be dependent on E2F3 concentration at all.

7. The authors present the TSS bound by HELLS and E2F in the supplementary table S3. Unfortunately, p107, which was bound by HELLS and E2F3 in a re-ChIP experiment in Figure 2C and is an essential internal positive control for the genome-wide ChIP experiment, was only identified as an E2F3 but not as a HELLS target. Additionally, it does not become clear, if all E2F target promotors used in the previously described experiments were also found in the ChIP-seq experiment. To support Chip-seq data and to discriminate between repressive or activating functions of the HELLS/E2F3 proteins, it would be necessary to complete the experiment with genome-wide mRNA expression data and for instance mapping of the H3K4 trimethylation mark.

8. HELLS knockdown leads to a roughly 50% reduction of Mll1 mRNA. Consistently, in E2F3-/- MEFs Mll1 mRNA levels are also mildly reduced (about 20%). This effect was also seen on protein levels upon HELLS knockdown but it was definitely not significant in E2F3-/- MEFs (Figure S7B). Although the authors show a cell-cycle dependent expression of Mll1 mRNA in T98G cells, they do not test the influence of HELLS and E2F3 downregulation under these circumstances. To strengthen the biological observations in the paper it would be useful to investigate the expression of Mll1 or other found HELLS/E2F3 targets in prostate cancer and in the transformation potential of prostate cancer cell lines.

Minor points

1. In figure 1B there are no GST-inputs for the respective experiments.

2. Figure S2C is mislabeled for E2F3.

Referee #3 (Remarks to the Author):

In this manuscript, von Eyss et al. have investigated the functional link between the SNF-2 like helicase HELLS and E2F3. The authors showed that HELLS interacts with E2F3 in several assays. Depletion of HELLS results in defects in S phase entry which are reminiscent of the phenotype of E2F3-/- cells. Consistently, E2F targets are not induced in HELLS depleted cells upon serum stimulation. Using ChIP, the authors showed that both E2F3 and HELLS are present on the same set of E2F target genes. Furthermore, HELLS appears to be recruited to the promoters in an E2F3 dependent manner, as overexpression of E2F3 leads to an increase of HELLS on the promoters. Conversely, HELLS is reduced at these promoters in E2F3-/- MEFs. The authors further show that HELLS itself is an E2F3 target which perhaps explains why HELLS, like E2F3, is overexpressed in tumors of prostate cancer patients. In the last part of the manuscript, the authors performed genome-wide analysis of HELLS and E2F3 targets and found that more the majority of HELLS targets are also bound by E2F3. However, HELLS binds to a region downstream of TSS while E2F3 is present at the TSS. Additionally, HELLS occupancy does depend on E2F3 as evident from ChIP-seq analysis in E2F3-/- MEFs.

Deciphering the role of chromatin remodeling proteins, such as HELLS, in E2F dependent transcription is an important topic. In this regard, the findings are likely to be of interest for readers working in the filed of cell cycle control. This manuscript clearly represents a large amount of work and the authors make an effort to dissect the functional link between HELLS and E2F3. However, there are several major issues which, in my opinion, preclude publication of this work in EMBO Journal.

Firstly, the authors fail to convincingly demonstrate direct protein-protein interaction between HELLS and E2F3. The authors identified HELLS in Mass Spec analysis of E2F3b associated proteins and confirmed the interaction by IP/western and in GST pull-down experiments. However, the results of the IP/western experiment are questionable since the interaction appears to be highly dependent on the presence of DNA as it is disrupted by EtBr (compare Fig. 1H and Fig. S1D). The interpretation that DNA mediates some or perhaps most of interaction between HELLS and E2F3 would be consistent with the ChIP-seq data where E2F3 and HELLS are present on the same target promoter but localized to distinct regions within these promoters (Fig. 6D). The authors need to either abandon their claim of direct protein-protein interaction between HELLS and E2F3 or provide more convincing data to support it. Specifically, it is important for authors to confirm interaction between endogenous E2F3 and HELLS in a reciprocal IP (IP through HELLS antibody and WB with E2F3 antibody) and to test the effect of EtBr in these settings. The authors should also consider using DNAse treatment in IP-Western as alternative to EtBr.

Secondly, from the analysis of HELLS and E2F3 binding to classical E2F targets in Fig. 2, the authors concluded that HELLS is recruited to these targets by E2F3 (Fig. 2) and is important for activation of E2F targets and S phase entry (Fig. 3). These conclusions are nicely supported by extensive experimental data presented in these figures. However, the ChIP-seq analysis of E2F3 and HELLS genome-wide localization paints a different picture. Although HELLS and E2F3 share >90% of common targets, the two proteins bind to distinct regions in the promoters. My even bigger concern is that in contrast to the results in Fig. 2, E2F3 has little if any role in recruitment of HELLS to its genomic targets (page 17). If this is the case, then the significance of E2F3-HELLS interaction becomes even more questionable. The authors need to reconcile their ChIP-seq data with their previous conclusions, perhaps, by examining the occupancy of HELLS on a larger set of shared targets, identified in ChIP-seq, in E2F3-/- cells.

Thirdly, it is not clear to me the significance of overexpression of HELLS in prostate cancer. The authors show that HELLS is an E2F3 target. Since E2F3 has been previously shown to be overexpressed in prostate cancer, the observation that HELLS is elevated seems rather trivial.

Fourthly, I feel that the authors are attempting to incorporate a large amount of experimental data into the manuscript and this dilutes the main message of the paper. While reading the manuscript I often found myself struggling through the logic of experiments and apparently contradicting conclusions. The manuscript needs to be streamlined. I also feel that the authors' model of how HELLS contributes to the E2F3 function changes as one reads through different sections of the results section.

Minor points:

a) There is an inconsistency in the use of two E2F3 isoforms in the experiments described in Fig. 1. The authors used E2F3b to identify interacting proteins by MS and in GST pull downs (Fig. 1A and B). However, in Fig 1F they switched to E2F3a. The authors need to use the same isoform E2F3b in this experiment to confirm the interaction (Fig. 1F). Importantly, the fact that E2F3 gene actually produces two isoforms is not described in either introduction or in the beginning of the results section. This is an important point since the two isoforms have opposing functions: one is a repressor and another is an activator. I also feel that the authors should include a rationale why they used E2F3b instead of E2F3a in the MS analysis.

b) The authors were using HA tagged E2F3a and b proteins in IP western in Figure 1G. I am puzzled why pan-E2F3 antibody was used in IP instead of anti-HA. Using pan-E2F3 antibody complicates interpretation of the results since this antibody recognizes transfected protein and both endogenous isoforms. In contrast, HA antibody would specifically recognize the transfected protein. I am also surprised by the lack of the band in the vector control lane which one expects to see if endogenous E2F3 and HELLS interact in these cells. Does it mean that such interaction is specific to HCT116 cells only (Fig. 1H)? It is also not clear from figure or figure legends which antibodies (pan-E2F3 or anti-HA) were used to detect E2F3 in the input lanes.

c) Is overexpression of HELLS sufficient to drive cells into S phase? Does HELLS drive E2F3-/- cells into S phase? If this is the case then these results might strengthen the authors' argument about functional cooperation between E2F3 and HELLS.

d) The authors mentioned that in prostate cancers E2F3 is amplified (page 3). I think that the cited paper (Foster et al 2004) refers to overexpression of E2F3. Is there evidence for increase in copy number of the E2F3 locus in human prostate cancer?

e) The authors mentioned that they detected pRB in their MS analysis of E2F3 interacting proteins and therefore their approach was suitable. Was DP, a well-known heterodimeric partner of E2F3, also present?

f) The authors used the DU145 prostate cancer cell line in colony formation assays. What is the level of E2F3 expression in this line? If E2F3 level is elevated then one expects that even without E2F3 overexpression depletion of HELLS would reduce colony formation. Perhaps, the authors should test the impact of the loss of HELLS in other prostate cancer cell lines with higher level of E2F3 overexpression.

g) It is not clear why the authors decided to choose the MLL1 gene for further analysis. The fact that there is a strong enrichment for HELLS at the MLL1 promoter does not necessarily imply that MLL1 is important for the HELLS function. Additionally, E2F3 appears to show a very weak binding to the MLL1 promoter (Figure 7B). Overall, I am not convinced that the MLL1 gene is the right model to study functional interaction between E2F3 and HELLS.

1st Revision - authors' response 27 October 2011

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The SNF2-like HELLS mediates E2F3-dependent transcription and cellular transformation

Björn von Eyss, Jonas Maaskola, Sebastian Memczak, Katharina Möllmann, Anja Schuetz, Christoph Loddenkemper, Mai-Dinh Tanh, Albrecht Otto, Kathrin Muegge, Udo Heinemann, Nikolaus Rajewsky and Ulrike Ziebold

Point-by-point responses to referees

Referee #1

1. The Title of the manuscript undersells the discoveries made: how about "The SNF2-like HELLs mediates E2F3-dependent transcription and cellular transformation".

* We are grateful for this suggestion and have used this new title instead of the old one.

2. On page 3 in the Intro, they state that "loss of Rb and amplification of E2F3 are obligate events for tumorigenesis", but this cannot be concluded from these associations.

* That is correct. We agree, that the remark from the text is only true for a subset of the human tumours, i.e. bladder tumours. It was suggested by Hurst *et al*, 2008 that in bladder cancer, tumour proliferation depends on inactivating pRB (or p16 instead) as well as overexpression of both E2F3-isoforms (achieved via amplification). Interestingly, also in this context it is necessary to inactivate both E2F3-isoforms to diminish tumour cell proliferation. Therefore, the incorrect sentence was altered into *"it was suggested that the loss of pRB as well as amplification of E2F3 are obligate events for tumourigenesis*". This expresses that it might not be fully understood and might not be true for all tumours.

3. Using chimeras between E2F1 and E2F3, they show that the 331333 chimera (with only the coiled-coil domain of E2F3 replaced by that of E2F1) fails to bind HELLS. Ideally, testing the 113111 chimera would determine whether the E2F3 coiled-coil domain is sufficient for HELLS binding. But in lieu of this, the authors need to recognize that the 331333 chimera could have structural defects. If they showed that the 331333 chimera could still bind Rb or DNA, this concern would be alleviated.

* The exchanges in the 113111 and the 331333 swapping-mutants are the same that were made by Halstrom (Halstrom and Nevins, 2003). The difference in the mutant proteins used by us is that we exchanged solely either the respective E2F-marked box (MBD) or coiled-coil domains (CC). Halstrom exchanged the CC and the MB at the same time. These "doubly" mutant E2Fs had no problem to facilitate S-phase entry or apoptosis (the latter feature is specific to the MBD). In addition, the same mutants were used for other studies, where they are also functional since they

ectopically induced target genes and forced cells into the cell cycle (Black *et al*, 2005; Halstrom and Nevins, 2006). Most if not all of these features depend on the ability to bind DNA via the DPs. To be able to conclude that *our* swapping mutant is functional we have used an *in vitro* system to assay the interaction of E2F3 peptides with DP2. Indeed, the co-expression of GST-E2F3Del6_331333 and HIS-DP2 was performed with a singular vector (Figure S1F-G). Using pulldowns precipitated both proteins with either GST- or Co^{2+}-beads. Combined, we conclude that a 331333:DP2 complex can be formed and together with the work of the *Nevins* group, that such E2F1/E2F3A swapping mutants can create functional proteins.

4. Page 8, top paragraph: they refer to Figure 1D in last line, but this should be to Figure 1H.

*We thank the reviewer for this remark. Due to the change in the figures we now refer to (Figure 1G) for this analysis.

5. In showing HELLS enrichment at promoters of E2F3-dependent target genes, Bmyb is shown in Figure 2E, but should also be referred to as a E2F3-dependent target gene in the body of text.

*This is an unintended omission, since *Bmyb* is a well-described E2F-target. Due to the change in the text we now refer to “Performing ChIP assays …we detected a two-fold reduced occupancy of Hells on the E2F-dependent promoters of *p107* or *Bmyb* as compared to wild-type (WT) MEFs (Figure 2G)”.

6. On page 13, referring to Suppl Fig S4A,B, they state that there "is a general trend for E2F3 and HELLS co-expression" in other cancers, but the data presented do not indicate which tumors show co-expression.

*To make our analyses better understood, we changed the sentence to: ”There is a general trend for E2F3 and HELLS co-expression in specific other solid tumour entities that we tested including bladder, retina, lung and ovary”. Supplementary Figure S4A gives a count of how many tumors we analyzed and stained. That is: We stained 15 non-small lung tumours for E2F3, of which 13 showed an overexpression for E2F3. It might not be immediately visible from the tumour sections, presented in Supplementary Figure S4B. Only the strong red staining is positive for E2F3 or HELLS, respectively. The overlap for the tumours is 100%; all HELLS-positive tumours are also E2F3-positive.

7. **The ChIP-seq data are important and appear quite supportive**, but the Results section describing these results is hard to follow. It is clear that E2F3 and HELLS "share a largely overlapping target set", which is important, but some of the other relationships are difficult to understand (such as with H3K27me3).

*We are aware of the quite technical description of the deep sequencing-based data and have taken into account the referee’s comments to strengthen our data. Firstly, we have omitted much of the H3K27me3 data as far as it is not necessary to

understand the conclusions. Secondly, as suggested by referee#2 we have added H3K4me3 data to perform an overlap between H3K4me3 enriched promoters and the E2f3:Hells co-associated promoters. The overlap is significant, suggesting that most of the E2f3:Hells co-associated promoters are active (Figure 6E), which also confirms that the anticorrelation of E2f3 to H3K27me3 mentioned in our previous draft is probably correct.

8. Another discrepancy that the authors should address is that HELLS knockdown cells do enter S-phase, but with substantial delay, and yet E2F3 target genes are not induced at timepoints that are after this delayed S-phase entry (Fig 3). So unless the authors want to argue that the cells make it into S phase without E2F targets like CDC6, they need to discuss how the cells are doing it (i.e. is a little CDC6 enough?).

*Yes, this is correct. HELLS depleted cells enter the cell cycle with a significant delay. The transcriptional activation of genes as CDC6 is much delayed but not fully abolished. We have removed western blots that seemed, as if there would be no more CDC6 in HELLS-depleted cells, which might cause ambiguity. To highlight how essential HELLS is, we performed new sets of BrdU-analyses (Figure 4C). Even after 26 hours, thus a prolonged exposure to 20% serum, the HELLS-depleted cells showed difficulties to replicate properly (based on BrdU-incorporation). This delay with a slow increase in BrdU-incorporation over time is remarkably similar to E2f3-deficient MEFs (Humbert *et al*, 2000). Also here, the strongly reduced *cdc6* (and *mcm3*) allows only a limited, retarded entry and progression through the S-phase.

9. There are multiple grammar/punctuation errors throughout.

*We corrected typographical or spelling errors, to avoid potential ambiguities.

Referee #2 (Remarks to the Author):

Major points

1. Despite a detailed study of the HELLS and E2F3B interaction domains, it does not become clear whether the interaction is direct or indirect. To clear that point, the authors will have to show a binding between recombinantly produced E2F3B and HELLS.

*In order to clarify the question if HELLS and E2F3 are capable to interact directly we used bacterial expressed proteins. A singular vector system was used, allowing the generation of simultaneous co-expression of proteins. The co-expression is not achieved via mixing the purified complex partners, but by co-expression, which may be favoured by proteins that may only form heterodimers upon co-expression, but not by mixing purified partners (Scheich *et al,* 2007). Using this approach, we clearly demonstrated that a minimal E2F3-peptide (that is identical in both E2F3A and E2F3B) containing both the E2F-DP interaction domain CC as well as MBD are capable to coprecipitate the Hells-N-terminus. Importantly, since both peptides were

tagged (Gst-E2F3_Del6 and His-Hells_CC) we can show that the interaction works well from either side (Figure 1E-F), something we could not achieve in *endogenous* immunoprecipitations (see **referee#3; first point**).
The importance of *in vitro* interaction studies was justifiably raised. We have also used the bacterial expression system to show that a ternary complex exists between E2F3, HELLS, and DP2. We feel that this information is relevant to the referees and readers, since it is expected that only E2Fs contain its facultative dimerization partner is able to bind to DNA. The proof that all three peptides can exist as a complex is adding substance to the finding that chromatin aids the E2F3:HELLS interaction (Figure 2C). The possibility that the E2F3:HELLS interaction may occur more efficiently on the chromatin is consistent with our model that is found in the Discussion.

2. The interaction domain in E2F3B is common to both E2F3A and E2F3B, and HA-tagged versions of both proteins were able to co-immunoprecipitate HELLS. Although both proteins have a highly related protein structure, the function overlaps only partially. Generally E2F3A is known to be expressed in a cell cycle dependent manner and to activate its target genes, whereas E2F3B is expressed during the whole cell cycle and appears to have a repressive function at specific loci (e.g. Arf). Since the authors use in all following experiments a "pan-E2F3" antibody that recognizes both E2F3A and E2F3B, there is no way to understand which one of these two proteins was ChIP-ed on which promoter. It is essential to know, which isoform - E2F3A or E2F3B - is cooperating with HELLS function and is critical for tumor proliferation.

*We agree with the referee that this is an important issue, which was therefore addressed in much greater detail throughout the text. We also adapted our discussion accordingly. Indeed, the discrimination between E2f3a and E2f3b activities and their individual functions is important and yet their differences are not fully understood. The E2f3 isoform-specific mouse knockouts showed that E2f3a and E2f3b make overlapping contributions to S-phase entry but affect the total E2f3 activity differently (Danielian *et al*, 2008). E2f3b is largely dispensable for survival and even p19ARF repression, but contributes to the proliferation defect (and repression of p19ARF). The group of Dynlacht specifically monitored the role of E2f3b specific transcriptional targets in cellular differentiation (Asp *et al*, 2008). In this study, E2f3b was suggested to attenuate the expression of genes required to promote differentiation. It is also shown that E2f3b associates with many classical E2F-regulated genes, such as *mcm3*. We have incorporated these aspects in our discussion and could show that E2f3a/b as well as Hells ChIP-Seq tags can be mapped to the p19ARF genomic context. MACS called this gene significant for Hells in one condition. We included with the manuscript the screenshots of several promoters that are weak according to MACS, but experimentally proven. We also state: “We are not adverse to the idea that Hells also acts as a repressor, since the ability to function as activator and repressor of transcription is also found for other SWI/SNF molecules (Bell et al, 2011)”. E2f3a/b both contribute to proliferation and Hells is capable to bind well to either E2f3a or E2f3b protein.

3. For the understanding of E2F3 - HELLS cooperation and its impact on the expression of E2F target genes it is important to connect promoter occupancy,

mRNA expression and protein levels in wild type cells and in HELLS knockdown experiments. The authors present in Figures 2, 3 and 4 different experiments in asynchronous and synchronized cell populations but for none of them the complete data set is shown and discussed. Additionally some results are inconsistent - results in western blots of Figure 3A and 4A for CDC6, E2F1, p107 are not the same; in Figure 3 the protein and mRNA expression of E2F1 and E2F3B do not correlate and it is impossible to compare the experiments shown in Figure 3A and 4A, since different time points after serum addition are shown.

*****Figure 3 has been restructured to accommodate to the referee's points. The BrdU incorporation was repeated and measured while cell re-entered the cell cycle and went into S-phase. Now, it includes the 8, 12 hours time points as in Figure 4A as well as the 16, 22 and 26 hour time points of Figure 3C. We have removed parts of the western analysis (**see referee#1; point 8**) that could lead to incorrect conclusions.

4. When HELLS is down-regulated by shRNA, T98G cells show a delayed S-phase entry compared to controls. Since the absence of HELLS leads also to a down-regulation of the E2F3 protein expression, it is not clear if the S-phase entry delay is a secondary effect. In Figure 4 the authors try to address this problem with an over-expression of HA-ER-E2F3A (but not E2F3B), but they show no analysis of the cell cycle progression by BrdU labeling in presence of the ectopically expressed E2F3A. It would be also important to rescue the phenotype with an ectopic HELLS expression and to show that the interaction with E2F3 is essential for the effect, e.g. use a mutant form of the HELLS protein, which is not able to interact with E2F3.

*This valid concern is related to point 3 (referee#2). We have expanded our data of Figure 4 by adding BrdU-analyses in T98G cells that strongly over-express the exogenous HA-ER-E2F3A construct. In addition, this BrdU-analysis is also perfomed in the presence or absence of HELLS (using shHELLS). As anticipated, cells fail to properly enter S-phase in the absence of HELLS, even if massive amounts of E2F3A are over-expressed. This is not due to the inability of E2F3 to bind to DNA.

5. E2F3 and HELLS expression levels are correlated in prostate cancer. The down-regulation of HELLS decreases the transformation capacity of overexpressed E2F3. This is interesting, but in order to epistatically order E2F3 and Hells in this assay additional experiments are needed, e.g. over-expression of HELLS alone and HELLS-E2F3 combination, over-expression of HELLS mutant, which is not able to interact with E2F3.

*The questions asked and the experiments suggested are very good and indeed they would be very helpful to clarify epistasis. Unfortunately, in addressing these questions we found out that Hells does not act as a classical co-activator, but more similar to other chromatin remodelers. The over-expression of either a HELLS wild-type or a mutant form is phenotype-less. We have added this analysis as a Figure.

6. In Figure 6 the authors show ChIP-seq data for HELLS, E2F3 and H3K27me3 from wild type MEFs and E2F3-/- MEFs. 22% of the HELLS occupied transcriptional

start sites (TSS) are also enriched for H3K27me3. 93% of these promoters are also bound by E2F3. Contradictory to the data presented in Figure 2E, where it is shown that HELLS occupancy on p107 (i.e. rbl1) and Bmyb promotors is decreased in E2F3-/- MEFs, there is no HELLS binding to neither to the p107 promoter nor to the Bmyb promotor in the ChIP-seq data set done in the same cell lines and HELLS binding does not appear to be dependent on E2F3 concentration at all.

***Point 1:** We agree, that this point was insufficiently addressed before. For a better judgment, we added screenshots of new, physiologically relevant Hells-targets (Supplementary Figure S5C-E). Additionally, we included the screenshot of several Hells-associated promoters with lesser ChIP-Seq reads. Some of these promoters were not identified as statistically significant (by MACS), but were tested successfully by us, and others, in conventional ChIPs (Supplementary Figure S6A-C). We added a discussion about what could appear like a contradiction. The peak-identifying algorithm MACS is very efficient in detecting promoters with a high significance. Other tools might be more applicable if the IgG-background at a promoter is very high.

Point 2: Indeed this is an important point. Our initial analysis gave the impression that Hells binds less well in the absence of E2f3 (Figure 2G). This impression is only true if small genomic loci are monitored. The whole genomic view of Hells binding to chromatin reveals that Hells binds well in the absence of E2f3. We find this fact remarkable, since Hells-bound genes still contain the "E2f3-like" motif (Supplementary Table S4). We present evidence to suggest that in E2f3-absence, Hells shifts its chromosomal position. Thus we could claim, "Hells senses the absence of E2f3". A finding of this kind would hardly been made using conventional ChIP. It highlights the importance of ChIP-Seq in addition to conventional ChIP to acquire an unambiguous picture.

7. The authors present the TSS bound by HELLS and E2F in the supplementary table S3. Unfortunately, p107, which was bound by HELLS and E2F3 in a re-ChIP experiment in Figure 2C and is an essential internal positive control for the genome-wide ChIP experiment, was only identified as an E2F3 but not as a HELLS target. Additionally, it does not become clear, if all E2F target promotors used in the previously described experiments were also found in the ChIP-seq experiment. To support Chip-seq data and to discriminate between repressive or activating functions of the HELLS/E2F3 proteins, it would be necessary to complete the experiment with genome-wide mRNA expression data and for instance mapping of the H3K4 trimethylation mark.

*We thank the referee for this really helpful suggestion. To clarify this critical point a new comparative analysis of HELLS and E2F3 bound promoters was performed and compared to studies that analyzed the active, trimethylated histone H3 Lysine 4 mark in MEFs (Mikkelsen et al., 2007). Importantly, the overlap of H3K4me3 with Hells:E2f3 co-bound promoters is extensive. This largely supports our findings that Hells:E2f3 co-bound may be in an active and not a repressed state. We have added these data (Figure 6E) and a discussion of it in the manuscript.

8. HELLS knockdown leads to a roughly 50% reduction of Mll1 mRNA. Consistently, in E2F3-/- MEFs Mll1 mRNA levels are also mildly reduced (about

20%). This effect was also seen on protein levels upon HELLS knockdown but it was definitely not significant in E2F3-/- MEFs (Figure S7B). Although the authors show a cell-cycle dependent expression of Mll1 mRNA in T98G cells, they do not test the influence of HELLS and E2F3 downregulation under these circumstances. To strengthen the biological observations in the paper it would be useful to investigate the expression of Mll1 or other found HELLS/E2F3 targets in prostate cancer and in the transformation potential of prostate cancer cell lines.

*This is a very helpful suggestion. Agreeing that this point is necessary for a better judgment of our findings, we performed ChIP experiments, expression analyses (qRT-PCR), shE2F3 and shHELLS knockdown experiments in prostate cancer (PCA) cells. Our results confirm that the Hells-targets derived from the mouse ChIP-Seq are also physiologically relevant in human PCA (Figure 7), in human glioblastoma cells as well as in mouse cells (Supplementary Figure S7). We also performed a specific comparison of *MLL1* expression in G0 versus S-phase PCA cells and added the western blots showing MLL1 reduction after HELLS knockdown in asynchronous PCA cells.

Minor points

1. In figure 1B there are no GST-inputs for the respective experiments.

We have addressed this issue and added the data for this Figure, now in Supplementary Figure S1.

2. Figure S2C is mislabeled for E2F3.

Referee #3 (Remarks to the Author):

In this manuscript, von Eyss et al. have investigated the functional link between the SNF-2 like helicase HELLS and E2F3. The authors showed that HELLS interacts with E2F3 in several assays. Depletion of HELLS results in defects in S phase entry which are reminiscent of the phenotype of E2F3-/- cells. Consistently, E2F targets are not induced in HELLS depleted cells upon serum stimulation. Using ChIP, the authors showed that both E2F3 and HELLS are present on the same set of E2F target genes. Furthermore, HELLS appears to be recruited to the promoters in an E2F3 dependent manner, as over-expression of E2F3 leads to an increase of HELLS on the promoters. Conversely, HELLS is reduced at these promoters in E2F3-/- MEFs. The authors further show that HELLS itself is an E2F3 target which perhaps explains why HELLS, like E2F3, is overexpressed in tumors of prostate cancer patients. In the last part of the manuscript, the authors performed genome-wide analysis of HELLS and E2F3 targets and found that more the majority of HELLS targets are also bound by E2F3. However, HELLS binds to a region downstream of TSS while E2F3 is present at the TSS. Additionally, HELLS occupancy does depend on E2F3 as evident from ChIP-seq analysis in E2F3-/- MEFs.

Deciphering the role of chromatin remodeling proteins, such as HELLS, in E2F dependent transcription is an important topic. In this regard, the findings are likely to

be of interest for readers working in the filed of cell cycle control. This manuscript clearly represents a large amount of work and the authors make an effort to dissect the functional link between HELLS and E2F3. However, there are several major issues which, in my opinion, preclude publication of this work in EMBO Journal.

Firstly, the authors fail to convincingly demonstrate direct protein-protein interaction between HELLS and E2F3. The authors identified HELLS in Mass Spec analysis of E2F3b associated proteins and confirmed the interaction by IP/western and in GST pull-down experiments. However, the results of the IP/western experiment are questionable since the interaction appears to be highly dependent on the presence of DNA as it is disrupted by EtBr (compare Fig. 1H and Fig. S1D). The interpretation that DNA mediates some or perhaps most of interaction between HELLS and E2F3 would be consistent with the ChIP-seq data where E2F3 and HELLS are present on the same target promoter but localized to distinct regions within these promoters (Fig. 6D). The authors need to either abandon their claim of direct protein-protein interaction between HELLS and E2F3 or provide more convincing data to support it. Specifically, it is important for authors to confirm interaction between endogenous E2F3 and HELLS in a reciprocal IP (IP through HELLS antibody and WB with E2F3 antibody) and to test the effect of EtBr in these settings. The authors should also consider using DNAse treatment in IP-Western as alternative to EtBr.

*We have performed a number of assays since all referees raised concerns about the direct nature of the Hells:E2f3 interaction. Direct protein-protein interactions are best studied by bacterial expression and partially purifying the proteins with subsequent complex formation. We added several experimental data using solely bacterial proteins expressed in a singular vector system (Figure 1E-F; Supplementary Figure S2E-G). Our experiments using EtBr to disrupt the protein: chromatin interaction is merely evidence to suggest that the DNA can help (maybe for bridging but maybe for stabilization) the interaction. To follow these helpful suggestions, we have also used DNAse and micrococcus nuclease treatment, instead of EtBr, to the same effect (*data not shown*). To show the endogenous interaction between HELLS:E2F3, largely depends on the antibodies. We have failed to produce the reciprocal endogenous IP, although a total of 5 HELLS antibodies (Muegge lab, Abcam, Santa Cruz, self made and Novus) and 4 E2F3 antibodies (Lees lab, 2 x Santa Cruz and 2x self made) were used. Not all antibodies worked well in endogenous IPs. Those that worked were relentlessly assayed. It might be that the antibodies that IP well, also target the critical domains for the protein:protein interaction. Instead, we have added data that the direct interaction works both ways *in vitro* (Figure 1E-F).

Secondly, from the analysis of HELLS and E2F3 binding to classical E2F targets in Fig. 2, the authors concluded that HELLS is recruited to these targets by E2F3 (Fig. 2) and is important for activation of E2F targets and S phase entry (Fig. 3). These conclusions are nicely supported by extensive experimental data presented in these figures. However, the ChIP-seq analysis of E2F3 and HELLS genome-wide localization paints a different picture. Although HELLS and E2F3 share >90% of common targets, the two proteins bind to distinct regions in the promoters. My even bigger concern is that in contrast to the results in Fig. 2, E2F3 has little if any role in recruitment of HELLS to its genomic targets (page 17). If this is the case, then the significance of E2F3-HELLS interaction becomes even more questionable. The authors need to reconcile their ChIP-seq data with their previous conclusions,

perhaps, by examining the occupancy of HELLS on a larger set of shared targets, identified in ChIP-seq, in E2F3-/- cells.

Similar concerns were raised by referee#2. We have addressed what appears to be a disparity in our data. In fact, these two experimental data sets nicely complement each other, which we explain in the “Discussion” (**see referee#2; point 6**). We have made changes throughout the text and the discussion.

Thirdly, it is not clear to me the significance of overexpression of HELLS in prostate cancer. The authors show that HELLS is an E2F3 target. Since E2F3 has been previously shown to be overexpressed in prostate cancer, the observation that HELLS is elevated seems rather trivial.

*We understand the concern, but feel that it is still an important point we would like to make. In might be considered trivial, but the finding that HELLS is over-expressed in a variety of solid tumours including PCA is not yet publishes. Thus, we decided to keep the data in the manuscript.

Fourthly, I feel that the authors are attempting to incorporate a large amount of experimental data into the manuscript and this dilutes the main message of the paper. While reading the manuscript I often found myself struggling through the logic of experiments and apparently contradicting conclusions. The manuscript needs to be streamlined. I also feel that the authors' model of how HELLS contributes to the E2F3 function changes as one reads through different sections of the results section.

*This comment is duly justified, since simply a lot of data is presented. We have significantly streamlined the manuscript and removed data that was unnecessary to follow the main messages. We have also improved the text and added a discussion on points that might appear as contradictions.

Minor points:

a) There is an inconsistency in the use of two E2F3 isoforms in the experiments described in Fig. 1. The authors used E2F3b to identify interacting proteins by MS and in GST pull downs (Fig. 1A and B). However, in Fig 1F they switched to E2F3a. The authors need to use the same isoform E2F3b in this experiment to confirm the interaction (Fig. 1F). Importantly, the fact that E2F3 gene actually produces two isoforms is not described in either introduction or in the beginning of the results section. This is an important point since the two isoforms have opposing functions: one is a repressor and another is an activator. I also feel that the authors should include a rationale why they used **E2F3b instead of E2F3a in the MS analysis.**

*Referee #2 mentioned also the fact that we did not explain our data in the light of two E2F3-isoforms. We made extensive changes throughout the text and most obviously in the discussion. If E2F3A and E2F3B are truly opposing is uncertain, since they both contribute to S-phase induction and proliferation. E2F3B was chosen as bait for the MS analysis for the sole reason that it is more soluble and stable after *in vitro* expression and purification. We have not been able to express sufficient quantities of full length E2F3A in bacteria, precluding us to perform the same assay

again. E2F3A and E2F3B are identical, apart from their respective N-termini (Leone *et al,* 2000). Thus, all relevant domains are identical. Both E2F3A as well as E2FB bind to HELLS. We have found no evidence to suggest that HELLS distinguishes between these isoforms. Simply put, there is much more HELLS in the S-phase as compared to G0 cells. In G0 E2F3B is the sole E2F3 isoform. So by quantity, most HELLS:E2F3 complexes will be formed in the S-phase (see also **referee#2; point2**).

b) The authors were using HA tagged E2F3a and b proteins in IP western in Figure 1G. I am puzzled why pan-E2F3 antibody was used in IP instead of anti-HA. Using pan-E2F3 antibody complicates interpretation of the results since this antibody recognizes transfected protein and both endogenous isoforms. In contrast, HA antibody would specifically recognize the transfected protein. I am also surprised by the lack of the band in the vector control lane which one expects to see if endogenous E2F3 and HELLS interact in these cells. Does it mean that such interaction is specific to HCT116 cells only (Fig. 1H)? It is also not clear from figure or figure legends which antibodies (pan-E2F3 or anti-HA) were used to detect E2F3 in the input lanes.

*We agree the IP for HA would have been another very good solution here. E2F3A/B can be over-expressed to a large extent (up to about 50 fold) in these cells. Therefore, performing an IP with over-expressed E2F3A/B largely favours the exogenous protein (Figure 1H is now Figure 2A). Under these conditions, it is hard or almost impossible to expect to see the endogenous E2F3:HELLS interaction. Due to this reason, we also performed the IP for endogenous proteins (Figure 2C). No, the E2F3:HELLS interaction is not specific for these cells and it was detected in other cells too, such as HeLa and Saos-2 (we also tested embryonal carcinomas cells, P19 and others; *data not shown*).

c) Is overexpression of HELLS sufficient to drive cells into S phase? *Does HELLS drive E2F3-/- cells into S phase*? If this is the case then these results might strengthen the authors' argument about functional cooperation between E2F3 and HELLS.

*These are very good suggestions, that were also brought to our attention by referee#2 (see **referee#2 point 5**).

d) The authors mentioned that in prostate cancers E2F3 is amplified (page 3). I think that the cited paper (Foster et al 2004) refers to overexpression of E2F3. Is there evidence for increase in copy number of the E2F3 locus in human prostate cancer?

*No indeed, this was a mistake on our behalf. In fact, there is ample evidence for the overexpression of E2F3 in prostate tumours. Amplification for E2F3 at 6p22 was shown in human bladder cancer where it correlates with E2F3 overexpression (Feber *et al*, 2004; Foster *et al*, 2004; Oeggerli *et al*, 2004; Oeggerli *et al*, 2006). We have changed the sentence in the text. In advanced prostate cancers E2F3 is highly overexpressed and the highest E2F3 levels determine the worst clinical outcome for the patient (Foster *et al*, 2004).

e) The authors mentioned that they detected pRB in their MS analysis of E2F3 interacting proteins and therefore their approach was suitable. Was DP, a well-known

heterodimeric partner of E2F3, also present?

*A valid question, but no. This is due to the fact that E2F3B weighs about 37kDalton (without the additional GST-tag), whereas DP1 is 45kDalton (similar to DP2 that weighs about 43 kDalton). Thus, in the SDS-PAGE all DPs run in or just below the GST-E2F3B that was used as bait, precluding the analysis of smaller proteins. Indeed, the MS-analysis is not comprehensive. We performed pulldowns demonstrating that the ternary HELLS:DP:E2F3 complex exists.

f) The authors used the DU145 prostate cancer cell line in colony formation assays. What is the level of E2F3 expression in this line? If E2F3 level is elevated then one expects that even without E2F3 overexpression depletion of HELLS would reduce colony formation. Perhaps, the authors should test the impact of the loss of HELLS in other prostate cancer cell lines with higher level of E2F3 overexpression.

*In order to clarify, what may appear as an inconsistency, we have performed additional knockdown experiments in DU145 but also PC3 cells. Both PCA cell lines were used. They have near equal amounts of E2F3 and only mildly different levels of HELLS (*data not shown*). Although, knockdown of HELLS leads to severe problems for S-phase entry (T98G cells; see Figure 4C) the impact on survival or transformation in soft agar was somewhat mild. Soft agar assays are measuring anchorage independent survival and malignant transformation of cells. It is less efficient to measure growth.

g) It is not clear why the authors decided to choose the MLL1 gene for further analysis. The fact that there is a strong enrichment for HELLS at the MLL1 promoter does not necessarily imply that MLL1 is important for the HELLS function. Additionally, E2F3 appears to show a very weak binding to the MLL1 promoter (Figure 7B). Overall, I am not convinced that the MLL1 gene is the right model to study functional interaction between E2F3 and HELLS.

*MLL1 was most interesting to us, since it is the highest-ranking target according to our Hells-ChIP Seq data. We confirmed that the gene expression changes after HELLS-depletion. MLL1 has been linked to E2F-action before (see Introduction). It is one of several highly interesting candidates that await further molecular exploration.

References:

Asp P, Acosta-Alvear D, Tsikitis M, van Oevelen C, Dynlacht BD. (2009) E2f3b plays an essential role in myogenic differentiation through isoform-specific gene regulation. *Genes Dev* **23:** 37-53

Black EP, Hallstrom T, Dressman HK, West M, Nevins JR (2005) Distinctions in the specificity of E2F function revealed by gene expression signatures. *Proc Natl Acad Sci USA* **102**: 15948-15953

Danielian PS, Friesenhahn LB, Faust AM, West JC, Caron AM, Bronson RT, Lees JA (2008) E2f3a and E2f3b make overlapping but different contributions to total E2f3 activity. *Oncogene* **27**: 6561-6570

Feber A, Clark J, Goodwin G, Dodson AR, Smith PH, Fletcher A, Edwards S, Flohr P, Falconer A, Roe T, Kovacs G, Dennis N, Fisher C, Wooster R, Huddart R, Foster CS, Cooper CS (2004) Amplification and overexpression of E2F3 in human bladder cancer. *Oncogene* **23:** 1627-1630

Foster CS, Falconer A, Dodson AR, Norman AR, Dennis N, Fletcher A, Southgate C, Dowe A, Dearnaley D, Jhavar S, Eeles R, Feber A, Cooper CS (2004) Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. *Oncogene* **23:** 5871-5879

Halstrom TC, Nevins, JR (2003) Specificity in the activation and control of transcription factor E2F-dependent apoptosis. *Proc Natl Acad Sci USA* **100**: 10848–10853

Halstrom TC, Nevins, JR (2006) Jab1 is a specificity factor for E2F1-induced apoptosis. *Genes Dev* **20**: 613-623

Humbert PO, Verona R, Trimarchi JM, Rogers C, Dandapani S, Lees JA (2000) E2f3 is critical for normal cellular proliferation. *Genes Dev* **14**: 690-703

Leone G, Nuckolls F, Ishida S, Adams M, Sears R, Jakoi L, Miron A, Nevins JR (2000) Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. Mol Cell Biol 20: 3626-3632

Oeggerli M, Tomovska S, Schraml P, Calvano-Forte D, Schafroth S, Simon R, Gasser T, Mihatsch MJ, Sauter G (2004) E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer. *Oncogene* **23:** 5616-5623

Oeggerli M, Schraml P, Ruiz C, Bloch M, Novotny H, Mirlacher M, Sauter G, Simon R (2006) E2F3 is the main target gene of the 6p22 amplicon with high specificity for human bladder cancer. *Oncogene* **25:** 6538-6543

Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE. (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**: 553-560

Scheich,C, Kümmel D, Soumailakakis D, Heinemann U, Büssow K (2007) Vectors for co-expression of an unrestricted number of proteins . *Nuc Acids Res* **35**: N6 e43

2nd Editorial Decision 10 November 2011

Thank you for submitting your revised manuscript on HELLS and E2F3 for our consideration. We have now received the feedback from two of the original referees, which you will find copied below. I am pleased to inform you that both of them consider the study substantially improved and now in principle suited for publication in The EMBO Journal, pending some minor textual revisions as detailed in the comments below. I am therefore returning the study to you for a final round of minor modification to deal with these comments.

When uploading your final version, please also take the following editorial issues into account:

- please submit all supplementary figures, legends, material (except for data sets!) in the form of one single PDF

- please modify panel D of Figure 2, to include a less contrast/brightness-adjusted image of the p107 row that will still allow the background to be visualized.

- we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to ask you if you would be willing to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and would be published online with the article as a supplementary "Source Data" file.

- finally, please add 'protein' or 'helicase' after 'The SNF2-like' to the title. I would also like to propose a few changes to the abstract to improve its flow and clarity, and to cut out one partially redundant sentence that would more appropriately belong into the discussion section of the manuscript. If you are happy with these proposed changes, please kindly incorporate them into the final version of the manuscript; or get back to me if you should have any alternative suggestions for title or abstract.

"The activating E2F-transcription factors are best known for their dependence on the Retinoblastoma protein and their role in cellular proliferation. E2F3 is uniquely amplified in specific human tumours, where its expression is inversely correlated with the survival of patients. Here, E2F3B interaction partners were identified by mass spectrometric analysis. We show that the SNF2-like HELICASE HELLS interacts with E2F3A in vivo and cooperates with its oncogenic functions. Depletion of HELLS severely perturbs the induction of E2F-target genes, HINDERING cell cycle re-entry and growth. Using chromatin immmunoprecipitation coupled to sequencing, we identified genome-wide targets of HELLS and E2F3A/B. [Our analysis revealed that] HELLS binds promoters of active genes, including the trithorax-related MLL1 GENE, and co-regulates E2F3-dependent genes. [Our analysis is the first to link HELLS with E2F-controlled processes that are critical to establish a proliferative tumour circuitry.] Strikingly, just as E2F3, HELLS is overexpressed in human tumours including prostate cancer, indicating that either factor may contribute to the malignant progression of tumours. Our work reveals that HELLS is important for E2F3 in tumour cell proliferation."

Following these final modifications, we shall be happy to swiftly proceed with the formal acceptance and production of the paper!

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript has been substantially improved, and the authors have adequately responded to the previous critiques. This report is comprehensive, novel and important, and sheds very new light on E2F3 dependent transcription.

I would make a couple of minor suggestions. The first section dealing with interactions between recombinant proteins could be made more succinct (as it is now, there is too much detail about domain requirements that detract from their major conclusions, and paragraphs are too long).
Finally, the final section describing the ChIP-seq experiments in E2f3-/- cells is confusing, and I didn't get the major point (that Hells still binds E2F3 regulated promoters even in the absence of E2F3) until I read the Discussion.
Still, all in all, the writing is clearer than in the previous version.

Referee #3 (Remarks to the Author):

In general, the authors done a satisfactory job in addressing the reviewers comments. My only remaining concern is that DNA appears to contribute significantly to the E2F3/HELLS interaction in vivo (Fig. 2C). Perhaps, the authors might want to downplay their idea of E2F3 and HELLS being in the same complex? Especially, since HELLS binding is not much dependent on E2F3 according to their data in E2F3-/- cells.

2nd Revision - authors' response 18 November 2011

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The SNF2-like helicase HELLS mediates E2F3-dependent transcription and cellular transformation

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Point-by-point responses to referees

Referee #1

I would make a couple of minor suggestions. The first section dealing with interactions between recombinant proteins could be made more succinct (as it is now, there is too much detail about domain requirements that detract from their major conclusions, and paragraphs are too long). Finally, the final section describing the ChIP-seq experiments in E2f3-/- cells is confusing, and I didn't get the major point (that Hells still binds E2F3 regulated promoters even in the absence of E2F3) until I read the Discussion.
Still, all in all, the writing is clearer than in the previous version.

**Point 1*
Indeed, the Figure 1 now consists mainly of *in vitro* interaction studies. We wish to keep this section intact, since two referees raised significant concerns or questions about the validity of the claim that E2F3 and HELLS interact directly. The best way to prove this is by showing that the *in vitro* produced proteins *can interact*. Moreover, it was referee#1 who questioned if the E2F3-E2F1 swapping proteins are functional. These confirmatory experiments that show that the swapping mutant is functional is an interesting, and integral part of this section. Thus, we would like that the readers are also able to judge these data provided in this first paragraph. Still, we are thankful for the hint that this paragraph is rather lengthy. In order, to make this paragraph slightly better readable, it was subdivided into sections.

*Point 2
Due to the fact that the ChIP-seq data in the prior submission was too lengthy and complicated to be able to follow our main point (that E2f3:Hells targets largely overlap) the description of the ChIP-seq experiments in the E2f3-/- cells was separated from the WT ChIP-seq data. That Hells still binds E2F3 regulated promoters even in the absence of E2F3, was so important, that we used it as a final conclusion. Despite the fact that Hells still binds to many targets, Hells senses the absence of E2f3! To make this point more obvious, we changed the title/first sentences of the last paragraph:

“**Hells still binds to targets in the absence of E2f3**
Since either E2f3 or Hells is essential for proper gene activation and the Hells association to certain E2F-targets seem to decline (Figure 2G), one hypothesis would be that the Hells association to E2F-targets depends on E2F3. Thus, to address this

question Hells ChIP-Seq were performed in E2f3-/- MEFs (Supplementary Tables S2-S3). Even in the absence of E2F3, Hells was found to localize to a large set of TSS (Supplementary Figure S5A)..."

*Point 3:
We are happy, that referee #1 finds that the text has improved in clarity.

Referee #3

In general, the authors done a satisfactory job in addressing the reviewers comments. My only remaining concern is that DNA appears to contribute significantly to the E2F3/HELLS interaction in vivo (Fig. 2C). Perhaps, the authors might want to downplay their idea of E2F3 and HELLS being in the same complex? Especially, since HELLS binding is not much dependent on E2F3 according to their data in E2F3-/- cells.

*Point 1:
We are thankful that referee #3 finds that most of the remaining questions were addressed satisfactorily.

*Point 2:
That chromatin contributes to the complex formation of E2F3:HELLS is a valid point, that was addressed in the text and the discussion. Since we are aware that this is an interesting but critical question, we have stated:
"These analyses were also performed in the presence of ethidium bromide resulting in decreased amounts of co-precipitated HELLS. This decrease is consistent with the idea that endogenous E2F3:HELLS complexes partly depend on or are bridged via chromatin"
Moreover, throughout the manuscript we are careful not to claim that the complex is completely independent of chromatin or only occurs on chromatin. At this time none of these conclusions can be drawn. Several arguments were brought forward, that the proteins *could* interact. First, apart from endogenous IPs, *in vitro* expressed domains of each protein can interact without bridging chromatin or co-factors. Second, the interaction shows specificity (for E2F3 and not E2F1). Exchanging amino acids in a conservative fashion within the E2F3 heptad or coiled coil-domain destroys the E2F3:HELLS binding. Third, that co-expressing DP2 alongside E2F3:HELLS makes the complex more stable makes it more likely that the E2F3/DP:HELLS complex occurs on the chromatin (as shown by Re-ChIP; Figure 2D-F). Forth, we do not exclude that other chromatin-associated factors associate with or are in contact with E2F3:HELLS. To emphasize this, a section about HDAC and DNMT-association is found in the discussion. Lastly, and most importantly, despite the fact that Hells binds to targets in the absence of E2f3, we observe that the Hells position can be shifted ("*Hells senses the E2F3-absence*")! How this is achieved remains to be further elucidated, but we believe it seems reasonable to assume that E2F3:HELLS complexes may be part of the explanation.