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Arabidopsis E2FA stimulates proliferation and endocycle separately through RBR-bound and RBR-free complexes

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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 19 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please excuse the fact that it has taken longer than usual to have it evaluated - one of the three referees that initially agreed to review had to withdraw at an advanced stage in the process due to personal circumstances, requiring us to look for an alternative expert. We have now finally received three sets of referee comments, which I am transmitting to you with this email. As you will see, the referees consider your findings potentially interesting and important, yet raise a number of major conceptual and technical issues with the potential of undermining the main conclusions. Following improvements especially along the lines suggested by referee 1, I feel we should be able to consider a revised manuscript further for eventual publication in The EMBO Journal. In this respect, not all points appear to equally important to support the main message, and others may require further work beyond the scope of a regular revision; I would therefore not expect all of them to be addressed experimentally. Nevertheless, for a revision to be ultimately successful in this case, it will be essential to satisfy the referees' concerns as completely as possible, by addressing all the technical/specific/textual points, and by diligent answering of the major points, including the addition of new experiments especially for cases with a good ratio of experimental feasibility to added insight/conclusiveness. As the latter points cannot all easily be identified without technical expertise, I would in this case also be happy to discuss the exact revision requirements further on the basis of a draft point-by-point response letter, as soon as you will have had a chance to consider the reviews and comments in detail - possible in additional consultations with one of the referees.

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

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revision.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Magyar et al. study here the functional relationship between E2FA and RBR1 in proliferating and endoreduplicating cells of Arabidopsis thaliana. The authors accomplish a significant amount of work using a variety of constructs that allowed them to manipulate the levels of E2FA, DP counterparts and RBR1. The claim that E2FA maintains proliferation in a complex with RBR1 that is not targeted by CYCD3;1. Also E2FA alone stimulates endocycle and cell expansion in a RBR1-independent manner. This manuscript describes a thorough biochemical study of E2FA-RBR1 interactions. Based on numerous IP experiments as well as gene expression and other phenotypic analysis, the authors draw conclusions about the functional role of specific E2FA-RBR1 complexes in cell cycle regulation.

The topic is very important, not only because it contributes to our general understanding of cell proliferation control in higher organisms but also because it challenges previous ideas about the functional relevance of cell cycle regulators. Also, the effort made by the authors to carry out a biochemical approach in a difficult model system is very valuable. Unfortunately, many of their conclusions are not fully supported by the data or have been drawn without a critical test of alternative hypothesis. The text is, in some parts, difficult to follow, but this might be inherent to the type of experiments described. The authors tend to use very general statements, not supported by experimental evidence, or jump into general conclusions, which have not been fully demonstrated.

In short, I have serious concerns about (i) the current depth of the study, (ii) the refinement of some experiments, (iii) the analysis of some of the tools generated in this study, and (iv) the manuscript (text) in its present form, which jeopardize the general validity of the conclusions claimed by the authors.

General comments
a. A general comment regarding plants lacking a particular E2F factor. How do the authors evaluate (or rule out) the possibility that in plants lacking one E2F factor, other E2F proteins can occupy target promoters which were not normally used (because they were perhaps preferentially occupied by the missing E2F)? It seems that most reagents and tools needed to do ChIP experiments to determine occupancy by various E2F proteins are available.

b. The conclusion that RBR1 in complex with E2FA might have a role in maintaining rather than repressing cell proliferation appears to be based on a kind of syllogism: E2FA overexpression increases complexes with RBR1; it also increases proliferation, then, RBR1-E2FA complexes stimulate cell proliferation. Since an excess of E2FA stimulates cell proliferation, that is, more cells are present in the sample, what is the relative amount of RBR1-E2FA complexes on a per cell basis? In fact, under these conditions it might occur that many more cells are developing a G1 phase where these complexes might be abundant. Could RBR1-E2F complexes be measured in a synchronized system, ideally in seedlings? This would provide, at least in part, direct information on complex formation throughout the cell cycle.

c. Most co-IP experiments are aimed at determining complexes between RBR1 with various E2F proteins. Then, these data are correlated with regulation of target gene expression. Strictly speaking, soluble complexes do not necessarily represent the same as chromatin-bound complexes. A major question is whether complexes detected in this study are functionally relevant regarding target gene expression control. ChIP experiments are required to draw strong conclusions.

Specific points.
1. Co-regulation analysis. Data in Suppl Table 1 should be extended below the cutoff of 0.7. Also,

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the use of this cutoff sounds arbitrary, at least is not sufficiently justified.

2. Fig 1A. A wt root is missing; this will allow the reader to compare the meristem size as an indication of proliferation rate of E2FA-GFP and RBR1-GFP plants. It seems that the size of the RBR1-GFP meristem is smaller than that of the E2FA-GFP plants. Also, it would be desirable to see some details of cell architecture in these lines compared to wt. Formally, the ability of E2FA-GFP to complement a mutant, as has been done for RBR1-GFP would be the ideal situation. Alternative ways of showing functionality are needed.

3. The E2FA RNAi plants should be described in more detail. There is a partial reduction of E2FA in E2FB RNAi cells. Is this significant and biologically relevant? What is the mRNA level of the six E2F in the E2FA RNAi lines (see Suppl Fig 1B). This is important because some other E2F family members have been implicated in regulating cell cycle and endoreduplication.

4. It seems that the stomatal pattern is significantly altered in the E2FA RNAi plants. Is this correct? Is cell proliferation in this lineage altered and, if so, is it consistent with the major conclusions about E2FA-RBR1 interactions?

5. Fig 2B. E2FB level are quite different in the two lines studied. Does this have any consequences on the phenotypes observed? It seems that E2F targets appear more drastically down-regulated in line 7.2 than in line 2.1, but line 7.2 presents ~50% reduction of E2FB transcripts. Does this different E2FB level affect target gene expression?

6. Fig 3A. Since the amount of RBR1 significantly diminishes at late times, experiments to detect RBR1-containing complexes might be taken with caution (the starting material does not contain the same amount of input, in this case RBR1).

7. Fig 3B-C. I think that the large differences in E2FA and E2FB amounts shed some doubts about the validity of the results. Are the interactions observed comparable?

8. Suppl Fig 3B and C. The differences between RBR1 levels between 2-weeks and 1-week WT-Col plants, respectively, are real? Do they represent experimental variation?

9. Since the truncated form of E2FA eliminates the RBR1 interactions and destroys the transactivation domain, how the up-regulation of target gene expression is explained? What is the basis to correlate the results exclusively with the loss of RBR1 binding, without considering the loss of transactivation capacity?

Referee #3 (Remarks to the Author):

The manuscript by Magyar et al reports on the mechanism by which a cell cycle gene in plants (E2FA) might influence both cell proliferation and differentiation via interaction with a plant Rb-related protein (RBR1). RB/E2F complexes have long been recognised as key players in the control of cell division, but exactly how they integrate the various aspects of proliferation/differentiation is still unclear- thus the paper addresses an important topic.
I found the paper to be densely written, particularly in the context of the numerous immunoblots shown. The description of the figures was often poor. Key to the whole paper is the requirement that the commercial antibody used to detect the phosphorylation status of the RBR1 protein produces a true and accurate reflection of the RBR protein status. I could find no evidence in the paper to verify this or reference to publications showing that this antibody is doing what it is meant to for a plant Rb-related protein. I think for EMBO J this would be a requirement, especially when the data obtained with this antibody are central to the paper.
Apart from this technical issue, there are some further points.
1) The authors make a great deal of the endocycle and its link with cell growth yet, as they admit on p8, their own data (and others) indicate that the link is often tenuous.
2) p7- an increase in G1 to G2 ratio could reflect a shorter G2 or a longer G1, I think concluding an arrest in G1 is not waranted.
3) Obviously quantitative analysis of such IP experiments is very tricky, nevertheless it would be good to have an indication of how many times the individual experiments were performed and an

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assessment of the variation in band intensity between experiments. A triplicate biological replication would be a minimal expectation.
4) There are various minor corrections to English and formatting required.
Overall, the story is novel (although perhaps mainly for a plant audience) but the data underpinning the story need to be stronger.

Referee #4 (Remarks to the Author):

This works build up on the previously reported observation that E2FA expression results in stimulation of both the mitotic cycle as well as the endoreplication cycle (DeVeylder et al). Now, Magyar et al. use exquisite biochemistry to show that this dual role of E2F is due to different complexes formed. In combination with RBR E2F stimulates cell proliferation and represses differentiation while in complexes without RBR it rather promotes differentiation and along with that endoreplication. This finding represents a major advancement in the field and resolves some of the open questions how cell proliferation and differentiation are connected.
The authors have very carefully conducted their experiments and one statement is back upped by several experiments. Indeed, this paper holds a wealth of information that will be very important for future studies in the field. The heart of this paper is the expression of an E2FA variant that cannot bind RBR and indeed this version together with DPa expression strongly promotes differentiation. A somewhat simpler experiment would have been to co-overexpress RBR and E2FA since this should stimulate then cell proliferation. I am wondering whether the authors have attempted this?
In any case, the here presented data makes a very strong case for the reasoning of the authors. Moreover, with these finding the situation in Arabidopsis might reflect the presence of E2F and Rb in the dDREAM complex in Drosophila giving rise to an interesting evolutionary comparison and justifying the publication in a journal that address a broad scientific community.

Minor points
p3, end of first paragraph. The authors may cite Larson-Rabin et al., Plant Phys, 2008, when introducing CCS52.

p4, middle of second paragraph. A reference to Chen et al, PNAS, 2009 should be added.
The nomenclature of cyclin D3;1 is not uniform. Sometimes the authors abbreviate CycD3 (for instance p4, forth line from below), sometime they write CYCD3;1 (p4, third line from below).

p5, second paragraph. The sentence starting with "...On the other hand..." is fragmented.

p6, middle of the page. The statements "...We confirmed the functionality..." and "...we detected functional E2F-GFP..." are too strong. Binding of E2F-GFP to RBR1 is certainly an important aspect of the fusion protein but it is not the a the proof of functionality.

p7, first line. The authors state that there are no publicly available T-DNA insertion lines for E2FA. However, four insertion lines within the coding frame of E2FA are listed in the SALK T-DNA express database. Presumably, the authors have tested them and found them not useful. This information would be interesting to disseminate to the community.

1st Revision - authors' response 11 July 2011

Answers to the reviewer's comments:

Referee #1 (Remarks to the Author):

Magyar et al. study here the functional relationship between E2FA and RBR1 in proliferating and endoreduplicating cells of Arabidopsis thaliana. The authors accomplish a significant amount of work using a variety of constructs that allowed them to manipulate the levels of E2FA, DP counterparts and RBR1. The claim that E2FA maintains proliferation in a complex with RBR1 that is not targeted by CYCD3;1. Also E2FA alone stimulates endocycle and cell expansion in a RBR1-independent manner. This manuscript describes a thorough biochemical study of E2FA-RBR1

interactions. Based on numerous IP experiments as well as gene expression and other phenotypic analysis, the authors draw conclusions about the functional role of specific E2FA-RBR1 complexes in cell cycle regulation.

We thank to referee #1 for the comprehensive reviewing work. The main findings of our work are well summarized above. It is made clear that our conclusions are based on multiple lines of evidence; phenotypic, biochemical and gene expression changes in *Arabidopsis* lines where we genetically altered E2FA, DPA, RBR1, CYCD3;1 levels, functions.

The topic is very important, not only because it contributes to our general understanding of cell proliferation control in higher organisms but also because it challenges previous ideas about the functional relevance of cell cycle regulators. Also, the effort made by the authors to carry out a biochemical approach in a difficult model system is very valuable.

Again we thank reviewer #1 in recognizing the importance of this topic; cell cycle regulation in a developmental context. Reviewer #1 also rightly acknowledged that molecular/biochemical analysis during developmental processes is challenging, as it relies on dissecting developing organs, in our case leaves from the earliest developmental stage possible. Reviewer #1 also rightly highlights the value of the manuscript in challenging previous ideas how E2FA functions. As we write in the manuscript, the model based on E2FA/DPA co-overexpression was "that the elevated E2FA levels escapes from RBR1 repression and promotes both cell proliferation and endocycle, dependent on the tissue-specific availability of mitosis inducing factors." Our biochemical work, and conclusions drawn by molecular and phenotypic analysis of 35S::E2FA$^{\Delta RB}$/DPA lines challenges this model.

Unfortunately, many of their conclusions are not fully supported by the data or have been drawn without a critical test of alternative hypothesis.
The text is, in some parts, difficult to follow, but this might be inherent to the type of experiments described. The authors tend to use very general statements, not supported by experimental evidence, or jump into general conclusions, which have not been fully demonstrated.
In short, I have serious concerns about (i) the current depth of the study, (ii) the refinement of some experiments, (iii) the analysis of some of the tools generated in this study, and (iv) the manuscript (text) in its present form, which jeopardize the general validity of the conclusions claimed by the authors.

We will address these general critical comments in more details under the specific points the reviewer raised.
Our response to the above general points; (i) both reviewer #1 and the other two reviewers acknowledged that the manuscript already contain a large amount of work, using multiple techniques and approaches to back up the findings. Within the same manuscript it is not realistic to expand much further with technically demanding new experiments (ii) we have refined some of the experiments as will be detailed below (iii) we have molecularly verified all the tools what we have used in the manuscript and provide experimental evidence that they work as they should, including the E2FA-RNAi, the pE2FA::gE2FA-GFP and pRBR1::gRBR1-GFP, 35S::E2FA$^{\Delta RB}$/DPA lines and antibodies (iv) we have substantially rewrote the manuscript, mostly the abstract, results, figure legends parts to take into account the criticism. The manuscript has been edited by two native English speakers. The major conclusions of this work are based on multiple lines of evidence some of these are indirect, but in the light of all the other results strengthen the model.

General comments

a, *A general comment regarding plants lacking a particular E2F factor. How do the authors evaluate (or rule out) the possibility that in plants lacking one E2F factor, other E2F proteins can occupy target promoters which were not normally used (because they were perhaps preferentially occupied by the missing E2F)? It seems that most reagents and tools needed to do ChIP experiments to determine occupancy by various E2F proteins are available.*

We have demonstrated that E2FA downregulation and elevation within its own expression domain have opposite phenotypic consequences on cell proliferation and endocycle. This is strong genetic evidence that the phenotypes we are studying are linked to E2FA and not to a pleiotropic

compensatory effect, such as the occupancy of E2F elements on promoters by the remaining E2Fs. This genetic work strengthens the hypothesis that E2FA indeed involved in the regulation of both proliferation and endocycle, but could not tell anything about the underlying mechanism. The focus of our work was to unravel the mechanism of E2FA function by biochemical analysis of RB-E2F complexes, and analysis of 35S::E2FA$^{\Delta RB}$/DPA line rather than to study the complex genetic interactions within the E2F-RB regulatory network. Reviewer #1 is, however right that E2Fs, DPs, RBR1 function in a complex interacting network with many crossregulation. Perturbation of E2FA (by up and downregulation) could result in compensatory mechanisms, e.g. replacement of E2Fs on promoters by any of the other E2Fs, formation of new complexes etc. We agree with referee #1 that the regulatory network is an interesting scientific question, but this is not the focus of the present study. Moreover, the experiments suggested by reviewer #1 are challenging, as it is likely that different target genes will be distinct in how they respond to the E2FA perturbation by replacement of any of the 5 remaining E2Fs. Therefore the suggested ChIP experiments would only likely to be conclusive by genome-wide ChIP with all the 6 plant E2Fs in WT and E2FA RNAi lines. Additionally, this would need to be done in the developmental system we have studied at different leaf developmental stages. For the time being, we think it is equally important to clarify the function of individual E2Fs (E2FA) during plant development. We think our biochemical and mutant analyses discovered a novel mechanism how E2FA regulates proliferation and differentiation in an RBR1 dependent manner.

b. The conclusion that RBR1 in complex with E2FA might have a role in maintaining rather than repressing cell proliferation appears to be based on a kind of syllogism: E2FA overexpression increases complexes with RBR1; it also increases proliferation, then, RBR1-E2FA complexes stimulate cell proliferation. Since an excess of E2FA stimulates cell proliferation, that is, more cells are present in the sample, what is the relative amount of RBR1-E2FA complexes on a per cell basis? In fact, under these conditions it might occur that many more cells are developing a G1 phase where these complexes might be abundant. Could RBR1-E2F complexes be measured in a synchronized system, ideally in seedlings? This would provide, at least in part, direct information on complex formation throughout the cell cycle.

While the experimental result that elevated E2FA level leads to a dose dependent increase in E2FA-RBR1 complex and a corresponding increase in cell proliferation is suggestive that these are linked, the reviewer is right that it could have an alternative explanation with the increased proportion of proliferating cells in these samples. However, what is not reflected in this question that our conclusion is not solely based on this finding but also on the 1. elevated presence of E2FA-RBR1 complex in CycD3;1 overexpression plants, 2. opposite regulation of E2FA and E2FB complexes in E2FA/DPA and CycD3;1 lines, 3. phenotypic and molecular analysis of the 35S::E2FA$^{\Delta RB}$/DPA where the RB recruitment to promoters is blocked. Further conflict to the existing model assuming that E2FA overexpression leads to proliferation because free E2FA escapes from RBR1 repression is the comparison of RBR1 amounts in inputs and E2FA-IPs in Fig. 3B and Fig. 4C, suggesting that RBR1 free of E2FA is present even in the E2FA overexpression lines, thus RBR1 is in excess.
Reviewer 1 suggests testing his/her alternative hypothesis of increased E2FA/RBR1 complex due to increased proportion of proliferating cells in the sample by a cell cycle synhronisation experiment in seedlings. Seedlings have a mixture of cells, most are non-cycling, and others may cycle with distinct cell cycle lengths that would not allow conducting synchronisation experiment. However, we did test the alternative hypothesis of reviewer #1 by genetically modifying plants that have abbreviated G1; in the CYCD3;1 overexpression line, and determined E2FA- and E2FB-RBR1 complexes in this line compared to WT. CYCD3;1 is known to lead to extra proliferation by not allowing cells to exit cell cycle in G1, abbreviating G1. We show that in spite of the expected shortened G1 and the increased phosphorylation of RBR1 that should not favor RBR1-E2F interaction, there is even more E2FA-RBR1 complex present in the CYCD3:1 overexpression line, while the amount of E2FB-RBR1 complex is reduced, as expected.

c. *Most co-IP experiments are aimed at determining complexes between RBR1 with various E2F proteins. Then, these data are correlated with regulation of target gene expression. Strictly speaking, soluble complexes do not necessarily represent the same as chromatin-bound complexes. A major question is whether complexes detected in this study are functionally relevant regarding target gene expression control. ChIP experiments are required to draw strong conclusions.*

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The reviewer is right to state that the function of the E2FA/RBR1 complex is manifested when it is chromatin bound, and we have not yet demonstrated directly that the E2FA/RBR1 complex is physically present on the promoters of relevant target genes. However, it is not straight forward to demonstrate protein complexes on promoters, this cannot be done with a simple ChIP. We could have attempted to set up ChIP individually with RBR1 and with E2FA, but this would not show whether they regulate these target genes when in complex together, whether they occupy these promoters at the same time at the same place, whether RBR1 is bound through E2FA or through E2FB or E2FC. Furthermore, this would be most informative in the developing leaves, as these complexes and their function changes as the leaf develops. ChIP experiments require a lot of material and not feasible in dissected young leaf samples in Arabidopsis. Therefore we decided to do alternative ways to demonstrate that recruitment of RBR1 to promoters through E2FA is functionally relevant to maintain proliferation. The 35S::E2FA$^{\Delta RB}$/DPA show a clear loss of meristematic cell proliferation, stimulated entry into endocycle and a simultaneous upregulation of E2F target genes, which suggest that RBR1 recruitment to promoters through E2FA is functionally relevant to maintain proliferation and to repress gene expression (Fig. 5, Suppl Fig 7, 8). To further demonstrate that E2FA and E2FA$^{\Delta RB}$ binding to promoters is functionally important we co-expressed E2FA with a mutant DPA that was mutated in its DNA binding domain, and thus could only form soluble E2F complexes. These plants show none of the phenotypes characteristic for the E2FA/DPA overexpression line (Suppl. Fig 9). We have added further results including all the combination of crosses we have done with E2FA and DPA wild type and mutant versions as Suppl Fig 9C). Additional indication that the E2FA/RBR1 complex is formed on the chromatin is that both RBR1-GFP and E2FA-GFP expressed under the control of their own promoters are predominantly present in the nucleus.

Specific points.

1. Co-regulation analysis. Data in Suppl Table 1 should be extended below the cutoff of 0.7. Also, the use of this cutoff sounds arbitrary, at least is not sufficiently justified.

We agree with the referee, that the 0.7 correlation is arbitrary, but can be considered as a strong correlation, which we have chosen for simplicity. We could have also compared the top 50 of top 100 of co-expressed genes, that would lead to the same message E2FA and RBR1 shows a marked co-regulation with each other and with genes involved in DNA synthesis, chromatin functions. To address this point we have performed an experiment by applying sliding cut off windows of 0.7, 0.6 and 0.5 to generate gene lists with correlated expression with E2FA and RBR1 and performed GO overrepresentation analysis using the BINGO tool that rely on robust statistical methods to determine the significance of genes overrepresented in gene lists. All three cut of values produced very similar GO categories, and we included the GO overrepresentation results for the gene list with 0.6 correlation coeff cut off. This is included in Supplementary Table 2.

2. Fig 1A. A wt root is missing; this will allow the reader to compare the meristem size as an indication of proliferation rate of E2FA-GFP and RBR1-GFP plants. It seems that the size of the RBR1-GFP meristem is smaller than that of the E2FA-GFP plants. Also, it would be desirable to see some details of cell architecture in these lines compared to wt. Formally, the ability of E2FA-GFP to complement a mutant, as has been done for RBR1-GFP would be the ideal situation. Alternative ways of showing functionality are needed.

In Fig. 1A we intended to show the expression domains of E2FA-GFP and RBR1-GFP when expressed under their own promoters in developing roots and leaves and have not analyzed the root meristem sizes. To capture different domains in developing organs we show low magnification images. However, we agree with the referee that including high magnification images provide additional information. We also agree with the referee, to include WT both as a negative control, and to allow comparison of root meristem sizes. We have done this in the revised Figure 1 and in Suppl. Figure 1, and show that pE2FA::gE2FA-GFP has a slightly larger while pRBR1::gRBR1-GFP slightly smaller root meristem compared to WT.

We have worked with E2FA RNAi lines that did not allow performing genetic complementation, which would be the most straight forward test for functionality of the E2FA-GFP fusion protein. However, we do provide both biochemical and genetic evidence, all suggesting that E2FA-GFP is functional: *i,* it is able to form complex with RBR1, *ii,* could efficiently dimerize with both DPs, *iii,*

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E2FA-GFP protein is localized in the nucleus, and *iv,* it could stimulate mitosis as well as endocycle in a dose dependent manner (Fig. 4), v, up-regulate target gene expression such as MCM3, and CDKB1;1. We added this information more clearly to the text.

3. The E2FA RNAi plants should be described in more detail. There is a partial reduction of E2FA in E2FB RNAi cells. Is this significant and biologically relevant? What is the mRNA level of the six E2F in the E2FA RNAi lines (see Suppl Fig 1B). This is important because some other E2F family members have been implicated in regulating cell cycle and endoreduplication.

To demonstrate the specificity of the E2FA RNAi construct we focused on the two E2Fs with closest sequence similarities, E2FA and E2FB. To minimize indirect effects not related to silencing such as e.g. cross-regulation through promoters, we coexpressed silencing and epitope-tagged E2FA and E2FB constructs under constitutive CaMV 35S promoters transformed into protoplasts and show that the E2FA RNAi construct efficiently silences E2FA but not at all E2FB. From this experiment we concluded that E2FA RNAi construct is specific. Though it is not entirely relevant to the work, as we did not use the E2FB RNAi construct in any of the experiments, in Suppl. Fig 1 we also included the test of silencing effect of E2FB RNAi construct, and show an efficient silencing of E2FB with this construct while only a minimal reduction in E2FA. We do not know whether this is due to some residual silencing effect of E2FB RNAi construct on E2FA, or a cross-regulation on the posttranscriptional level between E2FB and E2FA, e.g. silenced E2FB effects E2FA protein stability. However, as stated above this is not relevant to the work in the manuscript, as we did not use the E2FB RNAi construct in this work.
It has been shown that altering the level of one E2F can influence the level of other E2Fs (Sozzani et al, 2006), and that is what we also observed for E2FB in the E2FA silenced (Fig 2B) and E2FA-GFP lines (Fig 4A). We have commented on this in the revised manuscript. We have not extended this study for all the 6 E2Fs, as studying cross-regulations among E2Fs was not the aim of this study.

4. It seems that the stomatal pattern is significantly altered in the E2FA RNAi plants. Is this correct? Is cell proliferation in this lineage altered and, if so, is it consistent with the major conclusions about E2FA-RBR1 interactions?

Reviewer #1 rightly noticed that there is an apparent increase in the stomata number in the E2FA RNAi line compared to WT. This could partly be because stomata linage cells continue proliferation even when pavement cells prematurely exit. It is also visible, that stomata cells are pushed together by the abnormally enlarged pavement cells. We did not expand further on this observation to study cell proliferation in the stomata linage in more depth, as it would require crossing the E2FA RNAi lines with stomata linage markers. RBR-E2F has been linked with stomata patterning and stem cell maintenance in other works (Borghi et al, 2010, Xie et al 2010; Meskiene et al, 2011).

5. Fig 2B. E2FB level are quite different in the two lines studied. Does this have any consequences on the phenotypes observed? It seems that E2F targets appear more drastically down-regulated in line 7.2 than in line 2.1, but line 7.2 presents ~50% reduction of E2FB transcripts. Does this different E2FB level affect target gene expression?

It has been published that E2FA can cross-regulate E2FB expression (Sozzani et al, 2006). However, based on our unpublished work we know that *e2fb* null mutant does not have the same phenotype as E2FA RNAi, and thus unlikely that the phenotype we see in E2FA RNAi is down to changes in E2FB. We have not completed our work on E2FB, but we did comment on this unpublished result in the revised manuscript.

6. Fig 3A. Since the amount of RBR1 significantly diminishes at late times, experiments to detect RBR1-containing complexes might be taken with caution (the starting material does not contain the same amount of input, in this case RBR1) .

Our results that RBR1 amount in *Arabidopsis* is highest in proliferating cells is well in agreement with recent published works (Wildwater et al 2005; Borghi et al 2010), while in maize leaf it was published to increase as cells leave proliferation and differentiate (Huntley et al, 1998). The amount of RBR1 does not necessarily need to parallel RBR1-E2F complexes, as this interaction is posttranslationally regulated by phosphorylation in proliferating cells. However, what we show in Fig 3A RBR1 and RBR1-E2FA complex amounts correlate and both are highest in proliferating

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leaves, which is in agreement to the other results in the manuscript that E2FA-RBR1 complex is formed in proliferating cells.

7. Fig 3B-C. I think that the large differences in E2FA and E2FB amounts shed some doubts about the validity of the results. Are the interactions observed comparable?

The reviewer is right to say that in the E2FA/DPA line E2FA is largely overexpressed. This leads to a cross regulation and increase of E2FB that is much more modest. However, our conclusions are based on comparisons of E2FA IP in WT and E2FA/DPA in panel B first; showing that in accordance with the large increase of E2FA amount, there is a large increase in E2FA/RBR1 complex as well. This contradicts the existing published model that E2FA overexpression solely promotes cell proliferation by producing RBR1-free E2FA. Secondly, we compared in panel C IP with E2FB between WT and E2FA/DPA overexpression lines and show that in spite of the increase in E2FB amount, there is NO increase, but rather decrease in E2FB/RBR1 complex. This contradicts the possibility put forward by reviewer #1 that we see more E2FA/RBR1 complex, as there are more cells proliferating and therefore in general more RBR1-E2F complex can form when cells are in G1, as this is not true for E2FB.

8. *Suppl Fig 3B and C. The differences between RBR1 levels between 2-weeks and 1-week WT-Col plants, respectively, are real? Do they represent experimental variation?*

Yes, this is real, other cell cycle genes and CDK activities are also significantly elevated at 1 weeks compared to 2 weeks old seedlings, and that is why we have chosen these two different time points to contrast the up and down regulation of RBR1 phosphorylation by CycD3;1 and KRP1 bound CDKs, respectively.

9. *Since the truncated form of E2FA eliminates the RBR1 interactions and destroys the transactivation domain, how the up-regulation of target gene expression is explained? What is the basis to correlate the results exclusively with the loss of RBR1 binding, without considering the loss of transactivation capacity?*

We have considered both possibilities in the manuscript stating: "To study whether the E2FA$^{\Delta RB}$/DPA binding to DNA blocks the recruitment of innate E2FA to promoters for trans-activation, or prevents the RBR1 repression of these genes, we tested the expression of various E2FA target genes (Vandepoele et al., 2005). In the first scenario target gene expression is expected to be down-regulated, while in the second they would be up-regulated." We found all target genes tested to be upregulated, therefore accepted the second scenario; E2FA recruits RBR1 to repress these target genes, when RBR1 recruitement to target genes is prevented in the 35S::E2FA$^{\Delta RB}$/DPA through a dominant-negative effect, these genes can be expressed, presumably through other unknown TFs.

Referee #3 (Remarks to the Author):

The manuscript by Magyar et al reports on the mechanism by which a cell cycle gene in plants (E2FA) might influence both cell proliferation and differentiation via interaction with a plant Rb-related protein (RBR1). RB/E2F complexes have long been recognised as key players in the control of cell division, but exactly how they integrate the various aspects of proliferation/differentiation is still unclear- thus the paper addresses an important topic.

We thank the reviewer the positive comments, recognizing the importance of our work.

I found the paper to be densely written, particularly in the context of the numerous immunoblots shown. The description of the figures was often poor. Key to the whole paper is the requirement that the commercial antibody used to detect the phosphorylation status of the RBR1 protein produces a true and accurate reflection of the RBR protein status. I could find no evidence in the paper to verify this or reference to publications showing that this antibody is doing what it is meant to for a plant Rb-related protein. I think for EMBO J this would be a requirement, especially when the data obtained with this antibody are central to the paper.

We have shown that immunoprecipitation with the commercial phosphor-RB (P-RB) antibody

raised against conserved phosphorylation sites (Ser807/811) brings down a protein that is recognised by the Arabidopsis specific RBR1, from which we conclude that it is recognising a genuine RB in Arabidopsis (Suppl. Fig 3). We also have carried out immunoprecipitation with P-RB antibody in the pRBR1::RBR1-GFP plants (data added as Suppl Fig 4F), which was followed by immunoblot using the GFP specific antibody. The immonoprecipitated RBR1-GFP band was shifted according to the extra GFP-tag, further confirming the specificity of the P-RB antibody. We also show that in CYCD3;1 o/e the reactivity with P-RB antibody increases, while in KRP it is decreased as expected. This together with a recent publication using the same antibody (Abraham et al 2011), which shows that phosphatase treatment of extracts diminishes the cross-reactivity with the protein which is recognised by the RBR1 antibody shows that the commercial P-RB antibody does work in plants.

Apart from this technical issue, there are some further points.

1) *The authors make a great deal of the endocycle and its link with cell growth yet, as they admit on p8, their own data (and others) indicate that the link is often tenuous.*

It is well accepted that in some plants and cell types cell growth correlate with ploidy, but the reviewer is right that our work on E2FA RNAi shows, that this can be uncoupled, and cells can enlarge without increasing the DNA amount. We noted this in the manuscript: "Although endocycle has been correlated with cell enlargement (Breuer et al., 2010), in the E2FA RNAi this appears not to be the case, since leaf epidermal cells enlarged with only a modest increase in their DNA content." Nevertheless, we still think that both roles of E2FA; meristem maintenance and endoreduplication are important for plant growth, as E2FA RNAi plants are small.

2) *p7- an increase in G1 to G2 ratio could reflect a shorter G2 or a longer G1, I think concluding an arrest in G1 is not warranted.*

Reviewer #3 is right that solely from flow cytometry measurement it cannot be established whether the G1 and G2 distribution is changed due to longer G1 or shorter G2. We favor the first scenario because if G2 would be shortened that would result in cells dividing with smaller size, but we see larger cells in E2FA RNAi plants. We added this point to the revised manuscript.

3) *Obviously quantitative analysis of such IP experiments is very tricky, nevertheless it would be good to have an indication of how many times the individual experiments were performed and an assessment of the variation in band intensity between experiments. A triplicate biological replication would be a minimal expectation*.

We have included repeats of the biochemical experiments as follows; (i) E2FA interaction with RBR1 during leaf development have been shown 2 times within the manuscript by using E2FA antibody in Fig3A, Fig4C and we attach an additional 3rd repeat to this letter as Figure 1 for reviewers panel A. The same experimental question with identical results was also presented by using the GFP antibody from the E2FA-GFP line. We have purified complexes by using GFP specific antibody in Fig 4C, Supplementary Fig 4D and E. Thus altogether this was shown 3 times with E2FA and 3 times with the GFP antibody. (ii) We have demonstrated different interaction of E2FA and E2FB with RBR1 in the CycD3;1 overexpression line, which we show in the original manuscript Fig3 D,E. Cotyledons provide a unique experimental system, since normally there is minimal proliferation and largely endocycle, while in the CYCD3;1 overexpression line this is switched to robust overproliferation. We added a new experiment on cotyledons with co-IP of E2FA or E2FB with RBR1 as Suppl Fig5C. (iii) To demonstrate the increased E2FA-RBR1 complex when E2FA/DPA are co-overexpressed, and thereby promote proliferation we have shown co-IPs with identical results in Fig3B&C, and we attach an additional co-IP from this line to the letter as Figure 1 for reviewers panel B.
To include statistical test of variation is indeed very tricky for these kinds of biochemical experiments, but since both the repeats and the 3 experimental approaches are all fully consistent, we do feel confident on these biochemical results.

4) *There are various minor corrections to English and formatting required.*
Overall, the story is novel (although perhaps mainly for a plant audience) but the data underpinning the story need to be stronger.

We have substantially edited the text, and the manuscript was also corrected by two native English

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speakers. We do not agree with the referee that this story is mainly for plant audience, as it investigates a fundamental question in biology how cell proliferation and differentiation are interconnected through a conserved regulatory mechanism, the RB-E2F pathway. This has been recognized by the two other reviewers.

Referee #4 (Remarks to the Author):

Review : Arabidopsis E2FA stimulates proliferation and endocycle separately through RBR- bound and RBR-free complexes
by Zoltan Magyar et al.

The authors have very carefully conducted their experiments and one statement is back upped by several experiments. Indeed, this paper holds a wealth of information that will be very important for future studies in the field. The heart of this paper is the expression of an E2FA variant that cannot bind RBR and indeed this version together with DPa expression strongly promotes differentiation. A somewhat simpler experiment would have been to co-overexpress RBR and E2FA since this should stimulate then cell proliferation. I am wondering whether the authors have attempted this?

We thank reviewer #4 for the comments, which demonstrate that he/she has fully understood the importance of the E2FA$^{\Delta RB}$ part of the work that together with the other experiments of E2FA up and downregulation, biochemical analysis of the complex allowed us to develop a new model. The experiment of co-overexpression of RBR1 and E2FA is interesting. In the manuscript we show that an increase in E2FA amount under its own promoter leads to dose dependently more E2FA-RBR1 complex and proliferation. Within the meristem RBR1 is already abundant. We know that in the pRBR1::gRBR1-GFP line there is around 10 time higher RBR1 level, yet there is no dramatic effect on plant growth. This suggests that RBR1 might be present in excess in the meristem. In contrast when RBR1 level is ubiquitously increased by inducible overexpression through the dex-inducible system, differentiation became a dominant event which consequently ended up in loosing meristematic activity (Wildwater et al., 2005; Wyrzykowska et al., 2006). In these published works it was suggested that the extra RBR1 is sequestering the free E2F/DP dimmers which cause cell cycle block and in parallel promote differentiation. We have tested this by co-IP of RBR1 with DPA. This shows that the complex formation between RBR1 and E2F/DPA dimmers was not increased but actually reduced when RBR1 protein expression was induced by nearly 10 times using the dex-inducible system (see in the attached Figure 2). This unpublished result indicates that constitutively overexpressed RBR1 might induce differentiation by a different mechanism than through the expected association with the E2F/DP dimmers. RB is known to have many other targets besides E2Fs, and perhaps when expressed to high levels outside its native expression domain, it acts through these nonconventional targets. We still need to do much work to elaborate this idea. Therefore we think the cross between the dex-inducible RBR1-OE and E2FA-OE might not give the expected result. Can we further increase RBR1 and specifically make more RBR1/E2FA complex where they are normally abundant, within the meristem? We have started to generate crosses between pRBR1::gRBR1-GFP and pE2FA::gE2FA-GFP overexpression lines. Since both E2FA and RBR1 effects are dose dependent, we will need to generate homozygous lines for both, that will require more time than what is available for revision. However, as reviewer #4 agrees, these results are not essential for the main conclusions, but might give an additional angle/confirmation to our story, provide a 4th way (additional to the leaf developmental series, E2FA/DPA and CYCD3;1 lines) to show a correlation between elevated E2FA/RBR1 complex and proliferation. However, this experiment would not replace the dominant negative approach of the 35S::E2FA$^{\Delta RB}$/DPA line to block the recruitment of RBR1 to promoters, and thereby to assess the importance of an RBR1/E2FA repressor complex for proliferation, leaf development.

In any case, the here presented data makes a very strong case for the reasoning of the authors. Moreover, with these finding the situation in Arabidopsis might reflect the presence of E2F and Rb in the dDREAM complex in Drosophila giving rise to an interesting evolutionary comparison and justifying the publication in a journal that address a broad scientific community.

We thank again for reviewer #4 recognizing the importance of our work.

Minor points

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p3, end of first paragraph. The authors may cite Larson-Rabin et al., Plant Phys, 2008, when introducing CCS52.

Thanks for realizing this omission of reference, we cited other related works at this point, but included this reference.

p4, middle of second paragraph. A reference to Chen et al, PNAS, 2009 should be added.
The nomenclature of cyclin D3;1 is not uniform. Sometimes the authors abbreviate CycD3 (for instance p4, forth line from below), sometime they write CYCD3;1 (p4, third line from below).

We agree and made these changes.

p5, second paragraph. The sentence starting with "...On the other hand..." is fragmented.

We agree and corrected.

p6, middle of the page. The statements "...We confirmed the functionality..." and "...we detected functional E2F-GFP..." are too strong. Binding of E2F-GFP to RBR1 is certainly an important aspect of the fusion protein but it is not the a the proof of functionality.

Although we agree with reviewer #4, further data presented in our work indicate that E2FA-GFP is functional (see above in the response to reviewer #1, and our changes to the text of the manuscript).

p7, first line. The authors state that there are no publicly available T-DNA insertion lines for E2FA. However, four insertion lines within the coding frame of E2FA are listed in the SALK T-DNA express database. Presumably, the authors have tested them and found them not useful. This information would be interesting to disseminate to the community.

We apologize not being specific enough. There was only one publicly available T-DNA insertion mutant line for *E2FA* (SALK_034842) at the time of this work, which should be a true null mutant. However, we found no insert at the expected position with this line. We added this information to the text.

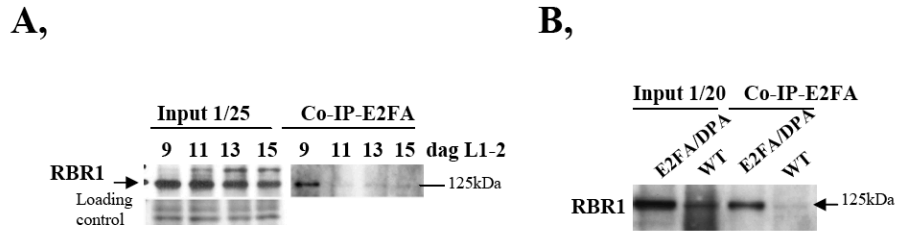

Figure 1 for reviewers. E2FA interacts with RBR1 in proliferating tissues. *A,* Interaction of E2FA with RBR1 during leaf development. Immunoprecipitation (IP) with antiE2FA antibodies from protein extracts prepared from the juvenile first leaf pair (L1-2) of wild type *Arabidopsis* plant at the indicated time points (days after germination - dag). Anti- RBR1 antibody was used to probe the co-IPs on Western blot. 1/25 of the extract was loaded as input. Coomassie-stained proteins on the same membranes are shown as loading control. *B,* IP with anti-E2FA antibody from protein extracts of WT-Col and E2FA/DPA seedlings one week after germination. The co-IPs were probed with antiRBR1 on Western blot. 1/20 of the extract was loaded as input to determine RBR1 level as indicated.

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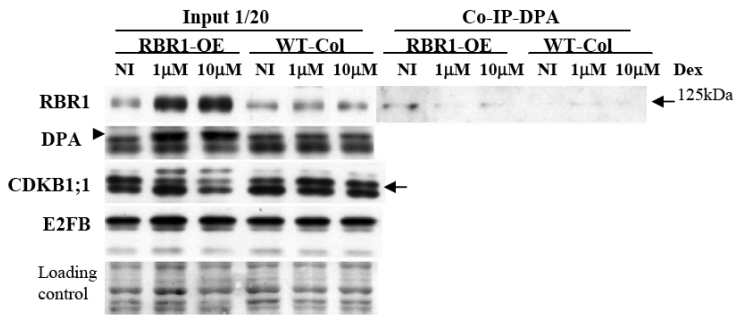

Figure 2 for reviewers. Ectopic RBR1 do not make more complex with E2F/DPA heterodimers. RBR1 expression was induced in RBR1 overexpressing line (RBR-OE; Wildwater et al., 2005) by adding dexamethasone (Dex) at the indicated concentrations into the growth medium of six days old transgenic seedlings or to the wild type control. Noninduced (NI) seedlings were prepared and used as contols. One day after incubation protein was extracted and probed with specific anibodies in Western blot as indicated on the left. Immunoprecipitation with anti DPA anti- body was carried out (Co-IP) and the presence of RBR1 protein in the immunocomplex were tested in Western blot assay by using RBR1 specific antibody. Input level was 1/20. Arrow indicates the position of RBR1 and CDKB1;1 proteins. Arrowhead marks a DPA form appeared only in the induced RBR-OE samples. The molecular weight of the RBR1 protein is indicated on the right.

2nd Editorial Decision 30 August 2011

Thank you for your patience with the re-review procedure of your E2FA manuscript. We have received reports from the original referees 1 and 4 (copied below). Referee 4 is satisfied with the revisions but referee 1 retains major reservations. We have now not only discussed these concerns in depth within our editorial team, but also taken the time to go back to the expert referees to assess the importance of clarifying these remaining issues. Our conclusion from these consultations is, I am afraid to say, that the paper is at this stage still not ready for publication in The EMBO Journal. The two main issues precluding publication are the unclear mode of action of the truncated E2Fa mutant unable to bind RBR1, and the unclear relative amounts of complexes being formed. In our view, these two points would minimally have to be addressed as important controls to support the current conclusions of the study.

i) some experimental data will be required to explain how E2FA lacking RBR1 binding (and transactivation domains) works in target gene regulation - also to rule out that the observed effects may be due to other causes.

ii) certain non-convincing co-IPs will need to be repeated, and existing/new IP data quantified to allow normalization by (re-)calculation of relative complex amounts. Relative amounts would also need to be determined in synchronized cells, e.g using an existing, published whole-meristem synchronization procedure.

Should you be able and willing to conduct these additional experiments - which are in our view not meant to expand the study into new directions but really to consolidate the current interpretations - then we could consider the manuscript through an exceptional second, final round of revision. I have to stress however that this will have to be the last round of revision in this case, and that acceptance or rejection will depend on convincing the critical referee 1 that you have added sufficient control data on these two issues to put the claims in question on a more solid basis and to offer stronger direct support for them. Therefore, I hope you will be able to address these points and return a re-revised manuscript to us as soon as possible. As usual, please do not hesitate to contact me should you require any further clarifications in this regard.

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Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Magyar et al. have resubmitted a revised version of their manuscript where they study the functional relationship between E2FA and RBR1 in proliferation and endoreduplication in Arabidopsis.
I have the following comments to the authors' reply to my original comments.

General concerns.
(i) Stating that "is not realistic to expand ... with technically demanding experiments" is not acceptable because the experiments suggested were intended to clarify and/or support the authors' conclusions, not extremely complex, and not to expand into new aspects of the study.

More importantly, I feel that some of the weak points of the work, expressed in paragraph b. of my comments, have not been addressed. They mostly refer to the amount of RBR1-E2FA complexes, in particular the relative amount of complexes (on a per cell basis).

The authors three arguments, which I do not find convincing.

1. The presence of E2FA-RBR1 complexes in D3;1 OE plants. It is true that this is shown in Suppl. Fig. 5 in the coIP with E2FA. But the input amount of RBR1 in D3;1 OE is much larger that in the control. Therefore, what is the relative amount of complexes formed in Ler and D3;1OE (ratio of IP to input?). I wonder whether there are really "more" complex (more efficiency in complex formation) when relative amounts are considered.

2. Opposite regulation of E2FA and E2FB. The claim that RBR1 decreases after IP with E2FB relative to Ler is not supported by the data.

3. Truncated E2FA. The use of E2FAdeltaRB is interesting but if this protein does not bind to RBR1 and does not transactivate, what does it when bound to promoters? It if does not transactivate how the up regulation of target genes is justified? The fact that endoreduplication is affected indicates the importance of E2FA for the endocycle, which is already known. Also, indirectly, the role of RBR in the endocycle, also known.

In addition:
- Fig. 3A. It would have been appropriate to show the amount of E2FA during leaf development and then consider the amount of complexes relative to the input amounts of both RBR1 and E2FA.

- The IPs shown in Fig. 3D and E are not convincing. The increase of RBR1 in D3;1 OE relative to Ler is clear in 3D (two middle lanes) but this is not the case in 3E (two middle lanes). They should look similar.

- The approach used by Cools et al. (2010) could be useful to determine E2FA-RBR1 complexes during the cell cycle. It is true that seedlings treated as described in this paper consist of a mixture of cells with different cycling characteristics. However, this is also the case in the coIP experiments described in this study.

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Referee #4 (Remarks to the Author):

In this revised version, Zoltan Magyar et al. have sufficiently addressed all of mine and, as far as I can see, all concerns of the other reviewers. In particular, the authors have verified the tools used, including the applicability of the used phospho-antibody against RBR, the E2FA/B RNAi lines and the pE2FA::gE2FA-GFP and pRBR1::gRBR1-GFP, 35S::E2FA RB/DPA lines. They have updated many figures and added supplementary information to corroborate their findings. The manuscript covers a complicated topic but as such the paper is well written now. With this, the authors present many interesting and important findings underpinned by strong biochemical data.

Additional correspondence (author) 9 September 2011

Thanks for your continued support on this manuscript. Understandably, the team is anxious of further delays publishing this work. We will aim to make the revision as quickly as possible. However, we are concerned with some of the points and suggested experiments, and would appreciate if we could get more details from you before we start working on the revision.

1. We think to a large extent we have provided data to question one. We have shown that the mutant E2FA (E2FAΔRB) is not able to transactivate (Supplementary Figure 7G) or to bind RBR1 (Supplementary Figure 7E), but binds to E2F sites (Supplementary Figure 7B,C), and by the co-expression of a mutant DPA (DPAΔDB), we show that its DNA binding activity is required for hyperactivation of endocycle (Supplementary Figure 8 and 9). Reviewer 1 is right that E2FA has already been implicated in regulating the endocycle but the underlying mechanism was not clear. DeVeylder et al (2002) overexpressed the full length E2FA and show that in certain cells it can activate endocycle.
They suggest a mechanism that E2FA when overexpressed becomes free of RBR1 and transactivate genes for S-phase both in mitosis and in endocycle. Overexpression of E2FAΔRB mutant form and biochemical and genetic studies clearly show that the mechanism must be different; proliferating cells contain E2FA-RBR1 complex and the transactivation of E2FA is not required for endocycle. The E2FAΔRB co-IPs and gene expression analysis work collectively suggest that the formation of E2FA-RBR1 repressor complex rather than transactivation is important to regulate the switch from mitosis to endocycle. We also up and downregulated E2FA within its own expression domain to provide further evidence for its function in regulating mitosis and endocycle. What we think the mechanism is that the overexpression of E2FAΔRB does not allow the formation of this repressor complex (dominant negative effect). As we discussed in our previous response letter, it is difficult to directly visualize repressor complexes on promoters by ChIP (the presence of RBR1 and E2FA together on promoters). We are glad that this has been accepted, as there were no comments on this point. One further experiment we can think of for demonstrating the importance of RBR1-E2F complex in endocycle is to genetically alter RBR1 levels and check endocycle onset.

2. We think that we have shown in a convincing manner that E2FA forms complex with RBR1 in dividing tissues. It is interesting, that RBR1 protein level is upregulated in tissues with more cell proliferation (such as young leaves, E2FA/DPA, CycD3oe lines). We also think, and the quantification of existing Western blots can show this that the complex formation is proportional between RBR1 and E2FA so where there are more RBR1 expressed in dividing tissues there are more RBR1/E2FA complex. This is not the case for E2FB. It is true that there are more cells proliferating in the E2FA/DPA, CycD3oe lines. There are no ways it would be possible to determine the amount of RB complexes in a per cell basis in this system. To keep the cell number/tissue organization comparable one needs and inducible system for CycD3;1, which we do not have. However, it is known that sucrose availability can regulate CycD3;1 amounts, and we can attempt to treat whole seedlings with sucrose, determine and quantitate the relative levels of E2FA- and E2FB-RBR1 complexes. To be completely comparable, we plan to use GFP tagged lines and the same GFP antibody across these experiments. We are skeptical to use the replication stress-induced synchronization method since it could trigger the formation of stable RB E2F complexes, as it has been published in animal cells regardless of the actual cell cycle phase (Ianari et al, Cancer Cell, 2009). Moreover, the work presented on the Cools et al 2010 paper is using root tips rather than whole seedlings for molecular and cell cycle analysis work, and this is not feasible for

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biochemical experiments.

In summary, what we suggest to do to further improve the manuscript in the revision is to 1. study the endocycle in RBR1 modified lines, 2. quantify the existing co-IP experiments and 3. carry out sucrose addition experiments and co-IPs in a system where we synchronously induce the CycD-RBR pathway.

We would appreciate your comments on this plan.

Additional correspondence 20 September 2011

Thank you for your enquiry regarding the planned experiments associated with your re-revision. Again please excuse the delay in getting back to you with a response, but not being an expert in the field I wanted to consult with the critical reviewer regarding your points. In the meantime I did receive his/her feedback, and the referee was also kind enough to provide some comments that I can pass on to you directly (see below). In essence, referee 1 feels that the experiment you propose (especially in points 1 and 3) sound reasonable, but obviously reserves final judgement about their adequacy depending on the eventual outcome of these experiments. At the same time, the referee also stresses the importance of trying to decisively answer the open questions directly wherever possible, and not just by indirect experiments. In this respect, especially points 2 and 4 below need to be decisively answered.

I hope you find these comments helpful, and that your revision experiments will be successful.

Sincerely,
Editor
The EMBO Journal

Comments from Referee 1:

I appreciate the authors' effort to design alternative ways of addressing my points, but I think that my previous report was sufficiently clear. I consider that some of the claims are not sufficiently supported by the data. Thus, there are a few questions that remain to be answered. I refer again to my last comments.

1. E2FA-RBR1 complexes in D3;1 OE plants. These experiments need a careful quantification and the values should be made relative to the amount of RBR1 in each case (they are quite different). My previous point of showing the amount of E2FA during leaf development is also relevant to this comment. The proposal of analyzing complex formation after sucrose treatment is interesting, and may shed some light.

2. Opposite regulation of E2FA and E2FB. The claim that RBR1 decreases after IP with E2FB relative to Ler is not supported by the data presented.

3. Truncated E2FA. The mechanism of upregulation of target genes should be delineated and discussed, since it is a major point of the story.

4. The IPs shown in Fig. 3D and E are not convincing. The increase of RBR1 in D3;1 OE relative to Ler is clear in 3D (two middle lanes) but this is not the case in 3E (two middle lanes). They should look similar.

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2nd Revision - authors' response 13 December 2011

Answers to Reviewer 1

We thanks to Reviewer 1 for all the criticism and for the editor to solicit further comments from reviewer 1 pointing out the most essential questions to be addressed during revision, and for the support to provide us the opportunity to revise the manuscript second time. During the revision we have considered all points of previous and present comments from the reviewers, performed new experiments that have added further data to substantiate our claims and rewrote the manuscript to make more clear our claims and model we propose. In the current manuscript we have 6 main figures, 13 Supplemental figures and 3 supplemental tables, in total 80 panels presenting the findings behind our claims. The following new experiments have been performed and added in figures during the revision:

1. We have performed synchronisation of seedlings with addition of 0% and 2% sucrose. We show alterred cell cycle parameters by flow cytometry of leaves 1-2 (Col-WT), and by expression analysis of cell cycle marker genes with Q-RT-PCR in seedlings (Suppl. Fig5 B and C). We monitorred the P-RBR1 6h after +/- sucrose addition in Western blots shown in the Supplementary Fig 5A in wild types (Col and Ler) and in the CYCD3;1 and KRP2-OE lines. These experiments show that + sucrose leads to RBR1 phosphorylation that depends on CYCD3;1/KRP2 levels. We have than utilised the alterred cell proliferation after adding 0% or 2% sucrose to seedlings to demonstarte the opposit regulation of RBR1 interaction with E2FA compared to E2FB as shown in figure 3D and E (IP from the RBR-GFP line, and from the CYCD3;1-OE, respectively).

2. To demonstrate how E2F target genes can be upregulated in the lack of transactivation capability of E2FA we focussed our work on the CCS52A with the aim to show that these genes are direct targets of the E2FA/RBR1 repressor complex. We show by QRT- PCR that in young developing leaves with cell proliferation active, the CCS52A1 and A2 genes are upregulated in the *e2fa-1 ko* line (as we have found in the E2FAΔRB/DPA) and downregulated with elevated E2FA expression in the E2FA-GFP lines (Fig 5C). We went on to show by ChIP with RBR1 antibody that RBR1 can bind at the vicinity of E2F sites at the promoters of CCS52A genes (Fig 5E).

3. We also have analysed by Q-RT-PCR the expression of all 6 E2Fs in the *e2fa-1-ko* line; Suppl. Figure 11D. This shows cross-regulatory links between E2FA and E2FC, DEL1-3. Interestingly, E2FB expression was alterred in a developmental-stage specific manner in the *e2fa-1* ko.

4. We have analysed the DNA content in the *e2fa-1* T-DNA insertion, and show that it similarily compromises endocycle than we have found in a number of E2FA RNAi lines (Suppl. Figure 3F).

5. We have analysed endocycle in the RBR-GFP (that has 10x elevated RBR1 level within its own expression domain) and found both proliferation and endocycle to be compromised– FCM analysis; Suppl Fig. 6E. 6. We provide a summary model in Fig. 6 to aid understanding.

Specific answers for reviewer 1 comments 2

1. *The presence of E2FA-RBR1 complexes in D3;1 OE plants. It is true that this is shown in Suppl. Fig. 5 in the coIP with E2FA. But the input amount of RBR1 in D3;1 OE is much larger that in the control. Therefore, what is the relative amount of complexes formed in Ler and D3;1OE (ratio of IP to input?). I wonder whether there are really "more" complex (more efficiency in complex formation) when relative amounts are considered.*

We have used cotyledon to demonstrate the opposite regulation of E2FA-RBR1 and E2FB-RBR1 complexes because it is a y a tissue with a high degree of endocycle. Interestingly in WT cotyledon there is hardly any detectable RBR1 (in agreement with RBR1`s role in repressing endocycle), while in CYCD3;1, RBR1 is abundantly present. We did not normalize the IP for the RBR1 amount (equal RBR1 input) but for equal protein amounts. What we find however in Suppl Fig 5 that there is no detectable E2FB association with the large amount of RBR1 in the CycD3;1, but there is clear association with E2FA. Because in other conditions (seedlings) we know that we can precipitate with E2FB antibody, we think this is not a technical problem.

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Fig. 3A. It would have been appropriate to show the amount of E2FA during leaf development and then consider the amount of complexes relative to the input amounts of both RBR1 and E2FA.

E2FA levels in most cases are too low to be specifically detected with our antibody in crude extracts, and this is the case in this particular experiment. However, we have tagged E2FA with GFP under the control of its own promoter and we show that it is most abundant in proliferating cells (Figure 4).

To provide further evidence that - The IPs shown in Fig. 3D and E are not convincing. The increase of RBR1 in D3;1 OE relative to Ler is clear in 3D (two middle lanes) but this is not the case in 3E (two middle lanes). They should look similar.

We decided to move Fig. 3D to supplementary Fig6A and remove Fig 3E and replace this Western blot on RBR1-E2FA and RBR1-E2FB interactions with experiments we have performed in different sucrose conditions. However, we quantitated this Western blot in Fig 3E, and do see a difference in the amount of RBR1-E2FA and RBR1-E2FB interactions in CYCD3;1 overexpression lines. As we have explained above, in equal protein loading there is more RBR1 in CYCD3:1 overexpression line than in Ler WT, yet less is in complex with E2FB, but not with E2FA (see quantified blot uploaded together with submission).

2. Opposite regulation of E2FA and E2FB. The claim that RBR1 decreases after IP with E2FB relative to Ler is not supported by the data.

We have performed experiments with +/- sucrose addition that synchronously induces RBR1 phosphorylation in a CYCD3;1 and KRP2 dependent manner and oppositely effect RBR1/E2FA (abundant in the presence of sucrose and RBR1-E2FB interactions (abundant in the absence of sucrose). Comparison of CYCD3;1 overexpression vs WT line in sucrose free condition clearly show that more E2FA-RBR1 and less E2FB-RBR1 complex is present in CycD3;1 compared to WT. We quantitated all these Western blots and provide it as a supplementary Figure 12.

The approach used by Cools et al. (2010) could be useful to determine E2FA-RBR1 complexes during the cell cycle. It is true that seedlings treated as described in this paper consist of a mixture of cells with different cycling characteristics. However, this is also the case in the coIP experiments described in this study.

We decided to use the sucrose addition to synchronously alter cell proliferation because it acts on the RBR1 controlled entry point to the cell cycle. Inhibition of DNA synthesis acts after this control point, and considering the low synchrony attainable in this system of whole seedlings we were not confident it would allow us to address the question the reviewer has asked. Additionally, the DNA damage 3 checkpoint induced by HU was shown to alter RB-E2F interaction on its own (Ianari et al, 2009), and therefore in a HU-induced synchronization we could not distinguish whether we look on cell cycle stage specific RB-E2F interaction or DNA damage checkpoint induced interaction. .

3. Truncated E2FA. The use of E2FAdeltaRB is interesting but if this protein does not bind to RBR1 and does not transactivate, what does it when bound to promoters? It if does not transactivate how the up regulation of target genes is justified? The fact that endoreduplication is affected indicates the importance of E2FA for the endocycle, which is already known. Also, indirectly, the role of RBR in the endocycle, also known. In addition: - -

It was published that E2FA/DPA overexpression in some tissues leads to endocycle, and it was proposed that this relies on the transactivation of S-phase genes in tissues where there are no mitotic genes expressed. However, in this manuscript we show that overexpression of E2FAΔRB/DPA leads to even more robust endocycle (yet it cannot transactivate), thus transactivation is not required for E2FA to stimulate endocycle. What does E2FA than do on promoters? We provide multiple evidence that in proliferating cells E2FA forms a repressor complex with RBR1 on promoters. We focussed during the revision on two such genes (CCS52A1 and A2) and show by ChIP that RBR1 in vivo binds to their promoters. We also show that in e2fa-1 KO as well as in the E2FAΔRB/DPA (where the RB binding to E2FA is abrogated) CCS52A1 and A2 are up while in E2FA-GFP with elevated E2FA expression these genes are down. Thus, it is the removal of E2FA-RBR1 repressor

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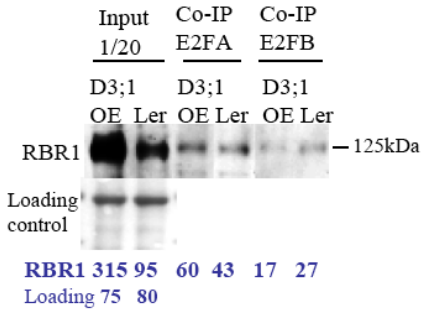
complex which activates these genes. We found other genes that are putative E2F targets to be upregulated in the E2FAΔRB/DPA line, but we do not yet know whether these are direct targets. To summarize how we think that E2FA functions in *Arabidopsis* we show a model in Figure 6.



3rd Editorial Decision 01 January 2012

Thank you for submitting your re-revised manuscript for our consideration. Referee 1 has now kindly looked at it once more, and I am happy to inform you that s/he now retains no further objections against publication in The EMBO Journal. There are nevertheless a few pending textual and presentational issues (see the report below) that I would kindly like to ask you to address. Because this affects both the manuscript text as well some of the main and supplementary figures, I am returning the manuscript to you for a final round of minor revision at this point, to allow you to modify and re-upload all the files affected. When doing so, please also make sure to upload all supplementary material in the form of one single PDF for easier access. At this stage, please also include signed copies of the relevant licenses and copyright forms (see below), in order to faciliate swift acceptance and production of the study.

Once we will have received the modified final version, we should then be able to swiftly proceed with formal acceptance and publication of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Magyar et al. have now resubmitted a new version of their manuscript trying to answer my concerns. I am pleased to see that they have accepted to run at least some of the experiments proposed with the aim of reinforcing the weak parts of the work. The authors argue that this manuscript contains a large amount of data both as regular multipanel figures and Supplementary figures, which is true. Some of my previous concerns were not really derived from a lack of information provided but, rather, on the lack of support of some of the authors' claims based on the data provided. Also, the manuscript contained some inconsistencies. In any case, I consider that the current version has ended up being a solid, though controversial, contribution to the field. However, as indicated above, I still detect a few points that need editorial modifications (see below).

1. The authors state that they "performed synchronisation of seedlings..:". Please change this term,

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since the treatment carried out (increase sucrose in the medium) is not the synchronization protocol that I suggested to use, as described by Cools et al. (2010).

2. Data described in Suppl Fig 12 clearly show that the expression of various E2F members is altered in e2fa-1 plants (increase of E2FC, first decrease of DEL1/E2FE, then increase of the three DELs/E2Fs at later times). This is described in a paragraph but is completely neglected in the model presented in Fig. 6A. In its current version, it is misleading since one can get the conclusion that E2FA is the only responsible for controlling the network, while it is very likely that other E2F members (based on the authors' own data and previous reports). It is also easy to include such information in the model without damaging the main conclusions of the work. Also related to Fig. 6, I think that eliminating a direct role of E2FA on proliferation is not consistent with the data and the title.

3. The use of the double terminology for DELs/E2Fs: E2FD/DEL2, E2FE/DEL1 and E2FF/DEL3, throughout the text, is recommended.

4. Check spelling of CYCD, throughout the text (not CycD).

5. Please check numbering of Suppl. Figures (there are two Suppl. Fig. 12 in the files that I got).

3rd Revision - authors' response 04 January 2012

We thanks again for Reviewer 1 for all her/his comments and criticisms and corrected the text according to his/her points, where it was relevant (point 3-4-5).

We do not need to change the term cited in point 1 since synchronisation was not stated in the manuscript but in the rebut letter. Actually we did present in Fig 6B a model based on the Q-PCR results shown in Supplementary Fig.12, but we further refined this (see below).

The direct role of E2FA on the regulation of cell cycle genes is indeed an interesting question. On the basis of our data presented in this manuscript we came to the conclusion that the transactivating function of E2FA TF is not rate limiting for the stimulation of DNA synthsesis (since the E2FA RB without transactivating domain can perform this function). We suggest a model where E2FA in complex with RBR1 directly represses genes such as CCS52A1 and CCS52A2 to control the switch from proliferation to endocycle. We have also seen that E2FC, another positive regulator of the endocyle, could also be repressed by E2FA (Supplementary Fig.12) but whether directly is not clear yet. Interestingly DEL1 expression is down regulated in the e2fa-ko plants (Supplementary Fig.12) indicating that E2FA could directly stimulate the expression of DEL1. DEL1 was shown previously to function as a transcriptional repressor of CCS52A2. However, it was recently demonstrated that DEL1 expression is not regulated directly by E2FA but by E2FB and E2FC (Berckmans et al., 2011a), which are two antagonistic E2F transcription factors in Arabidopsis. To be more specific we have changed the model in Figure 6B according to these data.

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