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Transcriptional repression of the APC/C activator CCS52A1 promotes active termination of cell growth

Christian Breuer, Kengo Morohashi, Ayako Kawamura, Naoki Takahashi, Takashi Ishida, Masaaki Umeda, Erich Grotewold and Keiko Sugimoto

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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 27 August 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the manuscript interesting and well suited for publication in the EMBO Journal. There raise relative minor concerns that shouldn't involve too much additional work to address. Given these positive comments, I would like to ask you to submit a suitably revised manuscript to us.

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

REFEREE REPORTS

Referee #1

The manuscript by Breuer et al deals with the mechanisms controlling endoreduplication, a process of major relevance during development and morphogenesis both in animals and particularly in plants. Here the authors focus on GTL1 to determine its role in regulating the extent of the endocycle program during trichome development in Arabidopsis leaves. Trichome development requires a strict coordination between successive endoreduplication cycles and cell expansion. Consequently, one key aspect of the whole process is how the endocycle terminates, in other words, how cells know how many endoreduplication cycles must occur before cessation of the program. A combination of genetic, ChIP-chip, expression and molecular interactions approaches conclusively led the authors claim that GTL1 is a master regulator of the endocycle program. Furthermore, they determine that GTL1 is largely acting as a repressor of CCS52A1, an activator of the APC, to terminate the endocycle program.
The work has been carefully conducted and the experiments are clearly described in the vast majority of the cases (see also below). The topic is of sufficient general relevance as to provoke the interest of scientists not only in the fields of plant development, endocycle control and gene expression, but also in endocycle-dependent cell growth in general.

Comments.
1) In results section "Ectopic GTL1 expression is sufficient to terminate ploidy-dependent cell growth", it would be helpful to find a more clear explanation/discussion of previous reports showing that GTL1 is expressed only at late stages of trichome development, probably justifying the use of GL2 and ATML1. Also, I would suggest finding an alternative to the term "ectopic" in this context since it refers to expression at a different time during development rather than at a different location. GTL1 is expressed also in pavement cells; this should be included in the discussion on the role played by GTL1 in regulating the endocycle, considering that pavement cells also endoreduplicate during leaf growth.

2) When Figure 1B is described, only the lack of trichomes and the epidermal pavement cell size decreased size of the transgenic plants is noted. Based on the image provided, it seems that a clear increase in the number of stomatal cells also occur, a point that need further attention, specially based on previous reports (Yoo et al., 2010; see also comment 3).

3) As a follow up of the previous comment, a differential behavior of leaf cell types to changes in cell cycle regulators has been previously reported for trichomes, pavement cells and stomata and should be commented and discussed here (Castellano et al., 2004; Park et al., 2005; Desvoyes et al., 2006, among others). Authors should consider whether it is appropriate to discuss these reports in regard to their finding of GTL1 action in different cell types and on certain cell cycle regulators.

4) Trichome ploidy level is measured in Figure S1C. Including glt1 mutant in the study could reinforce the data? Is pGL2:GFP:GTL1 not included because of its complete lack of trichomes? Is pATML1:GFP:GTL1 providing enough trichomes to do the measurements despite their decreased numbers? How was this problem solved? All this should be clarified in the text, in particular for the non-specialist.

5) In the Results section "Ectopic GTL1 expression interferes with trichome differentiation" it is stated, "These results suggest that ectopic overexpression of GTL1 also interferes with the full acquisition and/or maintenance of trichome cell fate." Is it really a matter of ectopic expression or just the early expression in the correct place? (see comment 1). If the large cells observed in panel 1B have a trichome cell fate, one would expect to see them as GL2-positive. Is this correct, and if so, this needs to be explained since they appear as GL2-negative in panel 1C.

6) When comparing GTL1 binding sites and genes that become up or down regulated it is important to note that these two experiments were done in different tissues and this might explain the apparent lack of correlation between their results. I wonder whether is feasible to carry out a ChIP-chip experiment with the purified preparation of trichomes? If so, it could shed some light on this section, although I do not see it as absolutely necessary based on results provided in other sections.

7) Figure 6 is very interesting and helps to show how CCS52A1 is down regulated in parallel with GTL1 expression, however because it has no statistical value I wonder if it could be a Supplementary Figure instead.

Referee #2

In this work, Breuer and colleagues study how the trihelix transcription factor GT2-LIKE 1 (GTL1) controls cell growth using Arabidopsis leaf hairs (trichomes) as a model system. Wild-type trichomes undergo approximately four rounds of an endocycle in which the nuclear DNA is replicated without subsequent cell divisions. In gtl1 mutants, trichomes undergo one additional endoreplication cycle and are larger. Previous experiments have also revealed a correlation between trichome growth and nuclear DNA content. However, the molecular networks controlling growth of trichomes and other cells are largely enigmatic and a key question is how the DNA content is regulated in the light of cell growth.

Combining genome-wide ChIP of plants expressing a GFP-tagged GTL1 with cell-type specific transcriptional profiling of gtl1 mutant trichomes, the authors follow a clever and powerful strategy to reach a mechanistic level in the analysis of cell growth and identify approximately 180 genes which expression is directly controlled by GTL1. Unexpectedly, the Arabidopsis Cdh1/Fizzy-related homolog CCS52A1 that serves as an activator of the APC/C is among these genes. Direct binding of GTL1 to CCS52A1 is confirmed by yeast one hybrid binding assays. Importantly, CCS52A1 was found to be upregulated in gtl1 mutant trichomes based on the analysis of a CCS52A1 reporter line. Thus, GTL1 acts as a transcriptional repressor of CCS52A1 and together with genetic evidence provided here and previous molecular analyses of CCS52 the authors provide convincing evidence that the delay in CCS52A1 expression causes one additional endoreplication cycle in trichomes.

With this paper, Breuer et al. make an important contribution to the question how cell growth is controlled - an important, yet not very well understood developmental question. The authors have identified the trihelix transcription factor GTL1 as a key regulator of trichome growth. Moreover, based on overexpression of GTL1 it seems more than likely that the authors have discovered a general mechanism of cell growth control. The authors demonstrate a nuclear DNA amount-dependent and a nuclear DNA amount-independent function of GTL1 and the many GTL1 targets identified here support a key and likely integrative role of GTL1 in cell growth control. The important discovery of this study is that at least in Arabidopsis the endocycle needs to be actively terminated through the transcriptional repression of CCS52A1. This finding of high interest for a large community of cell and developmental biologists.

The work is solid and sound and the results are clearly presented. I only have very few and minor comments.

Minor comments
p4 [...]One strategy to increase the ploidy level is to initiate an aberrant version of the mitotic cell cycle called endoreduplication cycle or endocycle. Endocycling cells skip the mitotic phase, thus they do not separate newly synthesized sister chromatids nor undergo cytokinesis, resulting in the duplication of the nuclear DNA content (Edgar & Orr-Weaver, 2001; Nagl, 1976). [...] Endoreplicating cells may or may not separate their sister chromatids. Thus, polyteny is not necessarily associated with endoreplication and for instance in Arabidopsis, sister chromatids appear to be even more distantly located to each other with higher levels of endoreplication, see Schubert, V., Berr, A., & Meister, A. (2012). Interphase chromatin organisation in Arabidopsis nuclei: constraints versus randomness. Chromosoma, 121(4), 369-387.

p8 [...] and that temporally regulated transcription of GTL1 is the key determinant of final cell size in Arabidopsis trichomes.[...] Certainly GTL1 is of a major regulator of cell size control in trichomes and other cells. However, given the strong effect of other transcription factors such as GL2 and GL3+EGL3 on cell size, it may be more appropriate to write "is a key determinant...".

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The GTL1 trihelix transcription factor was described previously to control growth and the DNA content of trichomes. This study was extended here by demonstrating that GTL1 might be a general (rather than a trichome-specific) regulator of growth. Additionally, the authors identified potential GTL1 targets through a combination of ChIP and microarray transcriptome analyses, yielding the CCS52A1 gene (encoding an activator of the APC/C E3-ubiqitin ligase) as a direct potential target. The latter was confirmed through Y1H and a genetic analysis, and allowed to disentangle the ploidy-independent and ploidy-dependent growth pathways controlled by GTL1, with CCS52A1 being the main target involved the ploidy-dependent pathway.

Plant CCS52A APC/C subunits have been characterized well before as important regulators of endocycle onset, but the knowledge on their transcriptional and post-transcriptional control remains scare. From that point of view, the identification of a novel transcription factor controlling CCS52A1 represents an interesting achievement. Moreover, indirectly the data suggest a role for CCS52A proteins not only in endocycle entry, but also during endocycle progression (although not discussed, see comments below), and opens the door to a more detailed temporal analysis of APC/C activation through an interplay of the E2F, DEL1, and GTL transcription factors.

Comments:

In abstract: "APCCDH1/FZR/CS52" should be replaced by "CDH1/FZR/CCS52" as the former denotes the protein complex.

As GTL1 is put forward as a late stage repressor of CCS52A1 during trichome development, it implicates that, next to endocycle onset, the plant APC/C is involved in endocycle progression. This agrees with observations made for Drosophila, but contradicts with observations published by Roodbarkelari et al. (2010). This discrepancy should be discussed.

In relation with the previous comment, it might be worthwhile to perform a time-course flow cytometry analysis on the leaves of the pATML1:GFP:GTL1 plants. This might allow to see whether the differences observed for the 21-day-old plants arise because of a late endocyle onset or early termination of the endocycle program. It would also be nice to correlate these data with the CCS52A1/2 transcription levels.

In the previous study on GTL1 it was mainly the CCS52A2 gene that was strongly upregulated in the trichomes of gtl1 mutant trichomes. This time no difference in transcription for this gene is reported (it appears to be absent in suppl dataset 2). How do the authors reconcile these data?

How was the P-value for the microarray analysis chosen (0.075)? This appears to me as a relative low stringent selection.

What is the GO enrichment of the 182 genes being common between the ChIP and microarray data?

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Figure 3D: "regulated" instead of "reglated"

1st Revision - authors' response 05 September 2012

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how cells know how many endoreduplication cycles must occur before cessation of the program. A combination of genetic, ChIP-chip, expression and molecular interactions approaches conclusively led the authors claim that GTL1 is a master regulator of the endocycle program. Furthermore, they determine that GTL1 is largely acting as a repressor of CCS52A1, an activator of the APC, to terminate the endocycle program.
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<our response>
Following these suggestions, we explained that GTL1 is expressed only at late stages of trichome development in Results (page 6 line 22). We replaced the term “ectopic” by “early” or “over” throughout the manuscript and we included that GTL1 is expressed in non-trichome leaf cells in Discussion (page 17 line 7).

2) When Figure 1B is described, only the lack of trichomes and the epidermal pavement cell size decreased size of the transgenic plants is noted. Based on the image provided, it seems that a clear increase in the number of stomatal cells also occur, a point that need further attention, specially based on previous reports (Yoo et al., 2010; see also comment 3).
<our response>
Although this is an interesting point and we looked into this, we did not find statistically significant differences in the stomata density between wild-type and GTL1 overexpressing plants. All pavement cells are small in pATML1:GFP-GTL1 plants and this may give the impression that they have more stomata. We did not comment on this in the manuscript as it is not the focus of our work.

3) As a follow up of the previous comment, a differential behavior of leaf cell types to changes in cell cycle regulators has been previously reported for trichomes, pavement cells and stomata and should be commented and discussed here (Castellano et al., 2004; Park et al., 2005; Desvoyes et al., 2006, among others). Authors should consider whether it is appropriate to discuss these reports in regard to their finding of GTL1 action in different cell types and on certain cell cycle regulators.
<our response>
We agree that this is an interesting area of work but we did not discuss this in the manuscript since we do not describe “differential behaviour of leaf cell types to changes in cell cycle regulators”.

4) Trichome ploidy level is measured in Figure S1C. Including glt1 mutant in the study could reinforce the data? Is pGL2:GFP:GTL1 not included because of its complete lack of trichomes? Is pATML1:GFP:GTL1 providing enough trichomes to do the measurements despite their decreased numbers? How was this problem solved? All this should be clarified in the text, in particular for the non-specialist.
<our response>
Following these suggestions, we described that the gtl1 mutants display trichome ploidy phenotypes opposite to GTL1 overexpressing plants in Results (page 8 line 1). We did not include pGL2:GFP:GTL1 as we could not isolate intact trichomes for ploidy measurements. As shown in Figure 1, pATML1:GFP:GTL1 plants do produce enough trichomes and we explained these details in Results (page 8 line 8).

5) In the Results section "Ectopic GTL1 expression interferes with trichome differentiation" it is stated, "These results suggest that ectopic overexpression of GTL1 also interferes with the full acquisition and/or maintenance of trichome cell fate." Is it really a matter of ectopic expression or just the early expression in the correct place? (see comment 1). If the large cells observed in panel

1B have a trichome cell fate, one would expect to see them as GL2-positive. Is this correct, and if so, this needs to be explained since they appear as GL2-negative in panel 1C.
<our response>
Sorry for the confusion, it is "early expression" and we revised the text accordingly. We found several types of large cells in GTL1 overexpressing plants and those that are out-grown and unbranched (marked with white arrows) clearly have trichome cell fate and are GL2-positive. In contrast, those that are abnormally enlarged but not out-grown (marked with black arrows and asterisks) are GL2-negative although they must have had the GL2 promoter active to express GTL1 eariler in development. Our current hypothesis is that the endocycle arrest caused by overexpression of GTL1 leads to the (partial) loss of trichome cell fate. We explained these details in Results (page 8 line 22).

6) When comparing GTL1 binding sites and genes that become up or down regulated it is important to note that these two experiments were done in different tissues and this might explain the apparent lack of correlation between their results. I wonder whether is feasible to carry out a ChIP-chip experiment with the purified preparation of trichomes? If so, it could shed some light on this section, although I do not see it as absolutely necessary based on results provided in other sections.
<our response>
We thank the reviewer for pointing this out. We noted in Results that the ChIP-chip and microarray experiments were indeed performed using different tissues and this may have contributed to the relatively low overlap between the two experiments (page 12 line 5). We agree that carrying out the ChIP-chip experiment with the purified preparation of trichomes is an excellent idea but this is not feasible with current techniques.

7) Figure 6 is very interesting and helps to show how CCS52A1 is down regulated in parallel with GTL1 expression, however because it has no statistical value I wonder if it could be a Supplementary Figure instead.
<our response>
We want to keep Figure 6 in the main manuscript since as the reviewer pointed out, it shows, very nicely, downregulation of CCS52A1 upon GTL1 expression. These data are complementary to the RTPCR data in Figure 4D where we demonstrated the statistically significant upregulation of CCS52A1 in the absence of GTL1. We noted that the images in Figure 6 are representative of our observation on these transgenic lines in the Figure legend.

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<our response>
We agree with the reviewer on this and we rephrased the sentence in Introduction (page 4 line 4). It now reads “Endocycling cells skip the mitotic phase, thus they re-enter into the S phase without cytokinesis, resulting in the duplication of the nuclear DNA content”.

p8 [...] and that temporally regulated transcription of GTL1 is the key determinant of final cell size in Arabidopsis trichomes.[...] Certainly GTL1 is of a major regulator of cell size control in trichomes and other cells. However, given the strong effect of other transcription factors such as GL2 and GL3+EGL3 on cell size, it may be more appropriate to write "is a key determinant...".
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Following this suggestion, we rephrased “the key determinant” to “one of the key determinants”.

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We fixed this error.

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Comments:

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Following this suggestion, we replaced "APCCDH1/FZR/CS52" by "CDH1/FZR/CCS52".

As GTL1 is put forward as a late stage repressor of CCS52A1 during trichome development, it implicates that, next to endocycle onset, the plant APC/C is involved in endocycle progression. This agrees with observations made for Drosophila, but contradicts with observations published by Roodbarkelari et al. (2010). This discrepancy should be discussed.
<our response>
Our data indeed suggest that the plant APC/C is involved in the endocycle progression. While this contradicts with the work by Roodbarkelari et al. (2010), it agrees with work in Drosophila by Narbonne-Reveau (2008) as well as work on the plant CCS52 by Larson-Rabin (2009), Kasili (2010) and Heyman (2011). We discussed these issues in Discussion (page 19 line 22).

In relation with the previous comment, it might be worthwhile to perform a time-course flow cytometry analysis on the leaves of the pATML1:GFP:GTL1 plants. This might allow to see whether the differences observed for the 21-day-old plants arise because of a late endocyle onset or early termination of the endocycle program. It would also be nice to correlate these data with the CCS52A1/2 transcription levels.
<our response>
This is indeed a very good idea to further investigate functional roles of GTL1 in general plant cell growth. However, we have several sets of experiments currently running in the lab to study this more extensively, e.g. we carry out not only gain-of-function studies of GTL1 but also loss-of-function studies of GTL1 and its close homologs. Thus, we think the suggested experiments are not complete on their own and fit better in our future manuscript.

In the previous study on GTL1 it was mainly the CCS52A2 gene that was strongly upregulated in the trichomes of gtl1 mutant trichomes. This time no difference in transcription for this gene is reported (it appears to be absent in suppl dataset 2). How do the authors reconcile these data?
<our response>
We only used technical replicates in our previous study while we used three independent biological replicates in this study. Therefore, we think the data described in this study are more robust and together with our ChIP-chip data, CCS52A2 is not a direct target of GTL1 in trichome cells. We added this information in Results (page 14 line 1).

How was the P-value for the microarray analysis chosen (0.075)? This appears to me as a relative low stringent selection.
<our response>
We set the P-value to 0.075 (instead of standard 0.05) to avoid discriminating real target genes due to possible variations in the trichome samples. Our strategy was to include as many potential targets as possible from the microarray and eliminate false positives by ChIP-chip and follow-up RTPCR.

What is the GO enrichment of the 182 genes being common between the ChIP and microarray data?
<our response>
Among the 182 genes, we had 79 genes positively regulated and 103 genes negatively regulated by GTL1. Both of these sets included genes of diverse functions and the BiNGO analysis did not identify any significant GO enrichment.

The last section of the discussion appear irrelevant to me in relation to the presented data, and might be deleted.
<our response>
We added further discussion on the role of APC/C in the endocycle progression (see also above) and we want to keep this section in the manuscript.

Figure 3D: "regulated" instead of "reglated"
<our response>
We fixed this error.

Accepted 02 October 2012

Thank you for submitting your revised manuscript to the EMBO journal. I asked referee #1 to review the revised version. I have now heard back from this referee. The referee appreciates the introduced changes and support publication here. I am therefore very please to accept your paper for publication in the EMBO Journal.

