Feedback regulation between atypical E2Fs and APC/CCdh1 coordinates cell cycle progression.

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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 20 August 2015

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have just now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I would prefer not to repeat the details of them here. What becomes clear is that while all referees in principle agree on the interest of the study, they also point out instances in which they feel the data needs to be strengthened (most obvious in the reports of referees 2 and 3) and they all provide feedback and suggestions on how to achieve this.

Given the potential interest of your findings, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees as listed above and in their reports should be addressed.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Formally, you have submitted your manuscript as a scientific report, rather than a full article. This is fine, as our scientific reports can contain up to 5 main figures. Should, during the revision, this number increase, you might want to consider turning the paper into a full article. In this case, the results and discussion sections can stay separate. In case you want to keep the article in the form of a
scientific report, it cannot have more than five figures and I would in this case ask you to combine the results and discussion section, as this is the normal format for our scientific reports.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS

Referee #1:

The paper entitled "Feedback regulation between atypical E2Fs and APC/CCdh1 coordinates cell cycle Progression" by Westendorp et. al. addresses the role and regulation of the atypical E2Fs E2F7/8. They present convincing evidence that E2F7/8 are degraded by APC/CCdh1 during mitotic exit and in early G1, and that this is required for S phase entry. A negative feedback loop in which E2F7/8 directly repress transcription of APC/CCdh1 inhibitors is also described.

This is a well written paper using a variety of experimental systems to present convincing evidence that the atypical E2Fs E2F 7/8 are degraded by APC/CCdh1, and that this event is critical for S phase entry. The results provide important insight into the critical molecular events controlling cell cycle progression, in particular providing additional clarity for the complicated interplay of EF2 family members in how cells "reset" for subsequent rounds of proliferation. The figures are well laid out, adequately controlled, and strongly support the conclusions. For these reasons I find the paper suitable for publication in EMBO reports with minor revision. Specific comments are below.

- In Figure 1A, the majority of E2F7/8 degradation appears to be completed in 1hr. The time course might be more evident after correcting for loading variation by standardizing relative to the tubulin control. Is the remaining E2F7/8 a more stable form or simply a fraction from cells not in G1?
- What is the rationale for describing APC as active in Figure 1F? Has its activity been specifically measured or is this this description based on the literature?
- In Figure 2A, the differential effect of CDH1 vs CD20 is not very dramatic, particularly with respect to E7. Is the transfection efficiency high enough to see effects on endogenous proteins? Figure 2B is more convincing in this regard.
- In Figure 5B, it would be nice to see a control transcript that doesn't change.
- In Figure 5E, I don't see the asterisk described in the legend.

Referee #2:

The manuscript by Westendorp et al. shows that E2F7 and E2F8 transcriptional repressors, important regulators of the cell cycle transcription programme, are themselves robustly regulated by APC/CCdh1-dependent proteolysis during G1 phase. They identify degron sequences within E2F7/8-GFP that determine targeting, and use this information to make stable versions of the proteins. Expressing stable versions of E2F7/8-GFP blocks entry to S phase and leads to cell death. Overexpression of E2F7/8 also causes transcriptional repression of FBXO5 (Emi1, inhibitor of APC/CCdh1), independent of cell cycle phase, supporting the proposed existence of a feedback loop by which Cdh1 - via targeting E2F7/8 - brings about its own inhibition at the end of G1 phase.

This is an interesting study and contributes valuable new understanding to regulation of the cell cycle. It is probably suitable for publication in EMBO Reports after appropriate revision. On the whole, experiments are well designed and tell a logical and concise story. However, some conclusions are not properly supported by the presented evidence, particularly given that functional studies rely entirely on use of ectopic overexpressed proteins.

For example, it is problematic that the consequences of expressing stabilized E2F7/8 are not the same as those of knocking down/out CDH1 (because Cdh1 is predicted to be activated by E2F7/8 stabilization, although not shown in this study). Experiments presented in Figure 4 show that CDH1
RNAi does not cause discernible effect on S-phase entry unless E2F7/8-GFP is overexpressed, yet are used to make the case that APC/CCdh1-dependent degradation of E2F7 or E2F8 during G1 is required for S phase. This is surely an over interpretation since endogenous E2F7/8 proteins are still present, and presumably not degraded during G1 phase, in CDH1 RNAi cells - yet do not significantly affect S phase entry. The authors do not address this point experimentally, only noting that accumulation of cyclin A and other Cdh1 substrates would be expected to override E2F7/8 in promoting S phase entry. Clearly, interpreting manipulations that disturb either APC/C or E2F factors - that have so many downstream effectors - is difficult, but additional data could support the conclusions drawn (see specific comments).

A further 'spanner in the works' of the proposed model of the E2F7/8-Cdh1 feedback loop is that much of the evidence in this manuscript points to E2F8 as a substrate for APC/CCdc20 rather than (or as well as) APC/CCdh1, a point that is not directly addressed. The IP showing apparently very strong interaction of Cdc20 with E2F8 should be moved into figure 2, and E2F7 should be included for comparison (see also specific comments).

It is somewhat unsatisfactory that experiments are conducted across several different cell lines (moving from RPE1-TERT to U2OS, to 293T to HeLa) with little cross-corroboration of results after Figure 1. Functional studies are carried out entirely in HeLa cells, which lack controls of G1-S present in cells such as U2OS or RPE1-TERT cells. How do these cells respond to overexpressed E2F7/8?

Specific comments:

1) Figure 2: Overexpression of Cdh1 in unsynchronized cells can produce strong effect on substrate levels because it is active in interphase cells. Cdc20 is not active in interphase, and it seems unlikely that dramatic effects on substrates could be reported by this method. The authors should either modify the conclusion that Cdc20 does not target these factors - or show how a known substrate of Cdc20 behaves in this assay.

2) Figure 2E: the profile of E2F8 degradation in response to CDH1 siRNA suggests it could be a target for both Cdc20 and Cdh1. Fig EV1C suggests that E2F8 should be a substrate of Cdc20. In Figure 3D the K/K mutant version of E2F8 is degraded with a profile remarkably similar to that of the wild-type protein after CDH1 siRNA, consistent with residual degradation by a different E3 via other degrons. Is residual E2F8 degradation Cdc20-dependent (or indeed, sensitive to APC/C inhibition)? This question is rather relevant to description of feedback between E2F7/8 and APC/C.

3) Figure 3C: FACS analyses appear to show that the dox-inducible E2F8 cell lines contain a high proportion of non-expressing cells, unlike the corresponding E2F7 clones. In particular, at least half of cells in the E2F8K/K mut cells may be non-expressing (comparing + dox to the vehicle control). Therefore the conclusions drawn from looking at unsorted cell populations in Figure 4 (and EV2), in particular the comparison of E2F7 versus 8, are probably not valid - although these are good quality data. To validate conclusions about the relative effects of E2F7 and 8 on BrdU incorporation (Fig4A), on cell proliferation (Fig4B), on S phase entry (Fig4C) etc. this figure should be controlled with evidence that every cell expresses the exogenous E2F7 or 8 in G1 phase (perhaps by FACS or IF {plus minus} proteasome inhibition). If indeed the E2F8 clones are diluted by non-expressing cells, cells could be sorted prior to analysis.

In addition, it is not clear from the figure legends to Figures 3 & 4 that the cell lines used are the same - I have assumed so.

4) As already noted, the authors draw conclusions about the role of E2F7/8 degradation (eg on p5 of results section "These data demonstrate that APC/CCdh1-dependent degradation of E2F7 or E2F8 during G1 is required for the initiation and progression of DNA replication") based only on the behaviour of the overexpressed protein. CDH1 RNAi has no discernible effect on S phase entry in the cell line tested, suggesting that degradation of endogenous E2F7/8 is not, in fact, required. More care should be taken in stating conclusions.

5) Discussion section p1 "Increasing atypical E2F levels through ablation of CDH1... resulted in
severely impaired S-phase entry. This sounds as if E2F7/8 RNAi should rescue CDH1 RNAi - except that in HeLa cells, there is no significant impairment without overexpression of E2F7/8. This experiment could be attempted in RPE1-TERT cells, for example.

6) Figure 5 is quite light on data, given the weight attached in this manuscript to the identification of the feedback loop between atypical E2Fs and APC/C. Certainly the data fit the idea of a 'novel feedback loop', but there is no test of this idea. Therefore although it is clear that overexpression of WT E27 or E28 strongly represses Emi1 (FBXO5), this is not the same thing as showing that destruction of E2F7/8 is required to allow Emi1 expression at the end of G1 phase. Does stabilization of endogenous E2F7/8 by Cdh1 RNAi in G1 phase also repress Emi1? Do stable versions of E2F7/8 mediate stronger repression than WT?

Can continued APC/C activity in presence E2F7/8-KENmt be confirmed by increased degradation of other APC/C substrates?

7) Knockdown of E2F7/8 after siRNA is not shown (Fig 5C). Authors should show how Cdh1 levels are affected under these conditions, as well as levels of an APC/CCdh1 substrate.

Referee #3:

The submitted manuscript by Westendorp et al. confirms the previously demonstrated APC/C-dependent instability in G1-phase of E2F7 and 8 (Cohen et al. 2013) and extends this finding by showing that this degradation is KEN-box motif dependent and requires CDH1, establishing E2F7 and 8 as CDH1 targets. It would be nice to see CDH1-dependent in vitro ubiquitylation but I think the data strongly support their claim even without this assay. The requirement of E2F7 and 8 degradation during exit from mitosis and G1-phase for cell cycle progression was demonstrated by analysis of stabilized versions of these proteins, which interfered with the onset of S-phase and caused cell death (at least in the case of the E2F7 KEN box mutant. E2F7 and 8 repress target genes that can inhibit CDH1, which creates a negative feedback loop that might be important for balancing the expression of E2F target genes as well as APC/C activity. Thus, this manuscript describes an important novel mechanism of cell cycle regulation.

I would however still raise a few questions and issues:

1. Overexpression of the wild-type versions of E2F7 and 8 do not reduce the numbers of BrdU positive cells but strongly impair proliferation of HeLa cells. In contrast, expression of the KEN box mutant of E2F7 strongly prevents BrdU incorporation and induces cell death. The stabilised version of E2F8, on the other hand, has a much milder effect on replication and does not induce cell death. These important differences are not further explored and might be subject for another manuscript but a few issues need to be clarified:

a. Cell death (Fig.4) is not clearly defined and should be determined by an additional method, because there is no evidence of a sub-G1 peak in the DNA histograms (Fig.3,4).

b. When do E2F7Kenmut cells die? By analysing the available time lapse videos, it could be determined whether cells die around the time point of initiation of DNS synthesis or much earlier. Why does the stabilized version of E2F8 (which seems to be expressed even stronger then E2F7Kenmut not induce cell death?

c. How do E2F7/8 mutants interfere with cell cycle progression? Fig.5 shows that the wild type versions of these proteins repress the expression of their target genes, when expressed during S-phase. It would be very interesting to know whether premature expression of these proteins by expressing them as stabilized proteins would prevent the induction of these genes. This could be readily determined by analyzing cells released from a mitotic block.

2. The suggested feedback loop should be interrogated by overexpressing CDH1 (the stabilized phospho-mutant) or Emi1-RNAi. The prediction is that this should reduce the levels of endogenous E2F7/8 (as shown by expressing wt CDH1 in Fig.2) while inducing endoreplication. In contrast, it would be interesting to know whether overexpression of E2F7/8 induces rereplication.
3. Figure 1 shows that E2F7 and 8 are unstable proteins, which are targeted for proteasome-dependent degradation. Cycloheximide treatment however only stabilize about 50% of the protein, which most likely reflects the cells in G1-phase of this asynchronous population of RPE cells. To further corroborate the degradation during exit from mitosis and G1-phase, cells should mitotically synchronised and released before addition of CHX. In addition CDH1 RNAi could clearly demonstrate the importance of this pathway during G1-phase degradation of these proteins.

4. Figure 2D shows the interaction between CDH1 and the E2Fs. These data could be strongly improved by demonstrating a KEN-box dependent interaction between the proteins. The evidence of an interaction between E2F8 and CDC20 is less convincing due to the weak expression of FLAG-Cdc20 and the strong contaminating bands and should therefore be repeated. In addition, the KEN-box mutant should be combined with the D-box mutant to show that this protein is now completely stable during exit from mitosis and in G1-phase. Also it would be interesting to know if the D-box mutants would prevent the initial fast kinetics of degradation (0 to 20 minutes, Fig.2E) of the GFP fusion protein.

5. Figure 3C shows the effect of E2F7/8 wild type and mutant overexpression on cell cycle progression. The effect on G1-phase accumulation should be quantified to match the claims in the text.

6. Similarly, the data shown in Figure 4G would be much easier to read if quantified. In addition, for comparison, the eGFP-negative cell population should be used as control.

7. Fig2A should include a CDC20 target, such as cyclin B1, as a positive control to show that overexpression of CDC20 is sufficient to destabilize target proteins in this assay.

Referee #1:

The paper entitled "Feedback regulation between atypical E2Fs and APC/CCdh1 coordinates cell cycle Progression" by Westendorp et. al. addresses the role and regulation of the atypical E2Fs E2F7/8. They present convincing evidence that E2F7/8 are degraded by APC/CCdh1 during mitotic exit and in early G1, and that this is required for S phase entry. A negative feedback loop in which E2F7/8 directly repress transcription of APC/CCdh1 inhibitors is also described.

This is a well written paper using a variety of experimental systems to present convincing evidence that the atypical E2Fs E2F 7/8 are degraded by APC/CCdh1 and that this event is critical for S phase entry. The results provide important insight into the critical molecular events controlling cell cycle progression, in particular providing additional clarity for the complicated interplay of EF2 family members in how cells "reset" for subsequent rounds of proliferation. The figures are well laid out, adequately controlled, and strongly support the conclusions. For these reasons I find the paper suitable for publication in EMBO reports with minor revision. Specific comments are below.

1. In Figure 1A, the majority of E2F7/8 degradation appears to be completed in 1hr. The time course might be more evident after correcting for loading variation by standardizing relative to the tubulin control. Is the remaining E2F7/8 a more stable form or simply a fraction from cells not in G1? We included the quantification of the three CHX experiments we performed (revised Fig 1B). We corrected the band densities of E2F proteins for loading by calculating ratios to tubulin. This shows a more gradual decrease in protein expression over time. We have no evidence for a stable form of E2F7/8, so we indeed think that the residual E2F7/8 is the fraction of cells not in G1. In figure panels 1C and 6F we convincingly show that E2F7/8 are completely absent in G1 cells.

2. What is the rationale for describing APC as active in Figure 1F? Has its activity been specifically measured or is this this description based on the literature? Its activity has not been measured specifically, but because geminin is a canonical APC/C substrate, the truncated version of geminin in the FUCCI system is an APC/C indicator. In
strong support of this claim, we included now Cyclin B1 transcript levels in revised Fig 1H. Although Cyclin B1 protein sharply drops in the (Geminin-negative/CDT1-negative) cells in late mitosis or early G1, the CCNB1 transcripts remain elevated. The absence of protein in spite of high mRNA levels strongly supports that the APC/C activity is very high in this condition. Furthermore, we have also treated RPE-FUCCI cells with an siRNA against the APC/C<sup>Cdh1</sup> inhibitor Emi1, and as expected, FACS analysis showed a dramatic reduction in the amount of geminin-azami green (data not shown).

3. In Figure 2A, the differential effect of CDH1 vs CD20 is not very dramatic, particularly with respect to E7. Is the transfection efficiency high enough to see effects on endogenous proteins? Figure 2B is more convincing in this regard. We usually hit ~50% of all cells in this kind of transfection experiments, which cannot be enough to completely remove all endogenous protein, but at least will show noticeable effects. With Fig.2B (revised Fig.2C), this is not an issue, because any cell transfected with CDC20 or CDH1 will also co-express E2F7/8. However, we saw a reproducible decrease in both endogenous E2F7 and E2F8, and to show this more clearly, we provide now quantifications of these blotting experiments (newFig.2B). Furthermore, we decided to remove the CDC20 overexpression condition from Fig.2A. It is not relevant because, as reviewer #2 pointed out, most cells are in interphase and overexpressed CDC20 is largely inactive. Indeed we found that the CDC20 substrate Cyclin B1 was not reduced. Instead, we have now included experiments to better analyze the role of CDC20, to address the points brought up by reviewers #2 and #3. These new data clearly suggested that CDH1 rather than CDC20 is responsible for E2F7 degradation during mitotic exit (see new Fig. 2G and I, 6F). Interestingly, CDC20 seemed to have a minor contribution to E2F8 degradation, indicating that the regulation of the two atypical E2Fs is not simply interchangeable.

4. In Figure 5B, it would be nice to see a control transcript that doesn't change. We have included now <i>AURKA</i> in the revised Figures 6B, D. Contrary to the 3 previously shown transcripts, this gene showed a non-significant trend of increase upon doxycycline treatment. RNAi against E2F7/8 also showed no significant change in <i>AURKA</i> levels.

5. In Figure 5E, I don't see the asterisk described in the legend. This description referred to an asterisk in an old version of the scheme: we corrected this small mistake.

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promoting S phase entry. Clearly, interpreting manipulations that disturb either APC/C or E2F factors - that have so many downstream effectors - is difficult, but additional data could support the conclusions drawn (see specific comments).

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Specific comments:

1) Figure 2: Overexpression of Cdh1 in unsynchronized cells can produce strong effect on substrate levels because it is active in interphase cells. Cdc20 is not active in interphase, and it seems unlikely that dramatic effects on substrates could be reported by this method. The authors should either modify the conclusion that Cdc20 does not target these factors - or show how a known substrate of Cdc20 behaves in this assay.

We thank the reviewer for this good suggestion. We immunoblotted cyclin B1 and indeed, its levels were not affected as most likely, the majority of cells are in interphase. Therefore we decided to remove CDC20 overexpression from the revised Fig 2, because it is not very meaningful. Instead, we have performed additional experiments address the role of CDC20, as discussed under point 2.

2) Figure 2E: the profile of E2F8 degradation in response to CDH1 siRNA suggests it could be a target for both Cdc20 and Cdh1. Fig EV1C suggests that E2F8 should be a substrate of Cdc20. In Figure 3D the K/K mutant version of E2F8 is degraded with a profile remarkably similar to that of the wild-type protein after CDH1 siRNA, consistent with residual degradation by a different E3 via other degrons. Is residual E2F8 degradation Cdc20-dependent (or indeed, sensitive to APC/C inhibition)? This question is rather relevant to description of feedback between E2F7/8 and APC/C.

We have now performed additional experiments with CDC20 and CDH1 RNAi in cells released from a mitotic arrest (new Fig. 2G/H). Briefly, we transfected cells, arrested them in prophase with overnight nocodazole, and release them from nocodazole, in presence of a CDK1 inhibitor and an Aurora A inhibitor to force the cells into mitotic exit. Although the CDC20 RNAi cells still didn’t exit from mitosis as well as their control or siCDH1 counterparts, we could draw several conclusions from these experiments:

- The remaining endogenous E2F7 is efficiently degraded within one hour after mitotic exit.
- CDH1 RNAi completely prevented the degradation of E2F7 during mitotic exit, to a similar extent as the well-described CDH1 substrates CDC6 and Aurora A. Importantly, the CDC20 substrate Cyclin B1 was completely degraded in this condition. This shows that CDC20 was highly active, but nevertheless E2F7 was not degraded.
- CDC20 RNAi completely stabilized a known CDC20 substrate (Cyclin B1), as well as known substrates strictly targeted by CDH1 (Aurora A and CDC6), showing that CDH1 was not yet active in the forced mitotic exit protocol.
- Endogenous E2F8 protein levels are very low after overnight nocodazole treatment, indicating that either their transcription rates are very low in this condition, or that an alternative E3 ligase, which is active during nocodazole treatment. Identification of
this putative E3 is beyond the scope of this study, but we show and discuss this issue in revised Fig 2.

- E2F8 bands were only visible after long exposure and showed evidence of posttranslational modifications, making quantification very difficult. Surprisingly, this residual bands appeared to partially disappear in both CDC20 and CDH1 RNAi-treated cells (revised Fig.2G). Keeping in mind that CDH1 is not activated in siCDC20 cells. To get a more conclusive picture, we also did the same experiment in HeLa cells expressing inducible E2F8-EGFP. Here, we observed a partial degradation of E2F8 during mitotic exit, which was partially rescued by CDH1 RNAi, but even stronger by CDC20 RNAi (revised Fig 2H). These findings indicate that in contrast to E2F7, E2F8 can be degraded via APC/C_{Cdc20}, although this might only involve a small fraction of the native E2F8 protein pool. We show additional evidence that E2F8, but not E2F7 is a substrate of APC/C_{Cdc20} in revised Fig EV3C/D. CDC20-Flag immunoprecipitates with overexpressed E2F8-EGFP, but not E2F7-EGFP.

3) Figure 3C: FACS analyses appear to show that the dox-inducible E2F8 cell lines contain a high proportion of non-expressing cells, unlike the corresponding E2F7 clones. In particular, at least half of cells in the E2F8K/K mut cells may be non-expressing (comparing +dox to the vehicle control). Therefore the conclusions drawn from looking at unsorted cell populations in Figure 4 (and EV2), in particular the comparison of E2F7 versus 8, are probably not valid - although these are good quality data. To validate conclusions about the relative effects of E2F7 and 8 on BrdU incorporation (Fig4A), on cell proliferation (Fig4B), on S phase entry (Fig4C) etc. this figure should be controlled with evidence that every cell expresses the exogenous E2F7 or 8 in G1 phase (perhaps by FACS or IF ± proteasome inhibition). If indeed the E2F8 clones are diluted by non-expressing cells, cells could be sorted prior to analysis. In addition, it is not clear from the figure legends to Figures 3 & 4 that the cell lines used are the same - I have assumed so.

Indeed, we used the same cell lines for figures 3 and 4; we now more clearly mention that we use these inducible HeLa cell lines where applicable. We acknowledge that it could be problematic to make direct comparison of differences between E2F7 and -8 if % of positive cells vary per clone. However, it was for us not possible to determine if indeed every cell from each line overexpresses E2F7/8 mRNA or that the percentage of E2F8-EGFP positive cell is simply lower because additional degradation mechanisms more efficiently clear the exogenous E2F8, similar to what our endogenous E2F8 data suggest.

Because our BrdU staining protocol involves a denaturation step, we lost the EGFP signal, so we cannot sort the cells in that particular experiment. This is the reason why we complemented the BrdU data with single-cell PCNA imaging data. These data show essentially the same, namely a much more severe S phase phenotype in the E2F7-KEN mutants compared to E2F8-KENmutant. We included now an extra quantification on cell fate in this assay to more clearly show the differences between the different constructs (revised Fig 4F and 5C). In addition, we show now quantification of cell cycle progression of ectopic mutant or wt E2F8 using FACS analysis of gated EGFP+ cells (revised Fig 4I, quantification of old Figure 4G in the manuscript). The advantage of HU synchronization is that the APC/C is inhibited at the start of the synchronization, and that percentages of E2F8 WT and E2F8 K/K are comparable (see quantification in revised Figure EV5B, C).

When comparing these data with the same experiment with E2F7 WT overexpressing cells (published in Westendorp et al. Nucl Acids Res 2012), it is very clear that E2F8 WT overexpression has a much less severe S-phase delay phenotype.

Finally, we added new data to confirm the apoptosis phenotype using Annexin V staining (revised figure EV5A), and gated only EGFP+ cells.

4) As already noted, the authors draw conclusions about the role of E2F7/8 degradation (eg on p5 of results section "These data demonstrate that APC/CCdh1-dependent degradation of E2F7 or E2F8 during G1 is required for the initiation and progression of DNA replication") based only on the behaviour of the overexpressed protein. CDH1 RNAi has no discernible effect on S phase entry in the cell line tested, suggesting that degradation of endogenous E2F7/8 is not, in fact, required. More care should be taken in stating conclusions.
This is correct, and we changed this sentence into “These data demonstrate that APC/C\textsuperscript{Cdh1} dependent degradation of ectopic E2F7 or E2F8 during G1 is required for the initiation and progression of DNA replication”.

CDH1 RNAi has many downstream effects, and stabilization of E2F7 is certainly not the only consequence. The optimal strategy to test whether E2F7 degradation is required for S phase entry would be to generate mice or cell lines with endogenously knocked-in KEN box mutations. This goes beyond the scope of the current paper, but we aim to perform this in the future.

5) Discussion section p1 "Increasing atypical E2F levels through ablation of CDH1.... resulted in severely impaired S-phase entry”. This sounds as if E2F7/8 RNAi should rescue CDH1 RNAi - except that in HeLa cells, there is no significant impairment without overexpression of E2F7/8. This experiment could be attempted in RPE1-TERT cells, for example.

We rephrased the sentence: “Combining E2F7/8 overexpression with ablation of CDH1 or expressing atypical E2F-KEN mutants resulted in severely impaired S-phase entry.” Previous work testing the consequences of CDH1 depletion mostly showed mild replication stress and mitotic defects. We do not think E2F7/8 can rescue this in any way. On the contrary, it is more likely that E2F7/8 depletion will cause further replication stress via unscheduled expression of E2F target genes.

6) Figure 5 is quite light on data, given the weight attached in this manuscript to the identification of the feedback loop between atypical E2Fs and APC/C. Certainly the data fit the idea of a 'novel feedback loop', but there is no test of this idea. Therefore although it is clear that overexpression of WT E27 or E28 strongly repress Emi1 (FBXO5), this is not the same thing as showing that destruction of E2F7/8 is required to allow Emi1 expression at the end of G1 phase.

We agree with this comment, and we have included experimental data that largely follow the suggestions brought up below. See revised Fig 6, and the point-by-point explanation below.

6a) Does stabilization of endogenous E2F7/8 by Cdh1 RNAi in G1 phase also repress Emi1?

Yes it does, at least in cells at the G1/S transition, released from a G1 arrest. We show this in RPE cells released from G1 arrest with PD0332991 in presence or absence of CDH1 RNAi (revised Fig 6F). Note that at the 6h and 8h time points, when Emi1 proteins become clearly detectable in the control cells, Emi1 expression is clearly reduced in the CDH1 RNAi condition, where E2F7 protein is increased. This difference could not simply be explained by differences in the amounts of cells that have entered S phase, as shown by the FACS analysis (see revised Fig 6E).

6b) Do stable versions of E2F7/8 mediate stronger repression than WT?

We are not sure here whether the reviewer specifically means repression of Emi1. Assuming so, we have now replaced the Emi1 blot in the old figure 5A with one that shows the mutant and wt overexpressing cell lines side-by-side (revised Fig 6A). This confirms stronger repression by the stable versions, particularly E2F8\textsuperscript{K/K}. Note that the stable mutants also resulted in stronger repression of other target genes, for example CDC6 and Cyclin A2 in figure 3B.

6c) Can continued APC/C activity in presence E2F7/8-KENmt be confirmed by increased degradation of other APC/C substrates?

This is indeed the case, as shown now in Figure 6A. Aurora A, although not transcriptionally repressed by E2F7/8 in this cell line, is clearly downregulated after E2F7/8 overexpression; in the KEN mutants more than their wild-type counterparts. We interpret this to be the consequence of continued APC/C activity, because induced expression of E2F7/8 did not repress AURKA transcription.

7) Knockdown of E2F7/8 after siRNA is not shown (Fig 5C). Authors should show how Cdh1 levels are affected under these conditions, as well as levels of an APC/C\textsuperscript{Cdh1} substrate.
We have included the requested data, Fig 6C in the revised manuscript. We show E2F7 and E2F8 RNAi in combination and separate on immunoblots, to clarify that they compensate for each other’s loss. Although E2F7 appeared to dominate over E2F8 in the HeLa cells, we generally find that combined knockdown causes the most robust depression of target genes. We found that E2F7/8 knockdown slightly decreased CDH1 protein levels and that Emi1 protein levels were increased (in accordance with deregulated Emi1 transcription). We found an increase in the levels of the APC/C-CDh1 substrate CDC6 under this condition, but not Aurora A (or Cyclin B1, data not shown). This difference can be explained by the notion that CDC6 is de-repressed on the transcriptional level after E2F7/8 depletion, whereas Aurora A is not. Most likely, the APC/C is already inhibited maximally in this experiment, because we harvested the cells in late S phase, 6h post-HU. A further increase in Emi1 levels or decrease in CDH1 protein levels does not have a dramatic effect.

Referee #3:

The submitted manuscript by Westendorp et al. confirms the previously demonstrated APC/C-dependent instability in G1-phase of E2F7 and 8 (Cohen et al. 2013) and extends this finding by showing that this degradation is KEN-box motif dependent and requires CDH1, establishing E2F7 and 8 as CDH1 targets. It would be nice to see CDH1-dependent in vitro ubiquitylation but I think the data strongly support their claim even without this assay. The requirement of E2F7 and 8 degradation during exit from mitosis and G1-phase for cell cycle progression was demonstrated by analysis of stabilized versions of these proteins, which interfered with the onset of S-phase and caused cell death (at least in the case of the E2F7 KEN box mutant. E2F7 and 8 repress target genes that can inhibit CDH1, which creates a negative feedback loop that might be important for balancing the expression of E2F target genes as well as APC/C activity. Thus, this manuscript describes an important novel mechanism of cell cycle regulation.

I would however still raise a few questions and issues:

1. Overexpression of the wild-type versions of E2F7 and 8 do not reduce the numbers of BrdU positive cells but strongly impair proliferation of HeLa cells. In contrast, expression of the KEN box mutant of E2F7 strongly prevents BrdU incorporation and induces cell death. The stabilised version of E2F8, on the other hand, has a much milder effect on replication and does not induce cell death. These important differences are not further explored and might be subject for another manuscript but a few issues need to be clarified:

1a) Cell death (Fig.4) is not clearly defined and should be determined by an additional method, because there is no evidence of a sub-G1 peak in the DNA histograms (Fig.3,4).

In our experience, sub-G1 peaks are not the most consistent measure for apoptosis. Therefore, we performed Annexin V staining followed by FACS analysis on the KEN mutants versus wild-type exogenous proteins. The FACS data were very consistent with the PCNA live imaging, showing again that cell death was clearly increased in cells expressing E2F7KEN, compared to cells expressing E2F8Kmut as well as wild type versions of E2F7/8 (see revised Figure EV5A). Secondly, we realize that cytosolic leakage of fluorescent PCNA is a rather unconventional way to define cell death. To let readers better appreciate the validity and clarity of this assay, we attached a supplementary movie showing examples of cells E2F7KEN-expressing cells that all clearly become apoptotic after cytosolic leakage of PCNA mCherry.

1b) When do E2F7Kenmut cells die? By analysing the available time lapse videos, it could be determined whether cells die around the time point of initiation of DNA synthesis or much earlier. Why does the stabilized version of E2F8 (which seems to be expressed even stronger then E2F7Kenmut not induce cell death?

These are great comments. We re-analyzed the time lapse videos, and the data are now included (Figure 4G and 5D). Because the figure became very large and busy, we split the old Figure 4 now into two separate figures (4&5) in the revised manuscript. Figure 4 deals with the consequences of KEN mutation, and 5 with the interaction between CDH1 depletion and
induced E2F7/8 expression. This additional analysis convincingly shows that the majority of E2F7\(^{ken}\) cells entered apoptosis without forming PCNA dots, which we interpret as by a failure to even begin S phase.

We were also surprised to see that E2F8\(^{K/K}\) did not show considerable apoptosis, with either live imaging or Annexin V staining, and we don’t have a clear explanation for this. It is possible that E2F7 and -8 do not have an entirely overlapping set of target genes. Secondly, our data show that E2F8\(^{ken}\) is not as strongly stabilized in G1 as the E2F7\(^{ken}\), and therefore has a less dramatic effect (see for example Fig 2E and 3D). We would like to point out that the E2F8K/K mutant is not higher expressed than the E2F7KENmut; in fact, it’s somewhat lower, even after correcting for percentage of positive cells (see quantification in revised Fig EV1B).

c. How do E2F7/8 mutants interfere with cell cycle progression? Fig.5 shows that the wild type versions of these proteins repress the expression of their target genes, when expressed during S-phase. It would be very interesting to know whether premature expression of these proteins by expressing them as stabilized proteins would prevent the induction of these genes. This could be readily determined by analyzing cells released from a mitotic block.

We thank the reviewer for this valuable suggestion, and we have performed this experiment. In brief, we have measured target gene expression using qPCR on doxycycline-induced HeLa cells lines expressing KEN-mutant E2F7/8 after a mitotic shake-off. Over the course of 12 hours, a strong induction of E2F target genes was seen in the untreated cells, reflecting an increasing number of S-phase cells. In accordance with the hypothesis, expression of the stable E2F7 mutants strongly repressed this induction. Consistent with the other assays, we again found that the E2F8K/K mutant was clearly less potent. Remarkably, the mRNA levels of mutant E2F7 and -8 were remarkably similar, indicating substantial KEN-domain independent degradation of E2F8. These data are shown in revised Figure 4C.

2. The suggested feedback loop should be interrogated by overexpressing CDH1 (the stabilized phospho-mutant) or Emi1-RNAi. The prediction is that this should reduce the levels of endogenous E2F7/8 (as shown by expressing wt CDH1 in Fig.2) while inducing endoreplication. In contrast, it would be interesting to know whether overexpression of E2F7/8 induces rereplication.

These are excellent suggestions, and we tested the effect of Emi1 RNAi. Although under unsynchronized conditions we did not see a decrease in E2F7, Emi1 depletion caused a partial degradation of E2F8 as well as the known APC/C\(^{cdh1}\) substrates. We then improved the experimental conditions by arresting the cells in S-phase with hydroxyurea, to avoid the effect of any transcriptional bias caused by the introduction of siEmi1 particularly in cells arrested in S phase with hydroxyurea. Even in this condition, we saw only a partial degradation of the classic APC/C\(^{cdh1}\) substrates, which we attributed to partial inhibition of APC/C\(^{cdh1}\) by high CDK2 activity. Hence, additional treatment with the CDK2 inhibitor NU6140 was done, and in this condition, we could demonstrate that APC/C substrates including E2F7/8 almost completely disappeared. We show these results in figure 6 of the revised manuscript.

As to whether re-replication would occur after E2F7/8 overexpression: this is absolutely an interesting possibility. However, it should be noted that most of the factors involved in origin licensing are E2F7/8 target genes (CDC6, CDT1, MCM proteins). Hence, E2F7/8 overexpression merely causes delayed replication, as seen by marked delay of proliferation. Nevertheless, it may still be possible that (modest) E2F7/8 overexpression may induce endocycles in tissues more prone to undergo endocycles; this will be a topic for our future studies.

3. Figure 1 shows that E2F7 and 8 are unstable proteins, which are targeted for proteasome-dependent degradation. Cycloheximide treatment die however only stabilize about 50% of the protein, which most likely reflects the cells in G1-phase of this asynchronous population of RPE cells. To further corroborate the degradation during exit from mitosis and G1-phase, cells should mitotically synchronised and released before addition of CHX. In addition CDH1 RNAi could clearly demonstrate the importance of this pathway during G1-phase degradation of these proteins.
To answer the first part of the question: it is correct that CHX only causes a partial degradation of E2F7/8 in unsynchronized cells, most likely due to the fact that part of the cells are in S/G2, where APC/C activity is low. However, in view of the fact that E2F7/8 proteins are very low and hardly detectable in G1 cells, it seems highly unlikely that CHX treatment will result in a detectable reduction of E2F7/8.

For the second suggestion we have done two different experiments to show the effects of CDH1 RNAi during G1 phase. First, we released CDH1RNAi-treated RPE cells from a nocodazole block. Secondly, we released cells from a G1 arrest with the CDK4/6 inhibitor PD0332991. In both instances, E2F7 behaved as a typical APC/C\(^{C\text{Cdhl}}\) substrate (see revised Figs 2G and 6F). Surprisingly, we detected only very low amounts E2F8 in these conditions, and we could not demonstrate stabilization of endogenous E2F8 in siCDH1 treated cells after mitotic exit or during G1 (revised figure 2G, I). We explain these findings by 1) transcriptional regulation mechanisms, or 2) an alternative E3 ligase pathway. Experiments with CDC20 RNAi and co-immunoprecipitation indicated that CDC20 indeed appears to target E2F8 (revised figure 2I). However, given that CDC20 is not active in nocodazole-arrested cells, additional –yet unidentified– E3 ligases are likely to play an important roles in E2F8 regulation. Although subject of ongoing research, this goes beyond the scope of the present paper. Nevertheless, we would like to point out that the assay shown in Fig2A, and the siEmi1 experiments show that E2F8 is an APC/C\(^{C\text{Cdhl}}\) substrate convincingly enough. We discuss our interpretation of these findings within the discussion section of the revised manuscript.

4. Figure 2D shows the interaction between CDH1 and the E2Fs. These data could be strongly improved by demonstrating a KEN-box dependent interaction between the proteins.

The evidence of an interaction between E2F8 and CDC20 is less convincing due to the weak expression of FLAG-Cdc20 and the strong contaminating bands and should therefore be repeated. In addition, the KEN-box mutant should be combined with the D-box mutant to show that this protein is now completely stable during exit from mitosis and in G1-phase. Also it would be interesting to know if the D-box mutants would prevent the initial fast kinetics of degradation (0 to 20 minutes, Fig.2E) of the GFP fusion protein.

We immunoprecipitated E2F7/8 using EGFP beads, and stained back for endogenous CDH (see revised Fig EV3), and showed for E2F7 that this interaction was completely dependent on its single KEN-box. Surprisingly, the interaction between E2F8 and CDH1 was clearly weaker. We could not detect a difference in CDH1 interaction between wild-type and KEN mutant E2F8. This looks consistent with our other data, showing that CDH1 is clearly not solely responsible for E2F8 degradation.

Concerning the interaction between E2F8 and CDC20, we have now repeated the co-IPs in a slightly different manner. We used GFP-binding beads to pull down E2F8 and then stain back the CDC20 construct with a flag antibody. This gave us much cleaner flag immunoblot, and again binding between E2F8 and CDC20 (see revised FigEV3C). Interestingly, we could not detect significant interaction between E2F7 and CDC20 using this same strategy, supporting our claim that E2F7 is not targeted by APC/C\(^{C\text{Cdcl}}\).

We did not generate D-box mutants of E2F8, for the following reasons. First, there is a risk that with so many mutated sites (2x KEN + 3x D-box) the E2F8 protein becomes dysfunctional; one D-box is located in the DNA binding domain of E2F8. Furthermore previous publications showed that binding via alternative atypical motifs is very well possible (He, J et al. Insights into degron recognition by APC/C coactivators from the structure of an Acm1-Cdh1 complex. Mol Cell. 2013;50:649-60). Finally, nocodazole treatment (when CDC20 is still inactive) already greatly reduced E2F8 expression, and it is highly likely that yet another E3 ligase plays an important role.

5. Figure 3C shows the effect of E2F7/8 wild type and mutant overexpression on cell cycle progression. The effect on G1-phase accumulation should be quantified to match the claims in the text.

We have quantified the effects on G1 phase accumulation by expressing fluorescence in G1...
cells as box plots, and performed statistical analysis on these data. These results are shown in Fig. EV2B.

6. Similarly, the data shown in Figure 4G would be much easier to read if quantified. In addition, for comparison, the eGFP-negative cell population should be used as control.

We have moved the histograms to the supplement and placed the quantifications in the main figure instead (revised Fig. 4I). Indeed this improves the clarity of the data; we plot in this figure the gated EGFP-positive cells, to address point 3 from Reviewer #2. We do not precisely understand why the reviewer thinks EGFP-negative populations should be used as a control, because this is why we have the vehicle condition. The numbers of EGFP-negative cells in each sample are low, but show similar cell cycle profiles as the vehicle conditions. We omitted them for now, but should the reviewer and editor still feel that these are essential data, we would be more than happy to provide these as an extended version figure.

7. Fig2A should include a CDC20 target, such as cyclin B1, as a positive control to show that overexpression of CDC20 is sufficient to destabilize target proteins in this assay.

We did immunoblots and found that cyclin B1 was not changed at all by CDC20 overexpression. The reason is that most cells are in interphase, where CDC20 is most likely inactive. We therefore concluded that this does not benefit the story in any way and removed this condition from the revised figure.

2nd Editorial Decision

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once the few minor issues/corrections listed in the referee reports have been addressed.

In addition, could you please also provide legends for the EV tables. As I have mentioned before, we have also started encouraging authors to submit the raw data of biochemical and/or microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but I encourage you to supply these files when submitting the final version of your study.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS

Referee #2:

The authors should be congratulated on the additional experiments they have carried out in response to reviewers' comments. The manuscript is strengthened in several places by new data (1) that clarify the question of E2F7/8 targeting by Cdc20 vs Cdh1 vs another E3 and (2) that test the functional consequences of E2F7/8 non-degradation - especially the experiments shown in Fig 4C and 6A.

Further proofreading is required to remove minor errors - examples I have come across include (1) legend to Fig 2G - schematic is fig EV1B not EV2B (2) occasional inconsistencies in protein names - e.g. AuroraA, CDH (3) Errors and poor grammar on 3rd page of results onwards, for example "Most likely, in the CDH1
activity was compromised in the CDC20 RNAi condition" "E2F7 appears to be stronger regulated""amino acid positions 31-33 position"
(4) last sentence of discussion
but otherwise I find this manuscript suitable for publication without further revision.

Referee #3:

The revised version of the manuscript entitled 'Feedback regulation between atypical E2Fs and APC/C-Cdh1' by Boekhut et al. is significantly improved and all specific question raised in my original evaluation have been sufficiently addressed. This work adds to our understanding of cell cycle transition and I congratulate the authors to their work.

Minor detail: ON page 7 in the Results section one sentence is unfinished. It reads now: Most likely, in the CDH1 activity was compromised in the CDC20 RNAi condition. Please correct this. 5 lines below CDH should be changed to CDH1.

2nd Revision - authors' response 07 January 2016

Because the remaining requested changes to the manuscript were only small and textual, we do not provide a point-by-point response. We corrected all of them according to the reviewer's instructions.

We now provide brief legends for EV tables 1 and 2 as per your request. We also noticed that we had omitted the primer sequences for AURKA in the previous version; we corrected this small mistake.

We chose to opt out from the possibility to show the raw biochemical images, because 1) we use well-characterized antibodies and in most cases show that the protein bands disappear in RNAi-treated conditions, and 2) we would have to cram >100(!) full blot scans in one supplementary figure.

3rd Editorial Decision 07 January 2016

We are pleased to inform you that your manuscript has been accepted for publication in EMBO reports.