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Chk1 and 14-3-3 proteins inhibit atypical E2Fs to prevent a permanent cell cycle arrest

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1st Editorial Decision 7 September 2017

Thank you for submitting your manuscript on Chk1 interplay with E2F7/8 to The EMBO Journal. After some delay linked to the summer vacation period, we have now received the reports from three expert referees, copied below for your information. As you will see, the referees acknowledge the interest and potential importance of your findings, but also point out several aspects that would require substantiation prior to eventual publication. In particular, two repeatedly raised concerns are the limited insight into E2F7/8 repression by 14-3-3 protein(s), and the missing information on a potential role of the new pathway also during unperturbed cell cycles.

Should you be able to adequately address these two key issues, as well as the various other more specific/straightforward requests, we would be happy to consider a revised manuscript further for publication in The EMBO Journal. Please do note that it is our policy to allow only a single round of major revision, making it important to carefully respond to all points raised at this stage. Should you have any additional questions/comments regarding the referee reports or the revision requirements, please therefore do not hesitate to get in touch with me ahead of resubmission. If needed, we might also extend the revision period, during which publication of any competing work elsewhere would have no negative impact on our final assessment of your own study.

Please refer to the sections below for additional information on preparing, formatting and uploading a revised manuscript.

Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to your revision.

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REFEREE REPORTS

Referee #1:

Yuan et al. describe a novel interaction between Chk1 and the atypical E2Fs, E2F7 and E2F8. The authors show that Chk1 phosphorylate E2F7/8 and inhibits the ability of these E2F proteins to repress transcription. After mapping and mutating the relevant phosphorylation sites, the authors show that mutant forms of E2F7/8 carrying Alanine substitution at this phosphorylation site have enhanced activity and delay S-phase progression, while phospho-mimicking mutations do not. Yuan et al show that knockdown of E2F7/8 suppresses the S-phase arrest caused by Chk1 inhibition, and they propose that Chk1 inhibition of E2F7/8 is important to allow cells to progress through S-phase following replication stress. To understand the underlying mechanism the authors profile proteins that associate with E2F7/8 and they demonstrate that 14-3-3 proteins are critical to this regulation by interacting with phosphorylated E2F7/8, and relieving E2F7/8-mediated repression. Curiously the 14-3-3 proteins do not appear to change the localization of E2F7/8 or their recruitment to promoters. Yuan et al propose that this set of interactions have a significant impact on the proliferation of many cancer cells. They show that copy gains that include YWHAZ (the gene encoding a 14-3-3 protein that binds to E2F7/8) are common in several types of cancer and correlate with high level expression of E2F7/8 target genes.

The de Bruin laboratory has previously demonstrated that E2F7/8 can act as act as tumor suppressors in mice, and that they are linked with DNA Damage responses, however the details of the underlying mechanism were not known. In a general sense, this study extends work that has linked Chk1 to the regulation of E2F6 (ref 6, 22), another atypical E2F, but the molecular mechanism that is proposed here is quite different. The experiments described here are convincing, and the model provides a very intriguing insight into the roles and regulation of E2F7 and E2F8.

Overall, this is an impressive piece of work and, given the obvious implications for cancer cell proliferation, I think that it will be of interest for the general EMBO J audience. I have a few minor questions and comments:

1. The authors propose that the 14-3-3 protein(s) bind to Chk1 phospho-sites of E2F 7/8 proteins to induce a series of transcriptional effects (Fig 5) without altering the subcellular localization of the E2F proteins (a mechanism that is different from that described for several other 14-3-3-binding proteins, ref 28). They show 14-3-3 is a cytosolic protein and later discuss 14-3-3 could cause conformational changes in E2F 7/8 (line 327). Where do the authors think that this interaction occurs, and do they have any evidence that either 14-3-3 or E2F7/8 relocate in response to replication stress?

2. The authors say that E2F 7/8 are not recruited to DSB sites in response to irradiation. In reference 13, others reported the opposite for E2F7. One difference between that paper and this manuscript is that Ref 13 used E2F7/8 GFP constructs, and in this manuscript, the authors use antibodies to track E2F 7/8. Have the authors done the experiment exactly as it was reported in reference 13, or have they done a different experiment? It is difficult to tell from the way that the text is written.

3. The model shown in Figure 6C is easy to understand but it only shows part of the picture. As the authors point out, Chk1 also affects E2F6 and E2F3 and it presumably alters the overall balance between E2F activators and repressors. It might be helpful to a general readership if the authors could add a second summary model in Fig 6D that summarizes the more complex impact of Chk1 on the overall family E2Fs.

4. The text says that E2F7S411A mutants are stalled in S phase and E2F8S395A stalled in G2 (Figure 3). What is the reason for this difference? Does one of these constructs give partial repression of E2F 7/8 targets? (Discussion Line 332).

5. In Table 1, the potential interactors are shown in alphabetical order. It took me awhile to find the enrichment of 14-3-3 relative to the other >80 proteins. It might be easier to digest this data if the proteins are listed by descending Fold change.

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Referee #2:

The study by Yuan et al. demonstrates that Chk1phosphoryates atypical E2Fs, E2F7 and E2F8, inhibiting their function as a transcriptional repressor through promoting the interaction with 14-3-3 proteins. Chk1-dependent phosphorylation of E2F7 and E2F8 suppresses DNA replication stress-induced cell cycle arrest and apoptosis. 14-3-3 expressions positively correlate with E2F target genes expression in several human cancers such as hepatocellular carcinoma, suggesting the reason why E2F7 and E2F8 transcripts are high in many cancer cells even though they function as a tumor suppressor. The mechanism by which loss of Chk1 induce a permanent cell cycle arrest is still missing. Therefore, the current study is of a significance and interest. The manuscript is clearly written and easy to follow. However, there are several important issues to be addressed before considering its publication. For example, the mechanistic insight into 14-3-3-induced repression of E2F7 and E2F8 activities is missing. Does Chk1-E2F7/8-14-3-3 axis regulate cell cycle progression under unperturbed condition? If these issues can be fully addressed, the paper will be suitable for publication.

Major comments:
1. The greatest weakness in this study is that the authors do not provide any mechanistic insight into the repression of E2F7 and E2F8 activities by 14-3-3. Given that 14-3-3 proteins have versatile roles in cell cycle regulation as the authors suggested. Therefore, it should be addressed to clearly support the conclusion.

2. Chk1 is essential for cell cycle progression under unperturbed conditions. However, the current study was mostly done under a DNA replication stress condition using only one cell line. They should examine whether Chk1-dependent phosphorylations of E2F7 and E2F8 also regulates normal cell cycle using multiple cell lines.

Minor comments:
1. The authors demonstrated that Chk1 phosphorylate E2F7 and E2F8 at S411 and S395 in vitro, respectively. However, there is no experimental evidence showing that Chk1 phosphorylates these sites in vivo. In addition, they should confirm whether there is a positive correlation between Chk1 activation, E2F7 and E2F8 phosphorylations, and the E2Fs target genes expression during S phase progression under unperturbed and replication stressed conditions.

2. In vitro kinase assay showed that phosphorylations of E2F7-S411A and E2F8-S395A was moderately reduced, suggesting that Chk1 phosphorylates E2F7 and E2F8 at the other site(s). Do E2F7 and E2F8 phosphorylations at the other sites also function in the regulation of their activities?

3. Knockdown of E2F7 and E2F8 reversed Chk1-induced cell cycle arrest under replication stressed condition. Rescue experiments using E2F7-S411A or -S411D and E2F8-S395A or -S395D) are required to strengthen their conclusions.

Referee #3:

The present manuscript from Yuan et al. is entitled "Chk1 and 14-3-3 proteins inhibit atypical E2Fs to prevent a permanent cell cycle arrest". Here, the authors explore the function of Chk1 signaling in regulating the activity of the transcriptional repressors E2F7 and E2F8. They uncovered that Chk1 mediated phosphorylation of E2F7/8 facilitates an association with 14-3-3 that inhibits the transcriptional repression of E2F7/8 target genes. This inhibition is required to allow cells to resume the cell cycle following DNA damage and prevent cell death.

Impact/novelty: This report establishes E2F7 and E2F8 as substrates of Chk1 and suggests that this phosphorylation mediates inhibition of the repressors' function. Chk1 has been linked with the E2F pathway in published work, however, these new observations provide relevant insight into Chk1 signaling beyond controlling an active cell cycle/replication checkpoint signal, i.e. Chk1 masters a

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transcriptional program that could prevent resumption of the cell cycle following repair of DNA damage. This is important as Chk1 is a major cell cycle regulator during S and G2 phase.

Overall impressions: Overall the quality of the article is fairly good and provides insight into a partly novel aspect of Chk1 signaling. However, a number of the experiments and conclusions need additional supportive evidence as indicated below.

Major points.
1) Please document that transcriptional control through the Chk1/14-3-3/E2F7-8 pathway impacts protein levels (for CDC6, RAD51, Cyclin E). Furthermore, are the protein changes functionally relevant, for example, is RAD51 function reduced when expressing the repressive E2F7/8 alanine-mutants?
2) A major question is if these findings are relevant for more unperturbed cell cycle progression and cell proliferation? As such, are the observations of E2F7/8 alanine mutants in Figure 3 a result of recovery from HU damage or a general cell cycle deficiency. In addition to HU, a standard double-thymidine release should also be conducted, as this exposes cells to much less fork collapse. Finally, E2F7/8 phosphorylation analysis should be done over the cell cycle (also in unperturbed cells) and after HU release.
3) The authors should address the role of ATR, the major upstream regulator of Chk1. Is ATR also required for E2F7/8 control?
4) The authors frequently mention permanent cell cycle arrest (driven by deregulated E2F7/8), however, clonogenic survival assays (+/- HU for the wt and mutant cell lines) were not performed or included. This should be included.
5) The authors should confirm that 14-3-3 is directly associating with phosphorylated E2F7/8 on the chromatin, is this the case?
6) Further, does loss of 14-3-3 or inhibition impact cell cycle reentry following marked replicative stress in a manner comparable to the E2F7/8 alanine mutants?
7) The authors present data that appears to conflict with the previously reported direct involvement of E2F7 in DNA damage repair (Zalmas et al. 2013). The authors should further discuss or reconcile this point.

Minor points:
1) Please ensure that Figure 3 panel c and d re: Rad51 qPCR have not been duplicated between E2F7 and E2F8.
2) Expression levels of endogenous protein vs the E2F7/8 transgenes should be documented. Could phenotypes be due to excess overexpression?

1st Revision - authors' response 6 December 2017

Referee #1:

Yuan et al. describe a novel interaction between Chk1 and the atypical E2Fs, E2F7 and E2F8. The authors show that Chk1 phosphorylate E2F7/8 and inhibits the ability of these E2F proteins to repress transcription. After mapping and mutating the relevant phosphorylation sites, the authors show that mutant forms of E2F7/8 carrying Alanine substitution at this phosphorylation site have enhanced activity and delay S-phase progression, while phospho-mimicking mutations do not. Yuan et al show that knockdown of E2F7/8 suppresses the S-phase arrest caused by Chk1 inhibition, and they propose that Chk1 inhibition of E2F7/8 is important to allow cells to progress through S-phase following replication stress. To understand the underlying mechanism the authors profile proteins that associate with E2F7/8 and they demonstrate that 14-3-3 proteins are critical to this regulation by interacting with phosphorylated E2F7/8, and relieving E2F7/8-mediated repression. Curiously the 14-3-3 proteins do not appear to change the localization of E2F7/8 or their recruitment to promoters. Yuan et al propose that this set of interactions have a significant impact on the proliferation of many cancer cells. They show that copy gains that include YWHAZ (the gene encoding a 14-3-3 protein that binds to E2F7/8) are common in several types of cancer and correlate with high level expression of E2F7/8 target genes.

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We think that the interaction between E2F7/8 and 14-3-3 occurs in the nucleus, when E2F7/8 is bound to DNA. We initially based this idea on the finding that the phosphorylated form of atypical E2Fs are predominantly located within the nucleus (Fig EV 2C), as well as the overall levels of atypical E2Fs (Fig EV 3C and EV 5C), and we now provide additional evidence supporting this idea. Although 14-3-3 is more abundant in the cytosol, it can also be found in the nucleus, where it can even bind to chromatin [Alasdair HM. et al. *EMBO J* 2006]. We now clearly demonstrate that 14-3-3 z and ε binds to E2F7/8 target gene promoters (Fig 5C and Fig EV 5E) using ChIP assays. Importantly, we did not find 14-3-3 enrichment on chromatin locations distal from E2F7/8 binding regions.

Secondly, it should be noted that under all the conditions tested throughout our study, E2F7/8 were strictly localized within the nucleus. For example in Figure EV2C, by using cytosol/nuclear separation and phospho-specific antibody against the E2F7^{S411} and E2F8^{S395}, we showed that the phosphorylated forms of atypical E2Fs are predominantly located within the nucleus. Furthermore, in our imaging data, including stripe assay (Fig 2E), camptothecin treatment (Fig EV 2D), live imaging (Fig EV 3C), and co-localization experiments with 14-3-3 (Fig EV 5D), we have observed clear nuclear localization of atypical E2Fs. Finally, we found very similar enrichments of phosphomimetic, non-phosphorylatable, and wild type E2F7/8 in our initial ChIP experiments (Fig 3E).

Considering all above evidence together, we conclude that the regulation of E2F7/8 by 14-3-3 occurs most likely at the chromatin level.

2. The authors say that E2F 7/8 are not recruited to DSB sites in response to irradiation. In reference 13, others reported the opposite for E2F7. One difference between that paper and this manuscript is that Ref 13 used E2F7/8 GFP constructs, and in this manuscript, the authors use antibodies to track E2F 7/8. Have the authors done the experiment exactly as it was reported in reference 13, or have they done a different experiment? It is difficult to tell from the way that the text is written.

We used GFP constructs as well in the current study. Based on the paper (Zalmas LP, et al. *Cell Cycle* 2013), we expected to observe binding of E2F7/8 to DNA binding sites in the stripe assay. We were surprised to see that this was not the case. Therefore, we took great care to perform the experiments in the same way as they described (Fig 3C of Zalmas paper). We took the same cell line (U2OS) and treatment (camptothecin, 24h) to perform the γ-H2AX immunofluorescence staining in combination with detection of EGFP-tagged E2F7. However we were not able to reproduce the experiment. We cannot explain the discrepancy, but it is obvious that in the previous paper, the cell nuclei seem quite disrupted by the camptothecin treatment, while we showed relatively intact morphology of the cell (Fig EV 2D). Perhaps the severity of the damage was different in the two studies, although we attempted to use the same concentration of camptothecin. We have now clarified on page 6, line 166-171 that we tried to completely reproduce their experiment.

3. The model shown in Figure 6C is easy to understand but it only shows part of the picture. As the

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authors point out, Chk1 also affects E2F6 and E2F3 and it presumably alters the overall balance between E2F activators and repressors. It might be helpful to a general readership if the authors could add a second summary model in Fig 6D that summarizes the more complex impact of Chk1 on the overall family E2Fs.

We thank the reviewer for this excellent suggestion to summarize our current understanding about Chk1-E2F interactions. We have generated a new scheme illustrated in Figure 6D replacing the original one, and the discussion about this model can be found in the manuscript discussion part page 13, line 382-385 and page14, line 396-403.

4. The text says that E2F7S411A mutants are stalled in S phase and E2F8S395A stalled in G2 (Figure 3). What is the reason for this difference? Does one of these constructs give partial repression of E2F 7/8 targets? (Discussion Line 332).

Our previous studies have shown that E2F7 and E2F8 repress a very similar set of target genes (Westendorp et al, *Nucleic Acids Res* 2012 and Kent LN et al, *J Clin Invest* 2016). This also makes sense if we consider the fact that E2F7 and E2F8 can bind DNA in the form of heterodimers (Li et al. Dev Cell 2008). We think this difference between E2F7 and -8 has to do with differences in their stability throughout the cell cycle. We previously published evidence that E2F8 is more efficiently degraded in G1 phase than E2F7 via APC/C^{Cdh1} (Boekhout M et al. *EMBO Rep* 2016). Therefore, we think that E2F7 overexpression will dominate the repression at the G1/S transition and early S phase, leading to (early) S phase arrest. On the other hand, exogenous E2F8 accumulates later and then contributes to late S-G2 phase arrest. We decided to not discuss these considerations in the manuscript, because they would distract from the main message of this story.

5. In Table 1, the potential interactors are shown in alphabetical order. It took me awhile to find the enrichment of 14-3-3 relative to the other >80 proteins. It might be easier to digest this data if the proteins are listed by descending Fold change.

We thank the reviewer for this feedback. We now list the potential interactors by descending average fold change.

Referee #2:

The study by Yuan et al. demonstrates that Chk1phosphoryates atypical E2Fs, E2F7 and E2F8, inhibiting their function as a transcriptional repressor through promoting the interaction with 14-3-3 proteins. Chk1-dependent phosphorylation of E2F7 and E2F8 suppresses DNA replication stress-induced cell cycle arrest and apoptosis. 14-3-3 expressions positively correlate with E2F target genes expression in several human cancers such as hepatocellular carcinoma, suggesting the reason why E2F7 and E2F8 transcripts are high in many cancer cells even though they function as a tumor suppressor. The mechanism by which loss of Chk1 induce a permanent cell cycle arrest is still missing. Therefore, the current study is of a significance and interest. The manuscript is clearly written and easy to follow. However, there are several important issues to be addressed before considering its publication. For example, the mechanistic insight into 14-3-3-induced repression of E2F7 and E2F8 activities is missing. Does Chk1-E2F7/8-14-3-3 axis regulate cell cycle progression under unperturbed condition? If these issues can be fully addressed, the paper will be suitable for publication.

Major comments:
1. The greatest weakness in this study is that the authors do not provide any mechanistic insight into the repression of E2F7 and E2F8 activities by 14-3-3. Given that 14-3-3 proteins have versatile roles in cell cycle regulation as the authors suggested. Therefore, it should be addressed to clearly support the conclusion.

Based on the literature, there are essentially four possible mechanisms of how 14-3-3 can regulate the function of its targets: 1) Relocation; 2) Degradation; 3) Preventing the interaction with yet another binding partner, such as a corepressor; or 4) Binding causing a

conformational change of the target and thereby altering its function. To provide more mechanistic insights we have performed several additional experiments to test whether 14-3-3 regulates atypical E2Fs via one these potential mechanisms.

1). Relocation. It has been reported that 14-3-3 proteins bind substrates including CDC25A to facility their export from the nucleus [Lopez-Girona A et al. *Nature* 1999]. We have analyzed this possibility by comparing nuclear and cytosolic levels of wild type and phospho-mutant E2F7/8, under normal and DNA damaging conditions, or after co-transfection with 14-3-3 using immunofluorescence microscopy (Fig 2E, Fig EV2C,D, EV3C and EV5C). All these experiments showed that the nuclear localization of E2F7 and E2F8 is not altered by 14-3-3. Furthermore we demonstrated that atypical E2Fs were not recruited to DNA damage sites (Fig 2E and Fig EV 2D). Together these studies suggest that 14-3-3 does not facilitate significant relocation of E2F7 and E2F8.

2). Degradation. Chk1 phosphorylation and subsequent binding of 14-3-3 could lead to enhanced degradation of E2F7/8. Our data in Figure 5 showed that 14-3-3s bind to Chk1-phosphorylated forms of E2F7 and E2F8, but not alanine forms. If 14-3-3 protein regulates the degradation, then alanine mutant E2F7/8 should be more stable than wildtypes. However, as shown in the Figure 2A, protein levels of wildtype and alanine mutants are expressed at similar levels with and without DNA damage. We have also performed experiments with cycloheximide to monitor degradation of wild type versus alanine-mutant E2F7, but their expression levels did not differ (data not shown). In addition, if 14-3-3 protein regulates the degradation, then we would expect that endogenous E2F7/8 would be more stabilized in DNA damage. However there was no difference in the half-life of endogenous E2F7/8 in response to DNA damage compared to untreated cells (cycloheximide experiment. Fig 2D and Fig EV 2B). Thus, these findings provide strong evidence that 14-3-3s do not mediate degradation of E2F7 and E2F8.

3). Preventing the interaction with yet another binding partner. Previous studies have shown that E2F7 and E2F8 form heterodimer and homodimer to repress target genes expression [Li et al. Dev Cell 2008]. In addition, E2F7/8 were found to interact with E2F1 and CtBP [Beiyu Liu. et al. *J Biol Chem* 2013]. Interestingly, 14-3-3 has been shown to be able regulate dimerization via binding to its targets [Xing H. et al. *EMBO J.* 2000]. Therefore, we first tested the hypothesis that 14-3-3 binding change the dimerization status among E2F1, E2F7 and E2F8. We performed co-immunoprecipitation assays and compared the binding of wildtype/alanine E2F7 to itself (homodimer), E2F8 (heterodimer) and E2F1 (heterodimer), and vice versa for E2F8 (Appendix Figure S1A). In the co-IP assay, we observed that both wildtype and alanine mutant versions of E2F7/8 bind equally to each other, as well as to E2F1. Based on these findings, we conclude that 14-3-3 binding does not change the dimerization of E2F7/8.

Moreover we evaluated whether altered co-repressor binding is involved to mediate the inhibition of E2F7/8 by 14-3-3. To test this hypothesis, we conducted a new SILAC experiment to identify interactors that bind differently to wildtype and alanine mutant E2F7. This experiment nicely confirmed that 14-3-3 binding is lost in alanine mutant E2F7 proteins However, other proteins that bound differentially did not include a clear candidate that could act as a co-repressor or transcription modulator (Appendix Table S1). Based on these experiments we have no clear evidence that 14-3-3 binding directly modulates binding of co-repressor to atypical E2Fs.

4). Conformational change. In the first version of the manuscript, we had already confirmed that the phospho-mutant and wild type E2F7/8 bind to target genes at very similar levels (Fig 3E). This strongly suggests that phosphorylation (and 14-3-3 binding) does not impair binding of E2F7/8 to their target genes. Interestingly, it was previously reported that 14-3-3 can also be recruited to promoter regions on chromatin (Alasdair HM, et al. *EMBO J.* 2006). We performed new ChIP assays to determine whether 14-3-3 z and ε are enriched at the promoter regions of E2F target genes. Indeed, as shown in the new Fig 5C and EV 5E, 14-3-3 z and ε were found at the *E2F1*, *CHEK1*, *RAD51*, *MCM2* and *CDC6* promoter regions, but not at the distal region in the *E2F1* gene (negative control). Since 14-3-3 is not known to directly bind to DNA by itself, our findings suggest that 14-3-3 might bind DNA via its interaction with E2Fs.

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It is still unknown how E2F7/8 exactly repress transcription at the molecular level. However, it is likely that the structure of the E2F7/8 dimers might be important for their repressor function. Interestingly, 14-3-3 binding can result in an extended conformation change of its targets [Michael B Yaffe, et al. *Cell*. 1997]. Since E2F7/8 represses transcription in the form of dimers, it is possible that 14-3-3 can structurally alter the conformation of the E2F7/8 dimers by positioning itself in between the two individual molecules. At this point we hypothesize that this is a potential mechanism of how 14-3-3 can regulate the function of the atypical E2Fs directly at the promotor levels. We have outlined this possible mechanism in the discussion part: page 14, line 410-418.

2. Chk1 is essential for cell cycle progression under unperturbed conditions. However, the current study was mostly done under a DNA replication stress condition using only one cell line. They should examine whether Chk1-dependent phosphorylations of E2F7 and E2F8 also regulates normal cell cycle using multiple cell lines.

To validate our findings in another cell line, we performed experiments using a non-transformed human cell line RPE (Retinal Pigment Epithelium Cells). As shown in the new expanded Figure EV 4A, knockdown of *Chk1* also stalled the cell cycle progression of RPE at G1/S after HU release, compared with scrambled condition. More importantly, Chk1-dependent cell cycle arrest was rescued by knockdown of *E2F7* and *E2F8*, showing progression to G2/M phase after HU release. These data suggested that Chk1-dependent inhibition on E2F7 and E2F8 was valid under DNA replication stress condition in two completely different cell lines.

To investigate cell cycle progression also under unperturbed conditions we treated HeLa and RPE cells with siRNA against *Chk1* without cell cycle synchronization and analyzed the cell cycle profiles with flow cytometry (Fig EV 4E). We found that si*Chk1* had no impact on cell cycle distribution in HeLa cells, while in the RPE cell line there was an increase in the number of G1 cells. Consistent with our result from HU synchronization experiments, the G1/S arrest phenotype in RPE cells was rescued by knockdown of *E2F7* and *E2F8*, suggesting that cell cycle arrest by Chk1 inhibition under unperturbed condition was also dependent on E2F7/8. However the phenotype in RPE cells under unperturbed condition was less severe than under DNA-damaging conditions. Together, our data in two completely different cell lines showed that the Chk1-E2F7/8 pathway is particularly important for regulating cell cycle progression under replication stress conditions and less critical for unperturbed conditions.

Minor comments:
1. The authors demonstrated that Chk1 phosphorylate E2F7 and E2F8 at S411 and S395 in vitro, respectively. However, there is no experimental evidence showing that Chk1 phosphorylates these sites in vivo. In addition, they should confirm whether there is a positive correlation between Chk1 activation, E2F7 and E2F8 phosphorylations, and the E2Fs target genes expression during S phase progression under unperturbed and replication stressed conditions.

Since the phospho-specific antibody against E2F7^{S411} and E2F8^{S395} cannot detect endogenous E2F7 and E2F8 phosphorylation, we performed studies with overexpressing E2F7/8. To demonstrate that endogenous Chk1 phosphorylates E2F7/8, we inhibit Chk1 by applying siRNA and UCN-01, and then determine whether the E2F7/8 phosphorylation vanished. As shown in the new Figure 1D, phosphorylation levels on E2F7^{S411} and E2F8^{S395} indeed decreased remarkably under si*Chk1* and UCN-01 treated conditions. These data suggested that atypical E2Fs are *bona fide* targets of Chk1 kinase *in vivo*.

In addition, we used the phosphor-specific antibodies to compare E2F7/8 phosphorylation under unperturbed and DNA damaging conditions. Since we had to overexpress E2F7/8, we were confronted with the problem that atypical E2Fs overexpression could cause DNA damage and S-phase arrest. To overcome this problem, we overexpressed the E2F7/8 DNA-binding domain mutant (DBD) constructs in HEK cells, to examine whether there is a positive correlation between Chk1 activation and atypical E2Fs phosphorylations. These DBD mutant constructs carry point mutations that prevent DNA binding, but are otherwise intact. As shown in Figure EV 1E, phosphorylation levels on E2F7^{S411} and E2F8^{S395} were low without

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DNA damage (before etoposide treatment). More importantly, phosphorylation on E2F7^{S411} and E2F8^{S395} increased substantially after 1-4 hours of etoposide treatment, while Chk1 kinase activity was also enhanced over the same time window. This positive correlation supports that Chk1 activation results in phosphorylation of atypical E2Fs in response to DNA damage.

2. In vitro kinase assay showed that phosphorylations of E2F7-S411A and E2F8-S395A was moderately reduced, suggesting that Chk1 phosphorylates E2F7 and E2F8 at the other site(s). Do E2F7 and E2F8 phosphorylations at the other sites also function in the regulation of their activities?

Actually only one strong putative candidate Chk1 phosphorylation site on human E2F7, Ser833 (in mouse it is Ser825), was identified in the mass-spectrometry (Fig EV 1B, Fold-change >21.9 with Chk1 incubation). We mutated this site to alanine in the mouse construct and tested whether the Chk1 phosphorylation will be abrogated. However, E2F7^{S825A} was still phosphorylated by Chk1 in the kinase assay (new Figure 1C), indicating that Ser825 was not a target residue of Chk1 kinase. We then decided to focus on Ser411 and S395 on E2F7/8, which resemble classical Chk1 target residues and locate at a parallel position of each other. Further investigation will be needed to address the functional relevance of potential other phosphorylation sites. However, one strong indication that serines 411 and 395 are the only physiologically relevant sites on E2F7 and -8, respectively, comes from our observation that 14-3-3 binding was completely lost when we mutated those sites. Also, we did not find evidence that etoposide treatment causes an increase in Ser833 phosphorylation.

3. Knockdown of E2F7 and E2F8 reversed Chk1-induced cell cycle arrest under replication stressed condition. Rescue experiments using E2F7-S411A or -S411D and E2F8-S395A or -S395D) are required to strengthen their conclusions.

This is a great suggestion to confirm our finding that Chk1-induced cell cycle arrest under replication stress condition was directly dependent on atypical E2Fs. As shown in the Figure 4B, si*Chk1* arrests the cell cycle at the start of S phase after 8h of HU release and si*E2F7/8* in combination with si*Chk1* rescued this phenotype, showing cell cycle progression to 4N population. Under the same condition of si*Chk1* + si*E2F7/8,* we now over-expressed the alanine and aspartic mutant versions of atypical E2Fs (Fig EV 4B). We found that alanine mutant forms of E2F7/8 strongly reverse the cell cycle rescue by si*Chk1* + si*E2F7/8*, showing an increased population in G1/S phase. Interestingly, compared with alanine mutants, we observed that a substantial population of aspartic mutants expressing cells was able to progress through S-phase and enter G2/M phase after HU release. Given that alanine mutants showed a stronger repressor capacity than aspartic mutant (Fig 3C and D), our results support and strengthen the conclusion that cell cycle arrest mediated by Chk1 inhibition was dependent on the repressor functions of atypical E2Fs.

Referee #3:

The present manuscript from Yuan et al. is entitled "Chk1 and 14-3-3 proteins inhibit atypical E2Fs to prevent a permanent cell cycle arrest". Here, the authors explore the function of Chk1 signaling in regulating the activity of the transcriptional repressors E2F7 and E2F8. They uncovered that Chk1 mediated phosphorylation of E2F7/8 facilitates an association with 14-3-3 that inhibits the transcriptional repression of E2F7/8 target genes. This inhibition is required to allow cells to resume the cell cycle following DNA damage and prevent cell death.

Impact/novelty: This report establishes E2F7 and E2F8 as substrates of Chk1 and suggests that this phosphorylation mediates inhibition of the repressors' function. Chk1 has been linked with the E2F pathway in published work, however, these new observations provide relevant insight into Chk1 signaling beyond controlling an active cell cycle/replication checkpoint signal, i.e. Chk1 masters a transcriptional program that could prevent resumption of the cell cycle following repair of DNA damage. This is important as Chk1 is a major cell cycle regulator during S and G2 phase.

Overall impressions: Overall the quality of the article is fairly good and provides insight into a partly novel aspect of Chk1 signaling. However, a number of the experiments and conclusions need

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additional supportive evidence as indicated below.

Major points.
1) Please document that transcriptional control through the Chk1/14-3-3/E2F7-8 pathway impacts protein levels (for CDC6, RAD51, Cyclin E). Furthermore, are the protein changes functionally relevant, for example, is RAD51 function reduced when expressing the repressive E2F7/8 alanine-mutants?

We agree on that this is an important point, and we thank the reviewer for this suggestion. We performed additional experiments and now show that protein levels of Rad51 and CDC6 nicely followed the mRNA levels in conditions of *Chk1* knockdown in presence or absence of *E2F7/8* knockdown; please see Figure 4A and 4C. Given that Rad51 plays a critical role in assisting DNA damage repair, Rad51 down-regulation during S phase could hypothetically lead to accumulation of unfixed DNA lesions, We, therefore, performed Rad51 and H2AX immunofluorescence staining on cells incubated with siRNA against *Chk1* and *E2F7/8* under HU conditions (Fig 4D and 4E). Indeed, we observed a significant down-regulation of Rad51 expression in the si*Chk1* condition compared with control. In addition, Rad51 decrease at the protein level was rescued under the si*Chk1*+*siE2F7/8* conditions, suggesting that Rad51 repression was controlled by the transcriptional activity of atypical E2Fs. Moreover, we found that si*Chk1* dramatically increase γ-H2AX accumulation, a marker for DNA damage, which was almost completely resolved by additional knockdown of E2F7 and -8. These data strengthen our conclusion that the Chk1/E2F7-8 pathway transcriptionally regulates its downstream targets such as Rad51, and eventually affects its protein levels as well as its function.

2) A major question is if these findings are relevant for more unperturbed cell cycle progression and cell proliferation? As such, are the observations of E2F7/8 alanine mutants in Figure 3 a result of recovery from HU damage or a general cell cycle deficiency. In addition to HU, a standard double-thymidine release should also be conducted, as this exposes cells to much less fork collapse. Finally, E2F7/8 phosphorylation analysis should be done over the cell cycle (also in unperturbed cells) and after HU release.

To address this suggestion, we synchronized cells with a standard double-thymidine release experiment (Fig EV 3B). Similar to our previous findings under HU conditions, we showed that overexpressing wildtype and aspartic mutant forms of atypical E2Fs allow cell cycle progression from G1 to S and G2/M phases after thymidine release, while the alanine mutants E2F7^{S411A} and E2F8^{S395A} dramatically delay the progression at S phase and G2/M phase respectively.

Furthermore we analyzed the cell cycle progression under unperturbed conditions in HeLa cells and in non-transformed human RPE (retinal pigmented epithelial) cells incubated with siRNA against *Chk1* and *E2F7/8*. As shown in the new Figure EV 4E si*Chk1* had little impact on cell cycle of HeLa, compared to scrambled controls. However, si*Chk1* in RPE cells partially stalled the cell cycle progression at G1/S, which was rescued by additional knockdown of *E2F7/8*. This data suggested that under unperturbed conditions, the Chk1/E2F7/8 pathway can regulate normal cell cycle progression in non-transformed cells. We described this data in the manuscript page 10, line 272-283.

Since our phosphorylation specific antibodies were only suitable to detect phosphorylation events on overexpressed atypical E2Fs we used the DNA binding domain mutant versions of E2F7/8, which do not cause any phenotype *in vivo* or *vitro* (Kent et al. *J Clin Invest*, 2016, Westendorp et al. *Nucleic Acids Res,* 2012). As shown in Figure EV 1E phosphorylation levels on E2F7^{S411} and E2F8^{S395} were low under unperturbed conditions, and increased in response to DNA damage by etoposide. Phosphorylation on E2F7^{S411} and E2F8^{S395}occurred at the same time when Chk1 was activated, suggesting that active Chk1 phosphorylates E2F7 and E2F8 in response to DNA damage.

3) The authors should address the role of ATR, the major upstream regulator of Chk1. Is ATR also required for E2F7/8 control?

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We investigated the function of ATR in regulation of E2F7/8 using siRNA against *ATR* under HU arrest/release conditions. We first confirmed the knockdown efficiency of *ATR* and *E2F7*, *E2F8* by immunoblotting (Appendix Figure S1B). Then cells were harvested at indicated timepoints and subjected to flow cytometry for cell cycle profile analysis. Interestingly, although ATR is commonly regarded as the upstream regulator of Chk1, si*ATR* did not affect the cell cycle progression (Appendix Figure S1B). Previous work showed that Chk1 can also be activated by ATM, DNA-PKcs and even Chk1 itself (auto-phosphorylation) [Zhang Y et al. *Int J Cancer*. 2014]. Therefore it can be speculated that these DNA damage machineries act as a backup system to maintain Chk1 function in the absence of ATR. We mentioned this data in the discussion part: page 15, line 419-421.

4) The authors frequently mention permanent cell cycle arrest (driven by deregulated E2F7/8), however, clonogenic survival assays (+/- HU for the wt and mutant cell lines) were not performed or included. This should be included.

We agree that it is important to evaluate the long-term effect of E2F7/8 alanine mutant on cell survival. We tested this idea using the HeLa/TO system, but we encountered the problem that over time the cells that showed very low levels or no expression of the construct tended to outcompete the cell lines with clearly detectable E2F7/8 expression. This skews the outgrowth in the clonogenic assays.

We therefore performed clonogenic assays in a different manner, by showing that over-activation of E2F7/8 by Chk1 deletion results in permanent arrest using RNAi knockdown. To this end, we transfected cells with siRNA, seeded them at low density and followed the cells for 4 days in presence or absence of hydroxyurea. Without hydroxyurea, the si*Chk1* condition displayed less cell colony formation compared with control condition (Fig EV 4F), indicating Chk1 loss causes a mild cell growth inhibition under normal growth conditions. However, loss of Chk1 resulted in a remarkable impact on cell survival under HU treated condition, with barely any colony formation. More importantly, we showed that the cell growth was rescued by knockdown of *E2F7/8*, with a comparable amount of colony formation compared to the control condition. Thus, our data support the conclusion that Chk1 inhibits atypical E2Fs to prevent permanent cell cycle arrest in response to DNA damage.

5) The authors should confirm that 14-3-3 is directly associating with phosphorylated E2F7/8 on the chromatin, is this case?
We now provide evidence that this is the case using ChIP assays. We confirmed that 14-3-3 z and ε are strongly enriched on proximal promoter regions of E2F7/8 target genes, but not on DNA regions remote from E2F binding motifs (Fig 5C and Fig EV 5E). Since we have shown that 14-3-3s bind specifically to phosphorylated E2F7/8, it is likely that once Chk1 phosphorylates atypical E2Fs, 14-3-3s are recruited to atypical E2Fs at the promoter regions.

6) Further, does loss of 14-3-3 or inhibition impact cell cycle reentry following marked replicative stress in a manner comparable to the E2F7/8 alanine mutants?

We have performed experiments to knockdown 14-3-3z with siRNA and the chemical inhibitor BV-02, and subsequently monitor the recovery from an HU block. However, we did not observe a clear effect on cell cycle reentry in either experiment. A possible explanation for this observation is that at least 4 different 14-3-3 isoforms appear to bind E2F7/8 (Fig 5A), and RNAi is not very suitable to knocking down efficiently all these isoforms in the same cells. In principle the chemical inhibitor would provide more complete inhibition. However, we have to take into account that 14-3-3 proteins have many targets. For instance, 14-3-3 can inactivate CDC25 phosphatases by relocating them to the cytosol. Because CDC25A/B/C activate Cyclin-CDK complexes, inhibition of 14-3-3 with BV-02 could lead to enhanced activity of CDC25 phosphatases and subsequent activation of Cyclin-Cdk activity. This would be a compensation mechanism why BV-02 treatment does not result in a severe cell cycle arrest, despite causing enhanced activity of E2F7/8 in a comparable manner to the alanine mutants.

7) The authors present data that appears to conflict with the previously reported direct involvement of E2F7 in DNA damage repair (Zalmas et al. 2013). The authors should further discuss or reconcile this point.

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We attempted to copy the experiment as shown in this pervious study (Zalmas et al. *Cell Cycle*. 2013). We transfected same cell line (U2OS) with E2F7/8 constructs and treated the cells with the same drug (Camptothecin, 24h). While the cell nuclei seem to be disrupted in their study, we showed relatively intact morphology of the cell (Fig EV2D). In addition, we measured the dynamics of MDC1 protein as a positive control in parallel with atypical E2Fs. As positive controls, MDC1 protein showed a clear re-localization to the DNA damage sites in the stripe assay and immunofluorescence staining, but not with atypical E2Fs (Fig 2E and Fig EV2D). We cannot explain the discrepancy, but perhaps the severity of the damage was different in the two studies. We have now clarified at line 166-172 that we tried to repeat their experiments, but with a different outcome.

Minor points:
1) Please ensure that Figure 3 panel c and d re: Rad51 qPCR have not been duplicated between E2F7 and E2F8.

We thank the reviewer for the careful inspection of the figures. We checked our qPCR data again and found that the standard deviations were indeed duplicated. We corrected this error, and now the qPCR results show the proper standard deviations for the E2F8 expressing cell lines.

2) Expression levels of endogenous protein vs the E2F7/8 transgenes should be documented. Could phenotypes be due to excess overexpression?

We documented the endogenous and exogenous levels of E2F8 of HeLa/TO cell lines in Figure EV 2A. This result shows that the exogenous and endogenous protein levels are quite similar. This suggests a modest and physiologically relevant increase in E2F8 expression. Unfortunately, we were not able to detect endogenous and exogenous E2F7 on the same bot, because E2F7/8 in our expression vectors are of mouse origin, and there is no antibody available that can cross-react with both mouse and human E2F7. Nonetheless, we believe that there was no excessive over-expression of the E2F7 constructs, because we previously have shown that $E2F7^{WT}$ and $E2F8^{WT}$ expressing cell lines have very similar expression levels analyzed on the same immunoblot (Boekhout et al. *EMBO Reports* 2016; Figure 6A). In addition, GFP immunoblots showed that expression levels of the alanine/aspartic mutant E2F7-EGFP constructs were quite similar (Fig EV 2A). In fact the alanine mutant appeared to have the lowest levels, despite showing the most severe cell cycle arrest. Based on the findings we conclude that the phenotypes are most likely not caused by excess overexpression, and that relative levels of mutant and wild type constructs do not explain the different phenotypes observed.

2nd Editorial Decision 21 December 2017

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by all two of the original referees, who generally consider their key criticisms satisfactorily addressed and the manuscript therefore in principle suitable for publication. As you will see from the comments copied below, referee 3 nevertheless retains some important concerns regarding data presentation and controls that would have to be dealt with before eventual acceptance. Related to this point, I notice that essentially all figure panels showing gels and blots appear to have been closely cropped and are often overly bright/contrasted, making it difficult to properly assess their relation to the primary data behind them. Therefore, I would ask you to provide figure source data for all the gels/blots/autoradiographs in the main, EV and Appendix figure. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all such panels displayed in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and they would be linked as such to the respective figures in the online publication of your article.

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I am therefore returning the manuscript to you for an additional round of minor revision, to allow you to respond to the remaining points and to upload the accordingly modified files. Please do not hesitate to get back to me should you have any further questions in this regard.

REFEREE REPORTS

Referee #2:

The revised version of the manuscript satisfactorily addresses the concerns raised by the reviewers. I recommend its publication in EMBO J.

Referee #3:

This is a revised manuscript version where the authors provide considerably increased experimental evidence to support their conclusions. Having stated this, a few points still need attention:

*The immunoblots in the new Figure 4A are not of acceptable standard as they clearly are assembled from different membranes, which means that several loading controls are needed. This figure contains an important point on protein level changes, hence, the authors should assemble a quality figure potentially including two loading controls if two membranes are needed.

*Figure EV4F needs quantification and normalization to scrambled control.

2nd Revision - authors' response 29 December 2017

Referee #3:

This is a revised manuscript version where the authors provide considerably increased experimental evidence to support their conclusions. Having stated this, a few points still need attention:

*The immunoblots in the new Figure 4A are not of acceptable standard as they clearly are assembled from different membranes, which means that several loading controls are needed. This figure contains an important point on protein level changes, hence, the authors should assemble a quality figure potentially including two loading controls if two membranes are needed.

Actually we used the same cell lysates, and loaded always the same amounts for these immunoblots. In such case, showing double loading controls is not a common practice. And we would like to point out that the newly added source data figure provides additional evidence of equal loading: the non-specific bands in the detection of Rad51 appear highly similar (Fig 4. Source data A).

*Figure EV4F needs quantification and normalization to scrambled control.

We quantified the percentage of violet staining in each condition, showing with a histogram in Fig EV4F. Because each image yields an actual percentage, it was not necessary to perform normalization to scrambled control. The quantification clearly confirms that si*Chk1* resulted in a significant reduction of cell growth under both untreated and HU treated conditions, and this phenotype was rescued by combinational knockdown of *E2F7* and *E2F8*.

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Corresponding Author Name: Alain de Bruin
Journal Submitted to: EMBO JOURNAL
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
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  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All immunoblots shown are highly representative for 3 independent experiments, unless stated otherwise in the figure legend. All immunoblots, co/immunoprecipitations, immunofluorescence, ChIP, reporter assay, FACS and qPCR results were replicated three times, unless stated otherwise in the figure legends. Statistical analysis on immunofluorescence staining, reporter assay, qPCR and FACS data was performed by Student's t-test or ANOVA followed by Dunnet's post-hoc individual group comparisons. Time lapse experiments were at least performed twice for each separate condition, the amount of cells analysed per condition is shown in the figure or figure legend.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Only technically interpretable data was omitted; smeared or abnormally weak bands for western blot, out of focus time lapse microscopy
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For drug-treatments of cell lines, we always prepared medium master mixes for all samples. Thus we avoided subjective / biased pippetting uL volumes of concentrated drug.
For animal studies, include a statement about randomization even if no randomization was used.	Otherwise: N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For immunofluorescence staining quantification, figures were obtained by another viewers who has no knowledge of the experimental settings.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, as described in Materials and methods: Immunofluorescence staining, reporter assay, ChIP assay, qPCR and FACS data analysis was performed by either Student's t-test or ANOVA, followed by Dunnet's post-hoc individual group comparison. If not indicated in the as significant in the figure, paires were tested as not significantly different.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We always test for normal distribution prior to statistical testing. If failed, we use non-parametric comparisons (Mann-Whitney / Kruskall Wallis). However, data passed normality tests in all statistical tests throughout the manuscript.
Is there an estimate of variation within each group of data?	We did not perform separate variance tests

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used in immunoblot, immunoprecipitation and immunofluorescence experiments are listed in Table 3 worksheet "4. Antibody", including provider, catalogue number and dilution. Many antibodies were indirectly re-validated, because the band disappeared after RNAi.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U2OS cells, HEK 293T cells, RPE-1 cells and HeLa cells originated from ATCC

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D- Animal Models

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E- Human Subjects

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