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MOLECULAR ARCHITECTURE OF THE DNA REPLICATION ORIGIN ACTIVATION CHECKPOINT

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Transaction Report:

(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 01 September 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal. I do apologize for the slight delay in getting back with a decision that was caused be one rather late incoming report and the fact that I did attend two subsequent very busy meetings. I now had the time to carefully read the referee comments that you will find enclosed below. Ref#2 and #3 certainly appreciate the molecular changes that you describe upon inhibiting replication initiation. At the same time, both express strong concerns related to the single approach employed here. They therefore demand significant additional experimental work to validate and expand the reported findings. Although both scientists also provide ample suggestions what could and should be done to confirm and extend this work, their comments unfortunately reflect the currently rather preliminary state of your analyses. Furthermore, ref#1's comments (single siRNA targeting in immortalized tissue culture with no confirmation in a more physiological context) make it rather clear that this scientist does currently not believe in proven significance of the current dataset and therefore does not provide any support for further consideration of your study at our journal. Consequently, we are at this very preliminary state of analysis not in a position to invite a single round of revision - and thereby essentially commit to your paper. This does unfortunately mean, and I am sorry to have to communicate this, that there is no choice than formal reject of the paper.

However, and appreciating the potential interest that has also been expressed from at least some of the referees, and on condition that you would be willing and able to significantly expand the current dataset, we would be able to reassess your findings at much more developed stage of analyses.
In case you might consider such an option, I have to stress that such a manuscript will NOT be

treated as a revision, but would be assessed as NEW SUBMISSION and would therefore be evaluated afresh with respect to the literature and the novelty of your findings at the time of submission and also without any obligation to send the paper out for peer-review or to involve the current set of referees.

Again, I am really sorry that we are unable to reach a more positive conclusion at this stage. Further, we would also understand that you might prefer to publish the results rather rapidly in a presumably less demanding publication. Overall, we still hope that you find the comments of our expert referees helpful in coming to a conclusion on how to proceed with your paper.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors provide an analysis of the effect of suppressing Cdc7 expression by RNAi, on cell division and apoptosis and identify FoxO3a, p15, p53 and Dkk3 as being all involved in regulating the Cdc7-dependent checkpoint. The work presented here provides very limited new information and is marginally advancing our understanding of this topic versus what has already been described. I am concerned about the following: a. the use of a single target cell strain (IMR90), with the possibility that the described phenotypes would not be generally seen; b. the use of a single approach, RNAi, for genetic interference; c. the not so uncommon assumption that under conditions such as cell culture on plastic, high O2 and growth factor milieu, one could drive conclusions on impact cell survival. Human cells in culture represent a truly unphysiological context, even when analyzing so-called cell autonoumous events. At best, one could consider this work as preliminary and in need of substantial additional mechanistic studies.

Referee #2 (Remarks to the Author):

In this manuscript Tudzarova and coworkers study the molecular mechanisms relevant to protect immortalized IMR90 fibroblasts from cell death caused by Cdc7 depletion by siRNA.
Cdc7 depletion was previously suggested to selectively induce apoptosis in cancer cells while it was shown to cause a G1 arrest in primary dermal fibroblasts and this was mediated by p53.
The authors confirm this observation in their cellular model and substantially expand our understanding of how cell cycle arrest and survival are mediated. With a combination of a candidate approach and gene expression profiling they identify Foxo3a, p15 p14 and Dkk3 as important cellular factors that protect human cells from Cdc7 inhibition. As mutations in these important tumor suppressor genes frequently occur in human cancers, this work further supports the notion that Cdc7 inhibition can be an important target for therapy and lays the basis for the molecular understanding of why.

These findings are novel and of importance to both a general and specialized scientific community as the signaling pathways underlying cellular responses to Cdc7 inhibition are to date obscure but have both important biological and clinical consequences.

Two main concerns from the reviewer:

1. Starting from the title the authors claim that the pathways that they have elucidated are activated by reduced origin firing on the grounds that one of the known functions of Cdc7 is the phosphorylation of MCM helicases leading to origin activation (clearly shown in budding yeast). According to this interpretation these pathways should be similarly activated by inhibition of other initiation proteins, but they do not address this point. This is important if this claim is to be maintained as many other functions have recently been suggested for Cdc7 including ATR signaling and cohesion. I would suggest to either assess the activation of this checkpoint in response to the inhibition of other initiation proteins or to change the title and the interpretation of their results throughout the manuscript into something like "Molecular architecture of the Cdc7 inhibition checkpoint".

2. This work suffers throughout from the fact that all the results have been generated with a single Cdc7 specific siRNA. As siRNAs also often have off target effects, it is normal practice to perform some specificity controls such as rescue experiments with siRNA resistant construct or with an alternative target specific siRNA. This is of particular importance when gene expression profiling is performed. The authors should make an effort to reconfirm their main findings with an alternative approach/siRNA.

Some inconsistencies across different experiments needs to be sorted out, see specific points.

Of minor importance: preparation and labeling of the figures could be greatly improved. A better separation of different panels in figurers would help the reader (i.e fig4 panel A contains a picture of cells and a western blot - these should be independently labeled panel A and panel B. similarly fig 3B contains Western and FACS analysis).

The manuscript can be shortened by reducing the data on the p53 dependency (that is very similar to previously published work) and focusing on the novel data (Foxo3a, p15, Mdm2, Dkk3)

Specific points

Introduction-

3. Authors should mention the newly proposed functions of Cdc7 kinase
4. Use official names: ASK is Dbf4

Results
5. A single Cdc7 siRNA was selected on the grounds of being the most efficient in downregulating Cdc7 expression and was used for all subsequent experiments. There was no mention of potential off target effects nor any effort to assess them with a different siRNA. The rational for choosing Cdc7 siRNA-A therefore is not sufficiently strong.
6. The marked decrease in MCM2 levels seen in figure 2A and widely discussed in the text are inconsistent with results in fig1A and fig 2C. What is the correct result?
7. Fig 2B is a combination of different films (possibly lanes 1-4 and lanes 3-7 and lane8) therefore results cannot be combined in single rows - separation should be marked . Also loading controls are different (this experiment should be repeated)
8. Fig2D cis-platinum has been included as a positive control for p53 ser15 phosphorylation, however it is a different blot and results cannot directly be compared.
9. Fig 3B: several reports have indicated that pS40/41 phosphorylation is Cdc7 dependent. I found it strange that the signal seen in the double Cdc7/p53 KD is interpreted as recovery of CDK activity. Is it possible instead that low levels of Cdc7 are reemerging? Authors should at least discuss this alternative possibility.
10. Fig 3C I also see abundant PARP cleavage and active caspase 3 in the Control +p53 siRNA double transfection. Can the authors quantify the difference in order to support the claim that this is due failure of activation of the Cdc7 dependent origin activation checkpoint?
11. Fig 3e what is the upper band seen with the anti-Dkk3 antibodies and why does it disappear at 84h (it does not disappear in the double Cdc7/Dkk3 KD- fig 4B)? How specific are these anti-Dkk3 antibodies?
12. Gene expression analysis would benefit from a second Cdc7siRNA in order to discriminate Cdc7 dependent vs Cdc7 independent changes.

13. p53 is stabilized by the lack of Mdm2. It is suggested that this is due to Mdm2 proteolysis. What happens to Mdm2 mRNA? Can the authors you rule out transcriptional down regulation due to off target effects?

Referee #3 (Remarks to the Author):

Overview: This paper entitled "Molecular Architecture of the DNA Replication Origin Activation Checkpoint", by Tudzarova et al, describes the molecular events comprising a checkpoint monitoring DNA replication initiation. It has previously been shown that reducing Cdc7 kinase levels to block replication initiation appears to activate a checkpoint preventing inappropriate S phase progression by causing G1 arrest. The authors take advantage of this observation to begin identifying the molecular interactions mediating this checkpoint. They conclude that blocking initiation activates the well known transition factor FoxO3a, which then coordinates a multi-pronged response involving the Arf/Mdm2/p53/p21, p15INK4B, and Dkk3/beta catenin pathways to ensure cells do not inappropriately proceed with the cell cycle. Experiments are carried out in tissue culture cells and utilize standard molecular biology techniques such as siRNA, western blotting, and immunofluorescence. A central conclusion from the presented results is that the lack of redundancy amongst the involved molecular pathways suggests a biochemical rationale for the efficacy of Cdc7 kinase inhibitor as possible chemotherapeutic agents.

Impact: The area of investigation is important and timely given its potential to help provide a molecular explanation for how targeting Cdc7 may be a viable strategy in cancer treatment. Results have broader implications for expanding current efforts to identify viable targets and develop additional methodologies for targeting DNA replication initiation. Results are for the most part clearly presented, convincing, and contain the proper controls. While not providing much in the way of a biochemical description of how the check point is activated, as indicated in the title they do describe the molecular architecture involved. Thus the paper makes an important contribution to the field that not only provides the framework for more mechanistic investigations, but should also contribute to develop of new therapeutic intervention strategies targeting replication initiation.

Data evaluation:
The data for the most part are understandable and convincing. There is rather an extensive amount of material presented that is fairly obvious and previously published (e.g. if replication initiation is blocked then clearly a wide array of downstream events will not take place). Since most of the observations are correlative, the key experiments indicating causality are the dual siRNA approaches which show that the replication initiation checkpoint induced by Cdc7 downregulation can be overcome by downregulating various pathways by which it is mediated. These are well done, but it might have been worthwhile to enhance their impact using complementary approaches (e.g. overexpression) to overcome the Cdc7 block. This issue is relevant in part because the control siRNA appears to have a significant impact on the flow cytometry profile (fig 1C). The authors need to provide quantitative data (% cells in various stages) to allow the reader to evaluate this effect and better determine the degree to which Cdc7 is causing G1 arrest. In regard to this latter point (the foundation on which the experimental approach rests), the significant G2/M fraction that remains after Cdc7 siRNA needs to be addressed. Are Cdc7 downregulated cells arrested in G2/M, and if so does this suggest that the origin activation checkpoint is active there as well? The concern is that the extensive time points at which cells are analyzed (discussed in more detail below), raise the potential for indirect effects peripheral to the topic at hand. One way to address this important issue (and provide further evidence that Cdc7 knock down is causing a G1 arrest) would be decrease Cdc7 in synchronized cells (e.g. serum starved/refeed, early S phase arrest with a DNA synthesis like aphidicolin followed by release, etc). While such an approach can be difficult using siRNA, it should be possible taking advantage of small molecule Cdc7 inhibitors.

2) There is some concern/confusion regarding the analysis of Dkk3 that needs to be addressed. In Fig 3E multiple bands (one prominent) are apparent in the Dkk3 western blot, but the Dkk3 arrow points to a very minor band. Are the other bands background, and if so why do they disappear under Cdc7/p53 knock down conditions?

3) There is some concern about the time points in which the majority of analyses take place given that the goal is to describe activation of a checkpoint, which must presumably be able to perform on

a much more rapid time scale. In contrast, described experiments indicate Cdc7 decrease takes more 48hrs or more, and then analyses of the molecular events involved often take place after an additional 48hrs (or more). What is the relevance/rationale of investigating a putative G1 checkpoint in this sort of time frame given that it must be much more responsive to the length of a typical cell cycle? To address these issues and help rule out the potential for indirect effects, the authors should take advantage of Cdc7 inhibitors to block replication initiation. This approach would allow analysis of immediate events involved in checkpoint activation and expand the types of experiments available (e.g. serum/starve release).

Conclusion evaluation:
1) The authors need to discuss/clarify the concepts of an origin activation checkpoint vs. an origin threshold checkpoint (i.e. a minimal number of origins required for cell cycle progression. In the case of Cdc7 downregulation (and as reflected in the title) they discuss the consequences of preventing replication initiation. In other instances, however, the discussion seems to revolve around the consequences of a reduction in the number of origin initiation complexes. The authors need to clarify whether they view these situations as analogous and whether they are thought to activate the same checkpoint.

Additional Correspondence 03 September 2009

Thank you for your interest in our study and your frank assessment of the reviewers' comments.

We are grateful for the reviewers' supportive comments and constructive criticisms. As pointed out in your response, the main concern raised by the reviewers, and in particular reviewer #1, relates to how broadly applicable our findings are to other human cell types and whether RNAi off-target effects can be excluded.

In anticipation of this criticism, we have already expanded our study to other epithelial and mesenchymal cells (primary and immortalized) derived from human breast and lung. Importantly, this work has confirmed our findings for IMR90 fibroblasts and thus has revealed the highly conserved nature of the checkpoint circuitry that we discuss in the present manuscript. These data which had not been generated at the time the manuscript was submitted can be made accessible to yourself and the reviewers immediately. Moreover, we have additional evidence which argues against RNAi off-target effects and are in a position to address most of the more specific points made by reviewer #2 and #3 with additional data already generated.

I would welcome the opportunity to discuss these new data which support and augment the significance of our findings over the phone. Perhaps you could suggest a convenient date/time for a conference call.

I look forward to your reply.

Additional Correspondence 03 September 2009

Thank you very much for your query. I do understand that you have in the meantime generated data that would address the critical concerns that were raised during peer-review. As I did explicitly offer submission of your work under such circumstances, and to speed-up the entire process, I would recommend you provide as with such a modified version (as new submission) that I would send for in depth-review to ref's #2 and #3.

Yours sincerely,

Editor
The EMBO Journal

Resubmission 29 March 2010

Main Points:

- *The main concern expressed by all three referees is related to the use of a single Cdc7-specific siRNA (possibility of RNAi off-target effects).*

  In the new submission we have included specificity controls with an alternative target specific siRNA (oligo CDC7-B; Supplementary Table 1, Supplementary Figure 1B and 1C). Oligo CDC7-B (i) showed comparable gene silencing efficacy (mRNA and protein reduction), (ii) induced similar phenotypic effects (accumulation of cells with G1 DNA content) and (iii) triggered the same molecular changes in the identified checkpoint pathways as CDC7 knock-down with oligo CDC7-A (used throughout the study). These specificity controls are shown in Supplementary Figure 2 and are described on pp 6 and 14.

- *Referee #3 pointed out that the control-siRNA appears to have an impact on the flow cytometry profile (Figure 1 of the original manuscript) and requested quantitative data (% cells in various stages) to allow the reader to evaluate this effect. The referee recommended enhancing the impact of the knock-down results through complementary approaches (Cdc7 over-expression to overcome the cell cycle arrest).*

  For the rescue experiments suggested by the referee, we inserted the full 1725 bp CDC7 cDNA sequence containing four silent, single base pair mutations in the 21 bp CDC7-siRNA (oligo A) interaction region into the pCMV6-AC expression vector (OriGene). Rescue experiments were performed in which the siRNA effect was abolished through expression of the CDC7 gene variant refractory to silencing by oligo CDC7-A. Under these conditions, the molecular changes in the Cdc7-depletion induced checkpoint pathways were reversed and IMR90 cells were able to recover from the cell cycle arrest as demonstrated by flow cytometry and BrdU-incorporation data. This additional specificity control is shown in Supplementary Figure 3 and the experimental results are described on pp 6 and 14. As requested by the referee, we have also added quantitative data (% cells in various stages) to all flow cytometry profiles shown in the manuscript.

- *Referees #2 and #3 requested additional experiments to support the claim that the checkpoint pathways are manifested by perturbed replication initiation rather than through loss of other functions recently suggested for Cdc7 (e.g. ATR signalling, cohesin loading). Referee #2 also asked for newly proposed functions of Cdc7 to be mentioned in the introduction.*

  To support our claim that the checkpoint pathways are activated by perturbed replication initiation, we followed referee #2's suggestion to test whether these pathways are similarly activated by inhibition of other replication initiation proteins. The cellular response to Cdc7 depletion was compared with that caused by RNAi against ORC2, an origin licensing factor that acts upstream of Cdc7 in the DNA replication initiation pathway. As predicted by the referee, ORC2 knockdown (i) led to an accumulation of cells with G1 DNA content, (ii) triggered the similar molecular changes in the identified checkpoint pathways as CDC7 knock-down, and (iii) resulted in downregulation of cyclin D1 and loss of CDK activity. We conclude that at least partially overlapping checkpoint pathways are activated by targeting either CDC7 or ORC2, reinforcing our claim that the Cdc7-depletion induced checkpoint is triggered by perturbed replication initiation. These data are shown in Supplementary Figure 13, the experimental results are described on pp14-15, and the implications discussed on pp20-21. As requested by referee #2 the newly proposed functions of Cdc7 are mentioned in the introduction (page 3).

- *Referee #3 asked us to discuss/clarify (a) the concepts of an origin activation checkpoint vs. an origin threshold checkpoint (i.e. a minimal number of origins required for cell cycle progression) and (b) whether these situations are thought to activate the same checkpoint.*

  Several different conditions (commonly separated into origin licensing and origin activation) must be met during G1 to ensure initiation of DNA synthesis. As pointed out by the referee, there is the possibility of multiple checkpoints, each detecting one of these conditions, or, alternatively, that a range of abnormalities may cause a single condition to be detected by just one checkpoint. Several abnormalities in the replication initiation

pathways are known to cause a G1 arrest phenotype (e.g. Machida et al. 2005, Shreeram et al. 2002, Montagnoli et al. (2004)) and were discussed in the original submission in the context of our new findings which address the consequences of preventing replication initiation (origin activation checkpoint). We agree with the referee that in the original submission we did not differentiate clearly between the concepts of an origin activation checkpoint vs an origin threshold checkpoint (reduction in the number of origin initiation complexes). Throughout the new submission we have aimed to clearly differentiate between these two concepts and have stressed that our studies address the consequences of perturbed replication initiation. With regard to the second question, in the course of additional experimental work for this new submission we discovered that the cellular effects of ORC2 knock-down are remarkably similar to those caused by CDC7 knock-down (Supplementary Figure 13, pp14-15). These new data suggest that at least partially overlapping checkpoint pathways are governing origin licensing and firing. We have discussed this new finding on pp20-21.

- *Referee #3 expressed some concern about the time points at which most of the analyses take place. The referee refers to the long half-life of the CDC7 message/protein (Cdc7 depletion takes at least 48 hours) and points out that the checkpoint must be more responsive to the length of a typical cell cycle. The use of small molecule Cdc7 inhibitors to block replication initiation is recommended to study immediate events involved in checkpoint activation.*

  We agree with the referee that due to the experimental limitations of transient RNAi gene silencing (half-life of the CDC7 message and/or protein) our study has been restricted to elucidating the transducer and effector mechanisms of the origin activation checkpoint. To overcome these experimental constraints and to investigate how perturbed replication initiation is sensed and signalled to transducer proteins (i.e. the molecular mechanisms upstream of FoxO3a nuclear accumulation), the referee makes the sensible suggestion to take advantage of emerging small molecule Cdc7 inhibitors. We considered the use of Cdc7 inhibitors at an early stage of the project, but were discouraged by off-target effects on Cdk9 and RNA polymerase II phosphorylation reported for the first in class group of Nerviano Cdc7 kinase inhibitors (lead compound PHA-767491; Montagnoli et al. 2008). More recently we have experienced major problems with off-target effects from early stage Cdc7 inhibitors at first hand in cell-based studies that we conducted for a Cdc7 drug discovery programme run by the technology transfer arm of our funding body (CRT Discovery Laboratories; see http://www.cancertechnology.co.uk/opportunities/companies_licensing_small.html#CDC7) . Since Bristol Meyer Squibb, Roche, Novartis, Pfizer and Sanofi-Aventis all have compounds in development, it is possible that more specific Cdc7 inhibitors will emerge in the future. However, few published data are available on the preclinical development of these molecules or indeed their structures. Thus although several lead compounds are being optimised for Cdc7 inhibition, at present there are no specific Cdc7 inhibitors available to study the immediate events involved in checkpoint activation. To address the referee's comment, in the new submission we have discussed the limitations of our RNAi-based experimental approach and addressed the current status of Cdc7 as a drug development target (pp16-17).

- *Referee #3 is concerned that the late time points (after Cdc7 depletion) at which analyses are undertaken raise the potential for indirect effects peripheral to the checkpoint. The referee specifically asked for the G2/M fraction that remains after CDC7 knock-down to be addressed. The use of Cdc7 inhibitors to block Cdc7 function in synchronized cells is recommended to address this issue.*

  The referee suggested depleting Cdc7 in synchronised cells (e.g. through release from early S phase arrest) to better determine the degree to which CDC7 knock-down is causing G1 arrest and to address the G2/M fraction (~10% of cells) that remains after Cdc7 downregulation. To address the referee's concern, we opted for double treatment of thymidine (which, in excess, is an inhibitor of DNA synthesis) as a method for synchronizing cells at the G1/S border. Without access to specific Cdc7 inhibitors (see point above), we decided to transfect cells with CDC7-siRNA directly upon release from the second thymidine block, accepting that, as acknowledged by the referee, "…such an approach can be difficult using siRNA,..". Flow cytometry was performed 48 hours post-

transfection. Figure 1E shows a tight synchrony achieved with the double treatment of thymidine (96% G1 fraction, 3% S fraction, 1% G2/M fraction). In synchronized Cdc7-depleted cells the G1 fraction was 90%, while the small G2/M peak (9%) noted for Cdc7-depleted asynchronous cells was lost, with remaining cells equally distributed in the S and G2/M fractions (5% each). Synchronized control-siRNA transfected cells, on the contrary, showed a flow cytometry profile similar to untreated asynchronous cells. Thus CDC7 knock-down is nearly as potent as double thymidine

treatment in inducing cell cycle arrest. In the new submission these data are included in Figure 1E and the experimental results are described on pp5-6.

- *Referees #2 and #3 questioned the specificity of the Dkk3 antibody used in the study. The referees requested an explanation of the additional bands detected with the antibody (Figure 3E in the original manuscript). They also asked why these bands (appearing as one prominent band) disappear under Cdc7/p53 knock-down conditions.*

  A previous study has demonstrated that Dkk3 protein is heavily N-glycosylated in human cells (Hsieh et al. 2004). Since preincubation with recombinant Dkk3 blocking peptide (1:1 w/w) abolished detection of the different bands recognized by the Dkk3 antibody, we reasoned that Dkk3 might be present in different glycosylated isoforms in our cell model. Nglycanase digestion of cytoplasmatic protein fractions prepared from CO and Cdc7KD cells showed that the digestion product has a decreased molecular weight in comparison to undigested extracts, indicating that the reduction in molecular weight is due to removal of N-linked oligosaccharides from the Dkk3 polypeptide backbone. Reducing the total amount of protein digested from 125 _g to 50 _g resulted in complete digestion and disappearance of the higher molecular weight bands as well as the faster migrating, Cdc7-depletion inducible Dkk3 isoform detected with the Dkk3 antibody. The loss of the prominent (heavily glycosylated) band(s) under Cdc7/p53 knock-down conditions (in addition to a significant decrease in the faster migrating, inducible Dkk3 isoform; Supplementary Figure 6G, formerly Figure 3E) can be explained by the late time point (84 hours post-transfection). At later time points (e.g. at 96 hours post-transfection) DKK3 knock-down in the Cdc7-depleted background abolished immunodetection of all Dkk3 isoforms, reinforcing the specificity of the Dkk3 antibody. These data demonstrate that the multiple bands detected with the Dkk3 antibody represent N-glycosylated Dkk3 isoforms. In the new submission these data are included in Supplementary Figure 10 and the experimental results are described on p10 and in the figure legend to Supplementary Figure 10. For greater clarity, we refer to the Cdc7depletion inducible Dkk3 isoform as the "inducible, faster migrating Dkk3 isoform" throughout the manuscript. We have also revised the labelling of Dkk3 bands in Figure 3C and in Supplementary Figures 2, 3, 6 and 13.

Minor Points:

- *Referee #2 asked for the figure layout to be improved. Specifically, the referee asked for a better separation of different panels in figures.*

  To help the reader, in the new submission different panels in figures are independently labelled.

- *Referee #2 pointed out that Figure 2B (in vitro kinase assay) has been constructed from different films and thus results cannot be combined in single rows – separation should be marked. The referee also questioned the use of different loading controls.*

  Lanes 1-8 shown in Figure 2B were run on the same polyacrylamide gel and proteins transferred to the same PVDF membrane by semi-dry electroblotting. The membrane was subsequently cut for optimized immunodetection of the indicated proteins. As requested by the referee, in the new submission the separation of different films is clearly marked in the figure and an explanatory sentence has been inserted in the figure legend (p36). The different loading controls (Rb and _-actin) are appropriate for the experimental design of the in vitro kinase assay. Lanes 1-4 are immunoblots of the eluate from the in vitro kinase reaction; the appropriate control is therefore recombinant Rb (the substrate). Lanes 5-8 are immunoblots of WCE prepared from different cell populations, hence the use of _-actin as loading control.

- *Referee #2 noted that the cis-platinum control for p53 Ser-15 phosphorylation (Figure 2D in the original manuscript) comes from a different blot and thus results cannot be compared with other western blot data shown in this panel.*

  To address the referee's criticism, the experiment has been repeated so that immunodetection of Cdc7, p53 and phosphorylation of p53 at Ser-15 in WCE prepared from untreated, controlsiRNA and CDC7-siRNA transfected cells and from cells treated with cisplatin can be directly compared. Phosphorylation of p53 at Ser-15 was not detected in Cdc7-depleted or control cells, confirming our earlier observation that the ATM/ATR checkpoint pathways were not activated. In the new submission these data are shown in Figure 2E, and the experimental results are described on p8 and discussed on p16.

- *Referee #2 noticed some inconsistencies in the extent of Mcm2 decrease during CDC7 knock-down shown in different panels (Figures 1A, 2A and 2C in the original manuscript) and asked for clarification.*

  Mcm2 total protein levels (hypo- and hyper-phosphorylated isoforms) consistently dropped in Cdc7-depleted cells. The extent of Mcm2 reduction, however, varied between experiments. To address the referees' comment, we performed Image J densitometry analysis to measure the intensities of Mcm2 bands in different experiments and calculated the average reduction in the intensity of Mcm2 protein bands relative to control-siRNA transfected cells. The following statement has been inserted on p7 of the new submission "The average reduction in the intensity of Mcm2 protein bands (relative to control-siRNA transfected cells) was 34% at 48 hours, 55% at 96 hours and 66% at 120 hours post-transfection (Image J densitometry analysis)".

- *Referee #2 pointed out that Mcm2 Ser-40/41 phosphorylation is Cdc7 dependent. In this context the referee questioned the interpretation of the Mcm2 Ser-40/41 phosphorylation signal seen in the double CDC7/p53 knock-down (Figure 3B in the original manuscript) as recovery of CDK activity and suggested recovery of low Cdc7 levels as an alternative explanation.*

  Montagnoli et al. (2006) showed by MALDI-MS that pre-phosphorylation of Mcm2 peptide 36–44 on Ser-41 by Cdk2 increases the efficiency of Cdc7 phosphorylation on Ser-40. The same authors report that Cdc7 activity was increased 3-fold in vitro when the phosphorylated pSer-41 peptide was used as substrate. They conclude that that the negatively charged residues may facilitate phosphorylation by Cdc7, i.e. there appears to be some cooperation between Cdk2 and Cdc7 at this phosphosite. The Mcm2 Ser-40/41 phosphospecific antibody used in our study detects phosphorylation at either of the two sites.

  In the western blots shown in Figure 3B of the original submission no signal is detected with this antibody in Cdc7-depleted cells, whereas a weak signal is detected upon co-depletion of Cdc7 and p53. Since Cdc7 is undetectable in doubly-depleted Cdc7/p53 cells, we interpreted the weak signal as recovery of CDK activity under conditions where the checkpoint itself is abrogated. However, we agree with referee that the use of this particular antibody which cannot differentiate between Cdc7- and Cdk2-dependent phosphorylation at this site is not ideal to support this claim. To address the referee's comment we have repeated the experiments using phosphospecific antibodies to Mcm2 pSer53 (a mapped Cdc7-specific phosphosite) and to Mcm2 pSer27 (a Cdk2-specific phosphosite). The results show that whereas Mcm2 phosphorylation at Ser-27 was abolished in Cdc7-depleted cells, phosphorylation at this mapped Cdk2-phosphosite was detectable in doubly-depleted Cdc7/p53 cells. On the contrary, Mcm2-phosphorylation at the mapped Cdc7-phosphosite was strongly reduced in both Cdc7- and Cdc7/p53 depleted cells. Thus these data confirm our claim that S-phase promoting CDK activity is restored in doubly-depleted Cdc7/p53 cells. In the new submission these data are shown in Supplementary Figure 9 and the experimental results are described on p10.

- *Referee #2 expressed concern over some PARP cleavage and active caspase 3 in the control/p53 double transfection (Figure 3C in the original submission). The referee asked for quantification of the difference to Cdc7/p53 doubly-depleted cells in support of the claim that induction of apoptosis is due to failure of the Cdc7-depletion induced checkpoint.*

As requested by the referee, we performed Image J densitometry analysis to measure the intensities of the p85 (PARP-1) and p17 (caspase 3) cleavage products in control/p53 and Cdc7/p53 double-transfections. While we agree with the referee that there is some PARP cleavage and active caspase 3 in control/p53 double transfections, the intensities of the bands for the p85 and p17 cleavage products were 3-fold and 7-fold lower compared to doubly-depleted Cdc7/p53 cells. These data support the claim that induction of apoptosis is due to abrogation of the origin activation checkpoint. To address this issue the following statement has been inserted in the legend to Supplementary Figure 6 (p7 in Supplementary Material section) "Note that while some PARP cleavage and caspase 3 activation were also evident in control- plus p53-siRNA double transfections, the intensities of the bands for the p85 and p17 cleavage products were 3-fold and 7-fold lower compared to doubly-depleted Cdc7/p53 cells (Image J densitometry analysis)".

- *Referee #2 questioned the interpretation that p53 stabilization in Cdc7-depleted cells is due to Mdm2 proteolysis (Figure 3A in the original manuscript) and asked us to show that Mdm2 loss is not caused by transcriptional downregulation due to off-target effects.*

  Mdm2 transcript levels increased two-fold in Cdc7-depleted cells relative to control-transfected cells 72 hours post-transfection (most likely due to p53 stabilization) and were comparable at later time points, arguing against transcriptional downregulation of Mdm2 due to siRNA off-target effects. In the new submission these data are included in Supplementary Figure 7 and the experimental results are described on p10.

- *Referee #2 recommended referring to ASK (Activator for S phase kinase in H. sapiens) as Dbf4.*

  As requested by the referee, in the new submission references to ASK have been replaced with "Dbf4"

- *Referees #2 and #3 recommended shortening of the manuscript by reducing the data on effects of blocked replication initiation on downstream events and data on the p53 dependency which are similar to previously published work.*

  Following the referees' suggestions we have moved the data on p53 dependency (Figure 3 in the original manuscript) to the supplementary section (new Supplementary Figure 6), which has reduced the total number of figures from 7 to 6. We have also significantly shortened the section discussing p53 dependency of the checkpoint. The new data on RNAi specificity controls, ORC2 knock-down, loss of CDK activity under conditions of checkpoint abrogation, and Mdm2 transcript levels have been inserted into the manuscript as supplementary figures to avoid increasing the length of the paper.

In following the referees' helpful and constructive comments, we have confirmed and significantly expanded the dataset included in the original manuscript. We trust that the new submission should now be acceptable for publication in the *EMBO Journal* and would be grateful if you were to support further consideration of our paper at your journal.

2nd Editorial Decision 12 April 2010

Thank you very much for submitting an improved version of your original research manuscript (EMBOJ-2009-71998) for consideration to The EMBO Journal editorial office.
The paper has been re-reviewed by two of the original and one so far unbiased scientist. As you can see, both of the original referees appreciated the amount of new data that went into the paper and ref#3 raises only one minor concern related to data-quantification. However, the novel ref#2, although also essentially in favor of your study, does request additional experimentation that should increase confidence in the reported relationships, particularly with respect to Dkk3 and FoxOA3. Given that the paper is editorially considered as a new submission rather than a sole revision, I kindly ask you to take the essential points of this referee into account. Specifically, this should include controls to rule out cell cycle position effects, stronger support for the truly novel link to Dkk3 and FoxOA3 and essential controls on single versus double depletion effects as indicated in the referee report. Conditioned on such further improvements, we would be happy to re-assess a revised version of your study in the near future. Finally, I have to remind you that the final decision

on acceptance or rejection would depend on the content and strength of the final version of your manuscript!

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors have done a good job addressing the major concerns expressed in previous reviews. As required by the referees, an alternative target siRNA has been examined and exhibits qualitatively similar effects. These experiments provide greater confidence in the conclusions, which are central to the paper. Quantitative data have been added to the flow analysis and the requested rescue experiments performed. The utilization of synchronized cells to evaluate CDC7 knockdown effects further supports the authors' interpretation of the data. In addition to addressing these major concerns, the authors have also responded to and corrected a substantial number of minor points, which taken together significantly improve the manuscript. Given the extensive revisions that have been undertaken, their significant contributions to the overall conclusions, and the novel contributions of this work to understanding the origin activation checkpoint, it is my opinion that the manuscript is now acceptable for publication in the EMBO Journal.

Referee #2 (Remarks to the Author):

In this study, the authors have explored the molecular phenotypes of IMR90 fibroblasts depleted of Cdc7 in an effort to establish the existence of an "origin firing checkpoint." If such a checkpoint operates in normal cells, then loss of Cdc7 should provoke a global response that suppresses cell cycle progression independently of the physical inability to replicate DNA. Cdc7 depleted cells stop proliferating with an apparent G1 DNA content, low Cdk4 and Cdk2 activity, and low expression of many E2F-regulated gene products. In support of a true checkpoint, cells depleted of Cdc7 have elevated levels of cell cycle inhibitors that are not direct participants in the process of DNA replication. Co-depletion of these inhibitors allows more replication to proceed than would otherwise take place in the Cdc7 knockdown alone. The involvement of p53 and Cdk inhibitors in delaying S phase entry in normal cells depleted of origin binding proteins is consistent with several prior publications. For the most part, the data are of high quality with the only criticism being that some of the protein changes are quite modest.

The most surprising and novel findings are the changes in Wnt signaling components, Dkk3 and FoxOA3. While this pathway is clearly linked to cell proliferation and survival, an intimate connection to DNA replication control has not been described before. This finding is intriguing, but has not been investigated sufficiently to have confidence that the relationship is as close as the authors interpret. The title and abstract imply the achievement of a much greater mechanistic understanding than can be supported by the data thus far. An advance beyond the descriptive nature of these findings is needed for publication in a broadly-read journal.

Major points

1. The interpretations of the results presented are limited due to the experimental approach-siRNA transfected into asynchronously growing cells for several days. Because the Cdc7 protein is long-lived, cells 'arrest' in G1 only after at least 2 days in the siRNA. It is thus difficult to determine which of the phenotypes are direct vs indirect. Nearly all of the proteins the authors observe upregulated in the Cdc7 knockdowns have been shown to be upregulated in G1 cells normally. Thus, some (and possibly all) of the changes in protein or mRNA levels could be indirect

consequences of cell cycle position rather than the cause of the arrest as the authors interpret. All of the experiments compare control knockdown to Cdc7 knockdown but not Cdc7 knockdown to control cells that are naturally in G1. Since the control cells are a combination of all cell cycle phases, but the Cdc7 knockdowns are not, how does one know which changes are part of the checkpoint and which are due to cell cycle position? Would Dkk3 and FoxOA3 levels be high in cell responding to other checkpoints that arrest in a similar phase, or are these responses truly specific to origin firing?

The authors should work to establish a more direct relationship between the knockdown and the phenotype using alternative or modified experimental approaches. The authors argue against the use of Cdc7 inhibitors (suggested by prior reviewers) on the basis of poor specificity, but if the drugs inhibit Cdc7 acutely in synchronized cells, then Wnt phenotypes should be induced. The synchronization in Figure 1E might be adapted to explore the more novel phenotypes directly. With regard to 1E specifically, is the 48 hour time point after the double thymidine release the first S phase in the control cells - seems unlikely, but that first S phase is the time that should have been examined.

2. The question of a checkpoint is particularly confounding in the double depletion experiments which typically lack a critical control showing the single knockdowns of p53, p15, Dkk3, or FoxO3a (the exception being Figure 4A). These single knockdowns could cause strong S phase stimulation on their own. Indeed, such effects have been reported in the literature, and one wonders if combining an S phase inhibitory effect (Cdc7 knockdown) with *any* S phase stimulation (e.g. p53 knockdown) would have the additive effects observed by the authors. As an example, FoxOA3 depletion alone reduced p15 expression relative to the control by what appears to be the same amount as the double depletion is reduced compared to the Cdc7 single knockdown. Could Dkk3 or FoxOA3 depletion over-ride unrelated checkpoints causing indiscriminate S phase entry independently of events at origins? The authors can provide greater confidence that these Wnt pathway changes are specific to replication origin events with additional controls and pathway specificity tests.

3. The major novel findings - particularly related to the Wnt pathway - need to be reproduced at least one other cell line.

4. The manuscript as presented is very frustrating to read. The convention of numbering lanes on autorads and then demanding that the reader look for identities of these lanes in the figure legend just forces the reader to relabel the figures on his/her own. "1,2,3..." mean different things in different panels throughout the paper. Also, the data are not presented in the order in which they are described, leading to much back and forth. These issues are easily addressed in a revision taking care to provide sufficient information in the legends but most especially, to label the figures appropriately.

Minor Points.
1. Could the activation of p15 and ARF be due to the origin in the INK4 locus (Gonzalez et al.)? If so, what implications does that have for the checkpoint model?

2. The results and discussion do not clearly take into account the difference between failure to activate any origins and the actual situation produced by siRNA in which a limited amount of Cdc7 is available, and small number of origins could be activated. RNAi should not be interpreted as the same as a complete loss.

3. The chromatin fractionation assay is not referenced/described - methods refers to a nucleolar fractionation assay.

4. PI staining for S phase content is heavily dependent on very careful gating - 2-dimensional BrdU + PI would have been more sensitive and quantitative

5. Figure 2B - what is "eluate"? The assay referenced in methods does not involve an elution step - unless SDS-PAGE sample buffer is what they mean? The Rb substrate was never "bound" so it isn't eluted. Rename please to avoid confusion.

Referee #3 (Remarks to the Author):

In this manuscript - a resubmission of previously submitted work-Tudzarova and coworkers describe the molecular mechanisms relevant to protect immortalized IMR90 fibroblasts from cell death caused by Cdc7 depletion by siRNA.
Cdc7 depletion was previously suggested to selectively induce apoptosis in cancer cells while it was shown to cause a G1 arrest in primary dermal fibroblasts and this was mediated by p53.
The authors confirm this observation in their cellular model and substantially expand our understanding of how cell cycle arrest and survival are mediated. With a combination of a candidate approach and gene expression profiling they identify Foxo3a, p15 p14 and Dkk3 as important cellular factors that protect human cells from Cdc7 inhibition. As mutations in these important tumor suppressor genes frequently occur in human cancers, this work further supports the notion that Cdc7 inhibition can be an important target for therapy and lays the basis for the molecular understanding of why.

These findings are novel and of importance to both a general and specialized scientific community as the signaling pathways underlying cellular responses to Cdc7 inhibition are to date obscure but have both important biological and clinical consequences.

The authors have reasonably addressed the main criticism that was raised by referees in the first submission related to possible off target effects of siRNA approach by repeating relevant experiments with a second specific Cdc7 siRNA.
They have included an ORC2 siRNA depletion experiment that suggests that molecular event triggered by Cdc7 inhibition are overlapping with the ones caused by depletion of a different protein also involved in DNA replication initiation.
Authors have satisfactory answered most of the other questions raised by reviewers.

As minor criticisms,
1. I still see an inconsistency that was present in the first submission and now is clearly observed when comparing text and figure 2A: reduction of MCM2 levels upon Cdc7 depletion is claimed to be reduced in average of 34% at 48h and 55% at 72h, but simply looking at the figure the effects seems much more marked (to my eye at least 90% reduction at 48h).
2. In Fig 2B it is not clear if the eluate used in the first four lanes was obtained from a CycE or CycA IP. Please clarify this in the labelling or in the figure legend.

1st Revision - Authors' Response 10 July 2010

Thank you for your offer to reassess a revised version of our study “MOLECULAR ARCHITECTURE OF THE DNA REPLICATION ORIGIN ACTIVATION CHECKPOINT”. We are grateful for the supportive and helpful comments raised by your expert referees. As requested in your decision letter, we have addressed the major points made by the novel referee #2, which specifically includes controls to rule out cell cycle position effects, stronger support for the truly novel link to Dkk3 and FoxO3a and essential controls on single versus double depletion effects. In addition to addressing these major points we have also responded to and corrected the few remaining minor points raised by referees #2 and #3, which, taken together, has significantly expanded the study findings and improved the manuscript.

Main Points:

- *The novel referee #2 is concerned about cell cycle position effects, specifically “…, some (and possibly all) of the changes in protein or mRNA levels could be indirect consequences of cell cycle position rather than the cause of the arrest as the authors interpret.” The referee suggested alternative or modified experimental approaches to exclude the possibility of cell cycle position effects:*

- *Cdc7 knockdown should be compared to control cells that are naturally in G1 to determine which changes are part of the checkpoint and which are due to cell cycle position. "The synchronisation in Figure 1E might be adapted … With regard to 1E specifically, is the 48 hour time point after the double thymidine release the first S phase in the control cells – seems unlikely, but that first S phase is the time that should have been examined."*

  To exclude the possibility of cell cycle position effects, we have taken two different experimental approaches to prepare control knock-down cells that are in G1 phase and to compare these cells to Cdc7 depleted cells. Firstly, we followed the referee's suggestion to adapt the synchronisation protocol that is the basis for the data shown in Figure 1E. IMR90 cells were released from double thymidine block and transfected with control-siRNA. Cell cycle progression after the release was monitored by flow cytometry. BrdU was added to the medium at the point when synchronized cells were in early G1 phase (between 19 and 21 hours after the release) and the time of first entry into the subsequent S phase (between 25 and 27 hours) was determined through detection of BrdU incorporation. Whole cell extracts were prepared from control knock-down cells at the G1-S boundary (25 hours after release from DTB) and Cdc7KD cells, and protein expression of checkpoint components was studied by immunoblotting.

  The second approach involved the preparation of G1 cell fractions from control knock-down and Cdc7KD cells by one-way sorting of propidium iodide stained cells with a DAKO/Beckman Coulter MoFlo High Speed Sorter. Flow cytometry profiles pre- and post-sorting were generated to control for the purity of the G1 cell populations, and the changes in checkpoint protein levels were again analyzed by western blotting of whole cell extracts prepared from the two different G1 cell fractions. Both experimental approaches showed that the increased FoxO3a levels, inducible Dkk3 expression, and increased protein levels of ARF, p53 and the CDK inhibitors p15, p21 and p27 in Cdc7-depleted cells are not an indirect consequence of cell cycle position. These essential control experiments are shown in Supplementary Figure 14 and are described on p14.

- *o Would Dkk3 and FoxOA3 levels be high in cells responding to other checkpoints that arrest in a similar phase, or are these responses truly specific to origin firing?*

  For the pathway specificity tests suggested by the referee, we carefully considered the available options for inducing G1 arrest in our experimental system. To address the referee's question, we decided on two separate experimental approaches.

  Firstly, we sought to specifically activate the p53 pathway in IMR90 cells to determine whether p53- dependent inducible Dkk3 expression in response to CDC7 knock-down is closely associated with origin firing or, alternatively, part of a common p53-induced cell cycle arrest pathway. For these experiments, we used low dose (1 nM) treatment with actinomycin D, which has recently been shown by David Lane's group to mimic the HDM2 inhibitor nutlin-3 in the highly specific activation of p53- dependent transcription and induction of a reversible cell cycle arrest in normal cells (Choong et al. Cell Cycle 2009; 8:17, 2810-2818). Notably, ActD's more commonly known DNA damaging and RNA synthesis inhibition effects are generated only at a much higher dosage (100-200 nM). The experiments, which are displayed in Supplementary Figure 11 and described on p11, showed that whilst p53 protein was stabilized and p21 levels raised in both Cdc7KD and ActD-treated cells, the inducible, faster migrating Dkk3 isoform and a marked reduction in nuclear b-catenin and cyclin D1 levels were only detectable in Cdc7-depleted cells.

  Secondly, we sought to determine whether the cellular responses elicited by FoxO3a in response to CDC7 knock-down are specific to origin firing or are overlapping with a common FoxO-induced cell cycle arrest pathway. Since reactive oxygen species are amongst the well known stress stimuli that trigger FoxO-induced cell cycle arrest, we compared the expression dynamics of checkpoint components in Cdc7-depleted and oxidatively stressed IMR90 cells. These experiments, which are displayed in Supplementary Figure 15 and described on p13, showed that of the FoxO3a target genes studied, ARF protein expression was markedly increased only in Cdc7-depleted

cells, whereas p15 and p27 levels were raised in cells arrested by either oxidative stress or CDC7 knock-down. Moreover the inducible, faster migrating Dkk3 isoform was also only detectable in Cdc7-depleted cells.

In the revised manuscript we conclude on p20 that the pathway specificity tests in fibroblasts arrested by oxidative stress or through specific activation of the p53 pathway by low dose actinomycin D show that while p15 and p27 upregulation in Cdc7-depleted cells overlap with the common FoxO-induced cell cycle arrest pathway, inducible ARF and Dkk3 expression are more specific events associated with DNA replication control.

- *Referee #2 is concerned that "single knockdowns of p53, p15, Dkk3, or FoxO3a …. could cause strong S phase stimulation on their own." The referee raised the question whether "… Dkk3 or FoxOA3 depletion [could] over-ride unrelated checkpoints causing indiscriminate S phase entry independently of events at origins?" and requested additional controls on single versus double depletion effects to be included in the manuscript.*

  In the revised manuscript we have included the requested essential controls on single versus double depletion effects. IMR90 cells were first transfected with control-siRNA and after 72 hours replated at low density and transfected with either control-siRNA (CO), control- and p53-siRNAs (p53KD), or control and DKK3 (Dkk3KD) or FOXO3A (FoxO3aKD) or CDKN2B (p15KD) oligos. Flow cytometry profiles and cell cycle phase distribution data demonstrate that single depletions of the studied checkpoint components p53, Dkk3, FoxO3a or p15 do not cause strong S phase stimulation on their own. This result is further supported by western blot data which show no significant increase in protein levels of the S/G2 phase markers cyclin A and geminin. Caspase 3 activation and PARP-1 cleavage were also not detected, indicating that single depletions of these checkpoint components do not cause apoptosis in this experimental system. These control experiments are shown in Supplementary Figure 16 and are discussed on pp14-15 and p20.

- *Referee #2 asked for the major novel findings to be reproduced in at least one other cell line.*

  As requested by the referee, the main study findings were reproduced in a different fibroblast strain. Consistent with Cdc7-depleted IMR90 cells, CDC7 knock-down in WI-38 cells resulted in FoxO3a nuclear accumulation, increased ARF and p53 protein levels, the appearance of the inducible, faster migrating Dkk3 isoform, and increased levels of the CDK inhibitors p15, p21 and p27. Low CDK activity in the Cdc7- depleted WI-38 cells was confirmed by the significant reduction in Mcm2 phosphorylation at the CDK phosphosite Ser-27. These data, which are shown in Supplementary Figure 17 and described on p15, show that the cell cycle arrest phenotype first discovered in IMR90 fibroblasts is fully reproducible in WI-38 cells.

- *Referee #2 recommended relabeling of the figures, in particular the lanes on autoradiographs, and providing sufficient information in the legends.*

  As requested by the referee, in the revised manuscript we have replaced the convention of numbering lanes on autoradiographs with new figure labels that no longer require the reader to look for identities of lanes in the figure legend. The figure legends have been revised in line with the changes made to the figure labels.

Minor Points:

- *Referee #2 questioned whether "the activation of p15 and ARF [could] be due to the origin in the INK4 locus (Gonzalez et al.)?" and what implications this might have for the checkpoint model.*

  The referee raises an interesting question which also touches upon the unconfirmed 'canary origin' theory that was to our knowledge originally formulated by Julian Blow. According to this theory, some replication origins might be positioned at or close to the promoter regions of S-phase inducing genes or genes encoding inhibitors of cell cycle progression. Assembly of pre-replication complexes at these origins is thought to positively or negatively affect the expression of adjacent cell cycle regulatory genes. Inhibition of the DNA replication initiation machinery prevents pre-RC assembly at these special replication origins which then act as 'canaries' (or 'finks'), informing the cell cycle engine to rest. Our results clearly show that p15 and ARF expression in response to Cdc7-depletion is dependent on FoxO3a activation. These findings neither confirm the canary origin theory nor do they disprove it. Thus it is theoretically

possible that canary origins exist in addition to the origin activation checkpoint discovered in this work.

- *Referee #2 pointed out that "the results and discussion do not clearly take into account the difference between failure to activate any origins and the actual situation produced by siRNA in which a limited amount of Cdc7 is available, and small number of origins could be activated." The referee cautioned that "RNAi should not be interpreted as the same as a complete loss."*

  The referee is entirely correct. We have further revised the results and discussion sections to avoid confusion over this issue. Specifically, in sentences where the wording could be misinterpreted as failure to activate any origins we have replaced the wording with a reference to impaired origin activation.

- *Referee #2 noted that "the chromatin fractionation assay is not referenced/described - methods refers to a nucleolar fractionation assay."*

  In the revised manuscript the sentence "Nucleoli and chromatin-bound protein fractions (CBF) were isolated as described (Muramatsu & Onishi, 1978; Kingsbury et al, 2005)" has been inserted on p25.

- *Referee #2 asked for the term "eluate" in panel B of Figure 2 to be renamed to avoid confusion.*

  As requested by the referee the term "eluate" has been replaced with "Cdk2 IP".

- *Referee #3 noted an inconsistency between the extent of Mcm2 protein reduction shown in Figure 2A and the average reduction figures stated in the text.*

  We are grateful to the referee for pointing out this inconsistency which has arisen as the result of a computational error in calculating the average reduction figures. The numbers have been recalculated as 45% reduction at 48 hours, 62% at 96 hours and 76% at 120 hours post-transfection and the corresponding text passage on p7 corrected.

- *Referee #3 asked us to clarify in the legend to Figure 2B which antibodies were used for the Cdk2 immunoprecipitation.*

  The figure legend has been revised in line with the referee's request (p37).

In following the referees' helpful and constructive comments, we have confirmed and significantly expanded the dataset included in the original manuscript. We trust that the revised manuscript is now acceptable for publication in the *EMBO Journal*.

3rd Editorial Decision 27 July 2010

I just received the final assessment from one of the original referees that is satisfied with the revisions provided. You will be pleased to learn that on the basis of this you will soon receive the official acceptance letter together with further instructions from our editorial assistance.

Yours sincerely,

Editor
The EMBO Journal

