THE EMBO JOURNAL

Manuscript EMBO-2017-96585

Initiation of DNA replication requires actin dynamics and formin activity

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Review timeline:

Submission date:	23 January 2017
Editorial Decision:	05 April 2017
Revision received:	06 July 2017
Editorial Decision:	09 August 2017
Revision received:	28 August 2017
Accepted:	07 September 2017

Editor: Hartmut Vodermaier

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.)

Additional correspondence (editor) 08 March 2017

Thank you again for submitting your manuscript on nuclear actin/formin and DNA replication for consideration by The EMBO Journal. We have now received the reports from three experts, which I am enclosing copied below. As you will see, the reviewers are very much divided in their opinions, and at least two of them raise a number of substantial experimental concerns, with the potential to confound key conclusions and interpretations of the study. I realize that in particular referee 3's report reads rather harsh, but I should also point out that it comes from a good and trusted referee of ours and that s/he appears to make a number of incisive specific points that we cannot simply dismiss.

Given the previous revision history of this work, we would prefer to avoid another drawn-out process at The EMBO Journal and instead look to make a clear-cut decision at this stage - meaning we would only invite revision if there would seem to be a reasonable chance to address the salient issues and convince the referees. I would therefore welcome your detailed responses to the referees' points at this stage, in the form of a point-by-point outline on how the major issues might be addressed/ clarified; as well as comments on the expected feasibility of such experiments as requested by the reviewers. These tentative response, which we may choose to share and discuss with (some of) the referees and possibly also additional arbitrating advisor(s), would then be taken into account when making our final decision on this manuscript. I would therefore appreciate if you could, following discussions with your co-authors, send us such a response in due time, ideally by early next week. Should you have any further questions in this regard, please do not hesitate to let me know.

REFEREE REPORTS

Referee #1

This paper describes a potential role for actin dynamics in nuclear transport and DNA replication. Although the conclusions are interesting, the following issues, some of which involve major technical concerns, would have to be addressed (listed in the order they come up in the paper).

1. In Figure 3A, there should be a loading control, given that the drugs added might affect nuclear morphology/integrity which could indirectly affect recovery of soluble and insoluble proteins.

2. There is no reference to Figure 3B in the Results section.

3. In EV2C, the control lane showing how much PCNA would normally load in the absence of SMIFH2 is overloaded, as evidenced by the loading control. This undermines the conclusion drawn.

4. Is the effect of SMIFH2 on S phase entry not explained by its inhibition of transcription (Figure 4B)?

5. No reference is made to the dnDiaNLS construct shown in Figure 4D.

6. Why does the R62D mutant decrease EdU incorporation (Figure 4E) but not increase the S phase population (Figure 4D)?

7. Line 169: gamm-H2AX staining provides evidence of DNA damage, but not necessarily dsDNA breaks.

8. Line 174: the authors need to explain what is gelsolin and why is it used.

9. Figures EV3C and D should indicate the drug being added.

10. Line 187: since PCNA is generally considered to mark replication sites, and actin appears to interact with PCNA, it is unclear why the authors conclude that actin is not present at replication sites. Is the actin-PCNA interaction replication-dependent? To address this, the authors could block initiation with a CDK inhibitor and repeat the experiment. As is, the data don't add anything and should be removed.

11. Contrary to the authors conclusion that NUP staining is disorganized (line 199), Figure 5C indicates it is greatly reduced. Also, it appears that a highly non-representative portion of the nucleus in 5C (top right panel) was shown in the inset that is devoid of NUP staining.

12. These papers should be cited and discussed:

J Cell Sci. 2004 May 15;117(Pt 12):2481-90. Epub 2004 May 5.
Actin- and protein-4.1-containing filaments link nuclear pore complexes to subnuclear organelles in Xenopus oocyte nuclei.
Kiseleva E1, Drummond SP, Goldberg MW, Rutherford SA, Allen TD, Wilson KL.

J Cell Sci. 1992 Jan;101 (Pt 1):43-53.
DNA replication in cell-free extracts from Xenopus eggs is prevented by disrupting nuclear envelope function.
Cox LS1.

13. Imp and CD conditions in Figure 7A appear to be overloaded (compare NUP62 blot), undermining the claim that CD promotes importin-NUP interaction. Were the two sections of the blot exposed equally?

14. Figure 7C lacks Ran loading control.

15. Although cytochalasin D and SMIFH2 both inhibit nuclear transport (Figure 6A), they appear to do so by different mechanisms (Figure 7). Some attempt should be made to unify the observations of these two actin modulators.

16. The authors should cite appropriate previous papers showing that WGA inhibits replication in egg extracts.

17. From Figure 8A, the authors conclude that once nuclei have formed, replication can proceed without further NPC formation. However, a critical control is missing, which is to show that in this experiment, NUP depletion was complete (which can be shown by placing sperm chromatin directly in this extract). Otherwise, it may be that the residual replication seen in WGA-bpdelta condition reflects residual de novo NPC formation. Conversely, it may be that the requirement for continued nuclear transport is due to nuclear disruption during the transfer experiment (this caveat affects all the nuclear transfer experiments shown). It would be better to do the experiment without transfer and to simply titrate away the Geminin with recombinant Cdt1.

18. Why does the second condition in Figure 8B not replicate better? Also, the logic underlying this triple transfer experiment is not explained well. Is the purpose of this experiment to address whether SIMULTANEOUS nuclear transport and formin activity are required for replication?

19. The failure of the PAWGA condition to replicate (Figure EV5D) might again be due to nuclear disruption during transfer? Also, why do the authors use this obscure CDK inhibitor? Roscovotine is much more widely used.

20. How do the authors know that in Figure 8C, nuclear transfer does not disrupt the nuclear envelope. If this is the case, all the CDK activity would leak out, imposing a requirement for new nuclear transport in the second extract to re-accumulate CDK activity.

21. Why does SMIFH2 inhibit DNA replication in 9D but barely at all in EV5E? The result in Figure 9E does not explain the total block in replication shown in Figure 9D. There must be a step that is inhibited downstream of Cdc45 loading. The authors should check GINS, RPA, polymerase etc to determine where the block occurs.

22. The discussion was not very informative, as it contained almost no discussion of how actin/formin might regulate replication.

Referee #2

Parisis et al. describe a novel role for actin and actin-assembling formins in DNA replication. The onset for the project is a mass spec study from Xenopus nuclear extracts, which identifies several actin-regulators in replicating nuclei. In their studies, the authors use both Xenopus egg extracts (XEE) and somatic mammalian cells. The XEE system is transcriptionally silent, which allows the authors to uncouple the potential role of actin in gene expression from its role in replication. Moreover, the XEE system permits elegant sequential inhibition experiments, which would not be possible in a whole cell model. The authors show that in mammalian cells, nuclear actin displays cell cycle-dependent dynamics, with actin filament formation at the early G1 phase of the cell cycle. By using actin-binding drugs, actin-regulators and formin-manipulation, the authors then show that nuclear actin dynamics (and formin activity) are required for DNA replication. Their studies further point to a role in both nucleo-cytoplasmic transport, which is critical for DNA replication, as well as in a subsequent steps of chromatin loading of replication factors and initiation of replication. Mechanistically actin seems to interact with Ran GTPase, with implications on cargo-release during nucleo-cytoplasmic transport.

Overall, this study is very interesting and reports a completely novel function for actin dynamics and formin activity in DNA replication. The use of two very different experimental systems supports the generality of the findings, and the XEE system permits very elegant experiments, such as the transfer of nuclei between differentially treated extracts. The experiments are carefully done, and link between actin/formin activity and efficient DNA replication is obvious. Also plausible mechanistic explanation regarding the nucleo-cytoplasmic transport is provided. This study thus broadens the functional implications of nuclear actin and its regulators, and is therefore very important to the field.

The major problem with the manuscript at the moment is that it is very difficult to read and follow,

due to the huge amount of data and juggling the two different experimental systems. I suggest that the authors simplify the figures, by carefully evaluating which experiments are absolutely necessary to prove their point. In addition, the figures should be labeled clearly so that it is immediately evident, which system (XEE or mammalian cells) is used. For example in figure 5, it is impossible to tell from the figure and figure legend, which system is used in each panel without reading the text. The introduction lacks intro to the replication process, which would make the manuscript easier to follow for readers not specialists in this topic.

One confusing fact is that some treatments, especially the formin inhibitor SMIFH2, seem to operate differently in the two systems. In XEE, SMIFH2 does not seem to affect nuclear actin amounts or polymerization, but in mammalian cells it seems to stabilize the nuclear filaments. So in one case, one would be looking at formin-inhibition that is not affecting actin, and in the other case, an indirect effect of stabilizing nuclear actin filaments? What effect does the other formin-inhibitor, 2.4, have on actin in these two systems? The interpretation of the results from SMIFH2-treated mammalian cells is problematic, since SMIFH2 most likely affects also cytoplasmic actin. For example in 5B, SMIFH2 treatment is shown to have an effect on nuclear morphology. Did the cells divide during the experiment?

The finding that actin dynamics plays a role in nucleo-cytoplasmic shuttling by regulating Ran-cargo release is very interesting, but the experiments in mammalian cells are not very convincing. In addition (or instead of) of looking at an endogenous shuttling protein, which can be regulated by cell morphology as pointed out by the authors, the authors should look at an engineered construct (like they use in XEE), such as NLS-GFP-GST-NES. As mentioned above, SMIFH2 likely also affects cytoplasmic actin. If the SMIFH2 functions here by stabilizing nuclear actin filaments, does expression of NLS-G15S/S14C actin mutants also impair nucleo-cytoplasmic shuttling?

The actin filament formation in early G1 is very interesting, but it is not clear how this fits with the rest of the story. In the discussion, the authors speculate that "deregulated nuclear actin dynamics, rather than an increase in nuclear actin levels or filament formation per se, prevents DNA replication". What is actually meant by this? In Serebryannyy et al, 2016, it was speculated that persistent nuclear actin filaments decrease nuclear actin monomer levels, and this causes the transcription defect in their system. Do the authors speculate that something like this is taking place also in their system?

Minor points:

SMIFH2 is abbreviated differently (SF or FH) in different figures.
In EV3C, Mical-2 text below the x-axis is not visible.

Referee #3

The authors of this study look into connections between DNA replication and the actin cytoskeleton. The make the claims that nuclear actin filaments form in G1 of human cells, that stabilisation of nuclear actin filaments blocks nuclear transport and DNA replication, that actin directly binds Ran-importin complexes and is required for cargo release, and that nuclear forming activity is required for DNA replication. Unfortunately, however, these claims are neither plausible nor are they supported by conclusive evidence. Instead, the study is an example of wishful thinking and inadequate experimental methods.

Specific points:

1. Introduction: it is remarkable that the authors introduce the issue of nucleocytoplasmic actin distribution without a single mentioning of exportin 6 (Xpo6), which excludes actin from nuclei in somatic cells. It is also remarkable that the authors did not mention that an unbiased phalloidin stain gives a bright cytoplasmic but no nuclear signal.

The authors also fail to introduce that Xenopus oocytes are special in that they lack Xpo6 and thus do not exclude actin from their nuclei. Xpo6 reaches normal cellular levels not before stage 7 of embryonic development (see figure 4, Bohnsack et al. (2006), doi: 10.1038/ncb1357). It is thus not surprising that nuclei reconstituted from Xenopus egg extract are still compromised in their ability

to exclude actin.

In fact, the lab of Rebecca Heald reported earlier that nuclei assembled in XEE contain nuclear actin (Krauss et al, 2003). Furthermore, Krauss et al. showed that latrunculin A interfered with nuclear assembly and DNA replication. It is unclear why this paper was cited only for a methodological detail.

The authors also fail to mention that a comprehensive study for identifying nuclear actin-binding proteins has been published before (Samwer et al., 2013, doi: 10.1038/emboj.2013.108).

2. Figure 1: The authors intend to identify intranuclear actin-binding proteins by a proteomics analysis of in vitro assembled nuclei. Although it is not detailed in the methods, I guess, the nuclei were simply isolated by centrifugation. Analyzing such pellet is, however, rather problematic: How do the authors distinguish proteins that are enclosed by nuclear envelope (true intra-nuclear proteins) from actin structures that associates from the outside or indeed simply polymerised actin? Without rigorous controls, such analysis is meaningless.

A similar concern applies to Figure 2B; 3A; 4J; EV3F; 7A, B, C, F; EV4A etc. In these experiments, the authors should have thoroughly tested if their fractionation procedures are suited to cleanly (!) discriminate between nuclear and cytoplasmic actin pools.

3. Figure 2A: What are the specificity controls for antibodies and IF-conditions used? Given that the authors suggest the Arp2/3 complex showed nuclear accumulation, while others clearly found that this complex is well excluded from Xenopus nuclei (see e.g. Samwer et al., 2013) I am having serious doubts. Indeed, the website of the manufacturer of the AB (AbCam) shows a clean cytoplasmic staining. The authors apparently miss the specific (cytoplasmic!) signal, because they look only at nuclei.

4. Statement 'We ... show that nuclear actin filaments form in early G1'. This is obviously an artifact of the detection methods. The expression of NLS-fused actin-binders (Lifeact or the anti actin chromobody) will necessarily cause nuclear import of actin and thus severly alter the nucleocytoplasmic distribution of actin.

Such bias can be avoided by first fixing the cells and then staining acti-filaments with fluorescent phalloidin. It has been known for decades that nuclei of undisturbed cells are phalloidin-negative. This applies also to S-phase.Another way of looking at the issue in an unbiased manner would be to expressed the chromobody of LifeAct without the NLS. Both actin-sensors would actually be small enough to enter nuclei by passive diffusion.

5. Statement: 'treatments that stabilize actin filaments abrogate nuclear transport'. This does not make any sense, and from my own experience I can tell that nuclear import works just perfectly fine in the presence of phalloidin (the most potent filament-stabilizing drug).

6. The authors state that staining of fluorescently labelled DNAse I (meant to detect actin) perfectly overlaps with the DNA staining. Are the authors able to exclude that the probe they used actually binds DNA?

7. If the authors want to make the point that nuclear actin filaments would be required for DNA-replication, then they should test this without messing with cytoplasmic actin structures. In fact, this experiment is very simple and requires just the addition of Xpo6 to the nuclear assembly reactions. It the authors' conclusion is correct, then one would expect a complete block of replication at sub-micromolar concentrations. Given however that these are conditions found in somatic cells, I doubt that nuclear exclusion of actin would compromise replication.

8. The authors claim that nuclear formin activity would be required for DNA replication. Which formin is this supposed to be and what is the evidence for such claim? The authors fail to recognize that a requirement cannot be proven by ectopic over-expression.

9. Figure 3: The finding that CytD has the same effect as Jasplakinolide, namely inducing nuclear actin polymerization is somewhat unexpected. My interpretation of the authors' explanation is that

CytD exclusively acts on cytoplasmic actin filaments, giving rise to a high level of monomeric actin that can enter assembled nuclei. What would prevent CytD from entering nuclei? And why do the authors add such a high concentration of the drug (400 micromolar)? Given that Cytochalasins are sparsely soluble in water this seems to be an inappropriate concentration. Why was latrunculin A not active at all, even at 100 µM? I am sorry to say, but such inconsistent pharmacology does not support the authors' conclusions.

10. Figure EV4A: The small differences in band intensities do not support the conclusions given in the main text.

11. Figure 7A: The western blot provided does not support the conclusion that CytD treatment increases binding of importins to FG Nups. The 'CD lane' is similar to the 'Ctrl lane'.

12. Figures 7B, C, F: How specific is the postulated interaction between actin and FG Nups (Figure 7B) or Ran (Figures 7C, 7F) in the presence of CytD? Already the input lanes contain elevated levels of actin. Furthermore, the Mock control for this specific condition is missing in all three experiments. This is a concern, since IPs were performed in PBS, i.e. under conditions that might favour actin (re-) polymerisation. It might be really hard to distinguish between specific binding and unspecific sticking of precipitated filamentous actin to the beads.

13. Figure 7D, E: I have serious doubts that the interaction between actin and Ran is specific and would occur under conditions found in the cytoplasm. As the authors show, actin also binds to GST control alone. It is known, however, that actin is part of a functional export complex that in addition to Ran depends on the presence of exportin 6 and profilin. In light of this a binary interaction between Ran and actin seems highly unlikely. Indeed, this would be first 'cargo' that shows direct interaction with Ran in the absence of its transport receptor. The authors do not provide any evidence for that, but rather show a low affinity and probably unspecific interaction between two proteins.

13. While it is interesting that both SMIFH2 and CytD affect nuclear transport, the experiments presented in Figure 7 illustrating that monomeric actin itself inhibits nuclear transport by binding to Ran are not convincing. There might be other targets and/or other factors, released upon actin depolymerization, that need to be identified. Alternatively, especially at the high concentrations used in the XEE system the drugs might have side-effects, even on NPC function. Overall, the mechanistic details as to how NPC assembly and nuclear transport are affected remain unsolved to me.

14. Figure 9A - C: The experiment is really difficult to understand, also because the rational behind it does not make much sense. Are all factors required for DNA replication shuttling proteins that would enrich in nuclei upon CRM1 inhibition? If yes, then leptomycin B treatment might indeed temporary uncouple replication from the necessity of having ongoing nuclear transport. In this case, nuclei should also replicate in the presence of WGA. Has this been tested?

Additional correspondence (author) 17 March 2017

Thank you for allowing us the opportunity to respond to all reviewers' comments. Reviewers 1 and 2 have carefully read our paper and make some sound and constructive points. In contrast, we feel that reviewer 3 has misunderstood our paper and his/her review contains a number of important flaws. Please find below our detailed response to all comments from the three reviewers.

Referee #1

This paper describes a potential role for actin dynamics in nuclear transport and DNA replication. Although the conclusions are interesting, the following issues, some of which involve major technical concerns, would have to be addressed (listed in the order they come up in the paper).

Our reply: we appreciate that this reviewer has carefully read the paper and finds the conclusions interesting, and that he/she provides constructive criticism to address technical issues in order to improve the paper.

1. In Figure 3A, there should be a loading control, given that the drugs added might affect nuclear morphology/integrity which could indirectly affect recovery of soluble and insoluble proteins.

Our reply: We should mention that equal numbers of nuclei were loaded in each lane. This is stated in the methods section. We will add a statement to this effect in the figure legend. It is the only appropriate way to load soluble/insoluble samples from nuclei purified from Xenopus egg extracts, because (the reviewer is correct) some of the drugs affect nuclear morphology as they affect nuclear transport, and it is therefore not possible that one can load equal numbers of nuclei *and* have equal protein amounts. Nevertheless, I appreciate that some kind of loading control would be useful, so we will show WB of additional proteins that we tested. We have shown however that the drugs do not affect nuclear integrity, so this is not an issue.

2. There is no reference to Figure 3B in the Results section.

Our reply: yes, there is, see lines 136/137.

3. In EV2C, the control lane showing how much PCNA would normally load in the absence of SMIFH2 is overloaded, as evidenced by the loading control. This undermines the conclusion drawn.

Our reply: It is true that there are some slight variations in loading. In the chromatin samples, the 13h control lane is more loaded than with SMIFH2, but it is clear that the differences in PCNA loading between these lanes are much more than the difference in loading. Moreover, the decrease in chromatin-bound PCNA is evident by independent approaches e.g. our timelapse experiments. We could present another western blot and provide normalised quantification values for each band. In any case, we do not draw any conclusions based on the blot, we simply state that PCNA loading onto chromatin was reduced. This does not undermine in any way the conclusion drawn from the FACS analysis, and which shows complete block of the S-phase entry. However, there is an interesting parallel with this result and the results shown in Figure 9 using nucleoplasmic extracts. In the latter case, PCNA was loaded onto chromatin in the presence of SMIFH2 but did not increase like in controls, and replication was abolished.

4. Is the effect of SMIFH2 on S phase entry not explained by its inhibition of transcription (Figure 4B)?

Our reply: It is indeed very likely that the effect on S-phase entry is due to inhibition of transcription, which is why we analysed it. We performed additional experiments using DAD mutants of mDia to address possible transcription-independent effects of altering actin dynamics on S-phase, as well as experiments in Xenopus, where the transcription is silenced. Perhaps we were not clear enough about our conclusions; this could be rewritten.

5. No reference is made to the dnDiaNLS construct shown in Figure 4D.
Our reply: We apologise for this oversight. The dnDiaNLS construct did show a slight increase in EdU postive cells, but this difference from the controls (DiaDAD and untransfected) was below the statistically significant threshold (p=0.08). This should be mentioned. Since the Dia.LG.NLS construct also causes an increase, it suggests that interfering with nuclear formin activity in either way impedes S-phase. However, overexpression of heterologous constructs and presumed "dominant negative" constructs does not allow much molecular insight, so we did not dwell on this. The most important point is that the cytoplasmic DiaDAD construct does not have any effect.

6. Why does the R62D mutant decrease EdU incorporation (Figure 4E) but not increase the S phase population (Figure 4D)?

Our reply: the WT and R62D mutants had little effect on the percentage of EdU positive cells, unlike the S14C and G15S mutants. It is true that we stated that the latter also reduce EdU intensity, as the variance of these samples was quite small. However, the variance being larger for the R62D mutant, we did not comment on this. Taken together, it is clear that effects of WT and R62D mutant were negligible, compared to obvious effects of S14C and G15S. We will alter the text accordingly.

7. Line 169: gamm-H2AX staining provides evidence of DNA damage, but not necessarily dsDNA

breaks.

Our reply: gamma-H2A.X certainly does stain dsDNA breaks and is widely used to identify them. Yet, technically the reviewer is correct as it has been reported that UV damage, which induces pyrimidine dimers, can give rise to gamma-H2A.X staining. However, it is likely that the latter is due to dsDNA breaks that arise indirectly during S-phase from the primary damage. Nevertheless, we will amend the text.

8. Line 174: the authors need to explain what is gelsolin and why is it used.

Our reply: gelsolin is a specific actin-binding protein that binds to the barbed end of filaments, arresting their dynamics, as well as severing filaments. Thus, it could be expected that it would have synergistic effects with agents like jasplakinolide, a peptide that stabilizes actin filaments. We will insert text to this effect.

9. Figures EV3C and D should indicate the drug being added.

Our reply: for EV3C, we apologise for the legend that was obliterated by the graph, we hadn't noticed this. It was the protein MICAL2 that was added. We will rectify this. For EV3D the legend already indicates the drug "2.4", although the reviewer might have overlooked the definition in line 177 of the text and not noticed the figure legend, line 1177; we could add "INH 2.4" in the figure to make it clearer.

10. Line 187: since PCNA is generally considered to mark replication sites, and actin appears to interact with PCNA, it is unclear why the authors conclude that actin is not present at replication sites. Is the actin-PCNA interaction replication-dependent? To address this, the authors could block initiation with a CDK inhibitor and repeat the experiment. As is, the data don't add anything and should be removed.

Our reply: we state in the lines 186 and 187 that unlike PCNA, actin does not interact with either RPA or MCMs, which would be expected if it was present at replication forks, for instance. We could rephrase this to make it clearer. But we accept that the data of actin-PCNA interaction by PLA, which probably reflects a different pool of PCNA (e.g., Stoimenov & Helleday, Biochem Soc Trans. 2009), might be confusing and could be removed. We do, however, think it important to show that actin does not interact with MCMs/RPA.

11. Contrary to the authors' conclusion that NUP staining is disorganized (line 199), Figure 5C indicates it is greatly reduced. Also, it appears that a highly non-representative portion of the nucleus in 5C (top right panel) was shown in the inset that is devoid of NUP staining.

Our reply: Figure EV4A, which is a Western blot with equal nuclei loaded, shows that NUP staining is not generally reduced overall. We deliberately showed a non-representative portion of the nucleus in 5C inset in the SMIFH2 sample to highlight an area where NUP staining is strongly reduced. Although we thought that the text on this was fairly clear "Upon SMIFH2 treatment, nucleoporin (NUP) staining was disorganised, with dense NUP clusters between NUP-free regions" (lines 198-199), this will also be indicated with explicit reference to the inset, in the text and in the legend or both. 3D-SIM was only an indicator of the possible effects on NPC structure, that was analysed directly by FEISEM.

12. These papers should be cited and discussed:

J Cell Sci. 2004 May 15;117(Pt 12):2481-90. Epub 2004 May 5. Actin- and protein-4.1-containing filaments link nuclear pore complexes to subnuclear organelles in Xenopus oocyte nuclei. Kiseleva E1, Drummond SP, Goldberg MW, Rutherford SA, Allen TD, Wilson KL.

J Cell Sci. 1992 Jan;101 (Pt 1):43-53. DNA replication in cell-free extracts from Xenopus eggs is prevented by disrupting nuclear envelope function. Cox LS1.

Our reply: we will cite these two relevant references.

13. Imp and CD conditions in Figure 7A appear to be overloaded (compare NUP62 blot), undermining the claim that CD promotes importin-NUP interaction. Were the two sections of the blot exposed equally?

Our reply: The two sections of the blot were indeed exposed equally and came from the same gel, we will mention this. Whereas NUP62 is hardly changed in Imp or CD compared to control, in controls there is no detectable importin (only background staining) but it is easily detectable in both Imp and CD conditions. It is, however, difficult to be extremely quantitative for interactions with IP-western blots. We would not say that CD promotes importin-Nup interactions, we would say that it rather reduces their turnover. If wished, we could tone down the affirmation in the text to "this provides evidence for", or something similar.

14. Figure 7C lacks Ran loading control.

Our reply: Ran was covalently coupled to the beads used in the pulldown so is not an appropriate control. Equal volumes of beads were used, as evidenced by the fact that RCC1 and importin-beta are pulled down to similar extents in the three experimental samples (and not in the control, for which obviously no loading control is possible). I don't really see what else we can do here other than amend the legend to explain this point.

15. Although cytochalasin D and SMIFH2 both inhibit nuclear transport (Figure 6A), they appear to do so by different mechanisms (Figure 7). Some attempt should be made to unify the observations of these two actin modulators.

Our reply: We have uncovered what we think is a possible mechanism for cytochalasin D. Indeed, SMIFH2 does appear to work by somewhat different mechanisms, but figuring out the molecular details of all of this is clearly beyond the scope of this paper and could take a long time. We will provide a better discussion of this, for example, actin-independent roles of formins.

16. The authors should cite appropriate previous papers showing that WGA inhibits replication in egg extracts.

Our reply: This reiterates point 12, it was a mistake on our part not to cite the Cox et al paper.

17. From Figure 8A, the authors conclude that once nuclei have formed, replication can proceed without further NPC formation. However, a critical control is missing, which is to show that in this experiment, NUP depletion was complete (which can be shown by placing sperm chromatin directly in this extract). Otherwise, it may be that the residual replication seen in WGA-bpdelta condition reflects residual de novo NPC formation.

Our reply: We already did the requested experiment; in Figure EV5B we have shown that sperm chromatin does not replicate in the WGA-depleted extracts. To make it easier to understand, it was not worded in the text such that it is perceived as the same experiment, but it was in fact the same experiment. We will change the wording of this. We could also include a WB of NUP depletion if wished, we did not include it as the data were already crowded.

Conversely, it may be that the requirement for continued nuclear transport is due to nuclear disruption during the transfer experiment (this caveat affects all the nuclear transfer experiments shown). It would be better to do the experiment without transfer and to simply titrate away the Geminin with recombinant Cdt1.

Our reply: this is not the case since we have shown controls (mock depletions), which replicate efficiently. It is impossible to do the experiment of letting the nuclei form properly before exposing them to conditions without NUPs by just titrating away geminin with Cdt1 (which does not work anyway, we have tried it) since the extract has to be depleted of NUPs – in which case the nuclei could not form properly. Nuclear transfers are the only way to perform the experiment.

18. Why does the second condition in Figure 8B not replicate better? Also, the logic underlying this triple transfer experiment is not explained well. Is the purpose of this experiment to address whether SIMULTANEOUS nuclear transport and formin activity are required for replication?

Our reply: we apologise for not having well enough explained this experiment, but indeed the reviewer worked it out. This was done to assess whether the roles of formins and nuclear transport are distinct or related. If they were distinct then the reciprocal transfers between SMIFH and WGA extracts should replicate, if they are involved in the same mechanism, they should not. Obviously the nuclei have to form in the first place, so this necessitates a double nuclear transfer. We will improve this explanation in the text. We should also mention that double nuclear transfer experiments are technically very challenging to achieve efficient replication and we are one of the few labs to do this. Therefore it cannot be expected to replicate to close to 100%. Thus, the single transfer from Geminin-> ctl replicates at close to 100% (figure 8C), but geminin-ctl-ctl in the double transfer (triple extract condition) only reaches 40%. The extract "gets tired"! Some other labs use "% of control" to express the efficiency of replication for their experimental samples; we do not do this, we always quantify the replication in absolute terms.

19. The failure of the PAWGA condition to replicate (Figure EV5D) might again be due to nuclear disruption during transfer?

Our reply: again, this is not the case as the controls replicate fine.

Also, why do the authors use this obscure CDK inhibitor? Roscovotine is much more widely used.

Our reply: Purvalanol A is hardly an obscure CDK inhibitor (it was first published in Gray et al., Science, 1998)! Roscovitine is more widely used since it has been around even longer and is used in clinical trials, but is less potent and less specific than purvalanol A. We have extensively characterised puvalanol A in kinetics terms against CDK1 and CDK2, the two main CDKs that promote origin firing in Xenopus egg extracts in a paper we cite (Echalier et al., Chem Biol., 2012). I don't feel we need to further justify this or cite all the original papers on purvalanol A.

20. How do the authors know that in Figure 8C, nuclear transfer does not disrupt the nuclear envelope. If this is the case, all the CDK activity would leak out, imposing a requirement for new nuclear transport in the second extract to re-accumulate CDK activity.

Our reply: again, we showed the controls, which replicate to virtually 100%. If the transfer disrupted the nuclear envelope, the control would not replicate.

21. Why does SMIFH2 inhibit DNA replication in 9D but barely at all in EV5E? The result in Figure 9E does not explain the total block in replication shown in Figure 9D.

Our reply: We explained in the methods that unless otherwise stated, SMIFH2 is used at 500μM to achieve a complete block. These were not the same concentrations that were used in EV5E (200μM; compound 2.4 is not very soluble and we could not achieve higher concentrations, so we compared it to SMIFH2 at the same concentration; text to this effect could be added in the methods section). Incidentally, this shows that the SMIFH2 effects are dose-dependent. We will amend the main text to clarify this point.

There must be a step that is inhibited downstream of Cdc45 loading. The authors should check GINS, RPA, polymerase etc to determine where the block occurs.

Our reply: we agree with the reviewer here, it is not clear what the roles of formins in replication are downstream of chromatin loading of replication proteins. However, nucleoplasmic extracts are also very challenging and performing chromatin blotting of samples from NPE is even more so. Although the suggestion is a good one, I do not feel that simply blotting all the replication proteins that we can think of, even if we had the antibodies available, would resolve this. Everything we looked at was already there. In future studies we will of course try to address further these points, but we cannot expect to have all the answers in the scope of a single paper. We can, however, propose some alternative scenarios in the discussion and will do so.

22. The discussion was not very informative, as it contained almost no discussion of how actin/formin might regulate replication.

Our reply: We will expand and improve the discussion.

Referee #2

Parisis et al. describe a novel role for actin and actin-assembling formins in DNA replication. The onset for the project is a mass spec study from Xenopus nuclear extracts, which identifies several actin-regulators in replicating nuclei. In their studies, the authors use both Xenopus egg extracts (XEE) and somatic mammalian cells. The XEE system is transcriptionally silent, which allows the authors to uncouple the potential role of actin in gene expression from its role in replication. Moreover, the XEE system permits elegant sequential inhibition experiments, which would not be possible in a whole cell model. The authors show that in mammalian cells, nuclear actin displays cell cycle-dependent dynamics, with actin filament formation at the early G1 phase of the cell cycle. By using actin-binding drugs, actin-regulators and formin-manipulation, the authors then show that nuclear actin dynamics (and formin activity) are required for DNA replication. Their studies further point to a role in both nucleo-cytoplasmic transport, which is critical for DNA replication, as well as in a subsequent steps of chromatin loading of replication factors and initiation of replication. Mechanistically actin seems to interact with Ran GTPase, with implications on cargo-release during nucleo-cytoplasmic transport.

Overall, this study is very interesting and reports a completely novel function for actin dynamics and formin activity in DNA replication. The use of two very different experimental systems supports the generality of the findings, and the XEE system permits very elegant experiments, such as the transfer of nuclei between differentially treated extracts. The experiments are carefully done, and link between actin/formin activity and efficient DNA replication is obvious. Also plausible mechanistic explanation regarding the nucleo-cytoplasmic transport is provided. This study thus broadens the functional implications of nuclear actin and its regulators, and is therefore very important to the field.

Our reply: We are glad that the reviewer perfectly understood the paper and its significance in the field and is convinced by our experimental demonstrations.

The major problem with the manuscript at the moment is that it is very difficult to read and follow, due to the huge amount of data and juggling the two different experimental systems. I suggest that the authors simplify the figures, by carefully evaluating which experiments are absolutely necessary to prove their point. In addition, the figures should be labeled clearly so that it is immediately evident, which system (XEE or mammalian cells) is used. For example in figure 5, it is impossible to tell from the figure and figure legend, which system is used in each panel without reading the text. The introduction lacks intro to the replication process, which would make the manuscript easier to follow for readers not specialists in this topic.

Our reply: These are good suggestions that we will take into account in a revised version.

One confusing fact is that some treatments, especially the formin inhibitor SMIFH2, seem to operate differently in the two systems. In XEE, SMIFH2 does not seem to affect nuclear actin amounts or polymerization, but in mammalian cells it seems to stabilize the nuclear filaments. So in one case, one would be looking at formin-inhibition that is not affecting actin, and in the other case, an indirect effect of stabilizing nuclear actin filaments?

Our reply: Again, this is more or less exactly what we believe. We do think that SMIFH2 will affect nuclear actin dynamics in XEE but since there are no actin filaments in these nuclei, they cannot be stabilised (see lines 126-133). As regards SMIFH2 response, XEE nuclei are similar to S-phase somatic nuclei. The apparent differences between somatic G1 nuclei and XEE nuclei in the intrinsic nature of nuclear actin, and hence the effects of SMIFH2, is likely related to their functional differences. Somatic nuclei in G1 are formed from post-mitotic chromatin, and are transcriptionally competent, whereas XEE nuclei are formed de novo from the virtually naked chromatin of sperm, and replicate but do not transcribe. The transient G1 nuclear actin filaments of somatic cells might well be a way of sequestering monomeric nuclear actin to allow transcriptional reactivation. We will improve the discussion on this point.

What effect does the other formin-inhibitor, 2.4, have on actin in these two systems?

Our reply: this is a good suggestion, we have not analysed this in detail but could do so.

The interpretation of the results from SMIFH2-treated mammalian cells is problematic, since SMIFH2 most likely affects also cytoplasmic actin. For example in 5B, SMIFH2 treatment is shown to have an effect on nuclear morphology. Did the cells divide during the experiment?

Our reply: we completely agree; this is why we did not analyse SMIFH2 much further in mammalian cells, especially since we found that it abolishes transcription which confounds a clean analysis of its effects on DNA replication. At no point in the manuscript do we attribute the observed effects of inhibitors exclusively to the nuclear actin. In the experiment in 5B, the treatment was for 4 hours only and then the cells were fixed. However, from other experiments shown we know that cells treated with SMIFH indeed do not divide, which is shown in Movies EV3 and EV4, but I agree that we should have mentioned this explicitly.

The finding that actin dynamics plays a role in nucleo-cytoplasmic shuttling by regulating Ran-cargo release is very interesting, but the experiments in mammalian cells are not very convincing. In addition (or instead of) of looking at an endogenous shuttling protein, which can be regulated by cell morphology as pointed out by the authors, the authors should look at an engineered construct (like they use in XEE), such as NLS-GFP-GST-NES.

Our reply: To obtain clear and quantifiable results, we needed an inducible system of nuclear transport. Originally we acquired and tested Rebecca Heald's ionomycin-inducible GFP-NFAT nuclear translocation system, but this did not work well at all. The ionomycin inducibility of the translocation was not obvious, it was also very leaky, and importazole did not inhibit it. This was why we looked at NFkB, which for us has the advantage of being a highly physiologically relevant nuclear transfer of an endogenous protein.

As mentioned above, SMIFH2 likely also affects cytoplasmic actin. If the SMIFH2 functions here by stabilizing nuclear actin filaments, does expression of NLS-G15S/S14C actin mutants also impair nucleo-cytoplasmic shuttling?

Our reply: I agree that if it worked, this would be ideal since then we could test whether it is nuclear or cytoplasmic actin dynamics that is affecting nuclear transport in cells. We indeed tried using the G15S/S14C mutants in HeLa cells and did not observe any effect of the mutants. There are probably technical issues here. At the moment we cannot say whether this is due to insufficient strength of the effects of the mutants compared to chemical blocks (which could in part be due to hypothetical effects of the nuclear actin on nuclear transport feeding back negatively on import of the actin mutants), or whether it is simply cytoplasmic actin that controls nuclear transport. As nuclear transport is very highly conserved, we felt that XEE were best suited for these experiments, but they do not allow to resolve this issue.

The actin filament formation in early G1 is very interesting, but it is not clear how this fits with the rest of the story.

Our reply: I understand why the reviewer would say that, but it is important if for no other reason than it allows us to better understand the effects of the drugs on actin dynamics, and the apparent differences between Xenopus and somatic cells. The S-phase nuclei, whether in somatic cells or Xenopus, do not form actin filaments when treated with SMIFH2. SMIFH2 probably only changes the dynamics of existing filaments, as shown in the Rizvi et al paper, which we cite.

In the discussion, the authors speculate that "deregulated nuclear actin dynamics, rather than an increase in nuclear actin levels or filament formation per se, prevents DNA replication". What is actually meant by this?

Our reply: we apologise for the rather terse discussion, which we will improve in a revised version. This, in particular, was not a well-worded sentence. We showed, for example, that jasplakinolide, or addition of purified Arp2/3 complex and GST-WAVE, cause a similar increase in nuclear actin levels to cytochalasin D, but does not induce formation of filaments nor block replication, whereas Arp2/3 complex and formin inhibition block do not promote filament formation either but do block

DNA replication. The common denominator is that all these agents disrupt actin dynamics: i.e. the polymerisation/depolymerisation equilibrium. Rebecca Heald's lab claimed in 2003 that latrunculins, which prevent actin polymerisation, block DNA replication, although the data were not shown in their paper. We could not reproduce this with the concentrations of latrunculins that they used (in fact we found rather the opposite, that we could rescue the block caused by cytochalasin D). We do, however, find a block with higher concentrations of latrunculins, and we could put this data in. The data that we did show could be perceived as being consistent with the possibility that nuclear actin filaments abolish replication, since under no circumstances in which we see strong nuclear actin filament formation do we see efficient DNA replication. But we cannot rule out that filaments simply sequester actin monomers that might promote DNA replication, directly or indirectly.

In Serebryannyy et al, 2016, it was speculated that persistent nuclear actin filaments decrease nuclear actin monomer levels, and this causes the transcription defect in their system. Do the authors speculate that something like this is taking place also in their system?

Our reply: Yes; see above.

Minor points
SMIFH2 is abbreviated differently (SF or FH) in different figures.
In EV3C, Mical-2 text below the x-axis is not visible.

Our reply: Thanks for pointing out these minor labelling and formatting errors, we will correct them.

Referee #3

The authors of this study look into connections between DNA replication and the actin cytoskeleton. The make the claims that nuclear actin filaments form in G1 of human cells, that stabilisation of nuclear actin filaments blocks nuclear transport and DNA replication, that actin directly binds Ran-importin complexes and is required for cargo release, and that nuclear forming activity is required for DNA replication. Unfortunately, however, these claims are neither plausible nor are they supported by conclusive evidence. Instead, the study is an example of wishful thinking and inadequate experimental methods.

Our reply: We are very surprised by these statements, which strongly contrast with those of reviewers 1 and 2.

Specific points:

Introduction: it is remarkable that the authors introduce the issue of nucleocytoplasmic actin distribution without a single mentioning of exportin 6 (Xpo6), which excludes actin from nuclei in somatic cells.

Our reply: We are well aware that Xpo6 exports actin from the nucleus of many cell types, but not in the giant nuclei (GV, or germinal vesicles) of Xenopus oocytes, which do not express it. But we disagree that Xpo6 expression means that there is no actin in the nucleus of other cells. For a long time, almost nobody believed in nuclear actin due to the difficulties in visualising it with the available technologies and the overwhelming amount of actin in the cytoplasm. However, there is now a large body of literature on nuclear actin, which has since been purified multiple times as a component of a variety of transcription complexes, chromatin remodeling complexes, etc; both import and export transporters have been identified; RNAi screens have revealed regulators of nuclear actin levels and polymerisation; numerous imaging probes have verified its existence (including phalloidin staining in certain circumstances such as serum stimulation in fibroblasts) and mobility, and so on. Although of course the nucleocytoplasmic shuttling of actin is relevant, our paper does not focus on this, but on functions of actin in DNA replication.

It is also remarkable that the authors did not mention that an unbiased phalloidin stain gives a bright cytoplasmic but no nuclear signal.

Our reply: We did not omit this. We stated in lines 63-65 in the text: "Physiological nuclear actin polymerisation remains poorly characterised due to difficulties in staining nuclear actin with

phalloidin". Indeed, this is probably why it was thought not to exist for so long. We cannot say that there is no nuclear signal because we can detect it in G1 cells using glutaraldehyde fixation with confocal microscopy, which we show in Figure 2I.

The authors also fail to introduce that Xenopus oocytes are special in that they lack Xpo6 and thus do not exclude actin from their nuclei. Xpo6 reaches normal cellular levels not before stage 7 of embryonic development (see figure 4, Bohnsack et al. (2006), doi: 10.1038/ncb1357). It is thus not surprising that nuclei reconstituted from Xenopus egg extract are still compromised in their ability to exclude actin.

Our reply: we are not working on Xenopus oocytes. We stated this explicitly in lines 65-69, where we point out the unusual structure of oocyte germinal vesicles and the presence of actin filaments. Xpo6 is abundant in Xenopus eggs (and therefore also in the egg extracts), as shown in the Figures 3 and 4 of the same paper that this reviewer refers to. Independently, there is overwhelming experimental evidence for nuclear actin in the literature.

In fact, the lab of Rebecca Heald reported earlier that nuclei assembled in XEE contain nuclear actin (Krauss et al, 2003). Furthermore, Krauss et al. showed that latrunculin A interfered with nuclear assembly and DNA replication. It is unclear why this paper was cited only for a methodological detail.

Our reply: We are very familiar with this paper. However, not only was there not even a beginning of an analysis of any mechanisms, and nuclear assembly and transport was not tested, no data on DNA replication was presented (it was mentioned as "data not shown"). Besides, only a single approach was used to manipulate actin (adding 0.1mM latrunculin A). We could not find any inhibitory effect of this concentration of latrunculin A – rather the opposite, in fact. Nevertheless, we of course cited this study.

The authors also fail to mention that a comprehensive study for identifying nuclear actin-binding proteins has been published before (Samwer et al., 2013, doi: 10.1038/emboj.2013.108).

Our reply: We felt that the Samwer et al. article was not very relevant to our study. This paper identified a number of actin binding proteins in Xenopus oocyte nuclei, which we are not working on. In contrast, we studied the entire nuclear structural proteome in the replication-competent nuclei assembled in egg extracts. We do cite a much more closely related study, that of Khoudoli et al., 2008.

2. Figure 1: The authors intend to identify intranuclear actin-binding proteins by a proteomics analysis of in vitro assembled nuclei.

Our reply: This is a misrepresentation of our experiment, which was not done to identify intranuclear actin binding proteins, but to assess possible cell cycle regulation of nuclear assembly (lines 92-97). As mentioned above, we report a very complete proteomics dataset of nuclear assembly in the presence or absence of CDK activity.

Although it is not detailed in the methods, I guess, the nuclei were simply isolated by centrifugation.

Our reply: The nuclei were isolated by differential sedimentation through a 24% sucrose cushion, as explained in the methods section for the nuclear transfer experiments (lines 553-559).

Analyzing such pellet is, however, rather problematic: How do the authors distinguish proteins that are enclosed by nuclear envelope (true intra-nuclear proteins) from actin structures that associates from the outside or indeed simply polymerised actin?
Without rigorous controls, such analysis is meaningless.

Our reply: We confirmed localisation of actin and actin regulators directly by imaging, including 3D imaging, as shown in Figure 2. Contamination by cytoplasm was negligible in both proteomics data (we could state the numbers in the text) and western blotting for tubulin (Figure 2B).

A similar concern applies to Figure 2B; 3A; 4J; EV3F; 7A, B, C, F; EV4A etc. In these experiments,

the authors should have thoroughly tested if their fractionation procedures are suited to cleanly (!) discriminate between nuclear and cytoplasmic actin pools.

Our reply: In Figure 2B we showed WB for tubulin to control for cytoplasmic contamination; there was none. In Figure 3 we show that trace labeling to image nuclei for actin in different conditions reproduces exactly the effects of drugs on WB for nuclear actin. We could also show immunofluorescence using the antibody 2G5 that recognises both nuclear and cytoplasmic actin. We also show PLA for endogenous actin (EV3G). In EV3F and 7A we did not look at actin at all. In Figure 7B we performed immunoprecipitations from lysed nuclei with NUPs, so it is not possible to discrimate any actin that could associate with NUPs on the cytoplasmic side of the envelope from the nuclear side. In Figures 7C and 7F we performed Ran or active Ran pulldowns and also found actin. Figure 2A shows evidence for nuclear actin regulators by direct immunofluorescence of purified nuclei. Thus, taken together, there is ample evidence for actin and actin regulators in nuclei in Xenopus egg extracts.

3. Figure 2A: What are the specificity controls for antibodies and IF-conditions used? Given that the authors suggest the Arp2/3 complex showed nuclear accumulation, while others clearly found that this complex is well excluded from Xenopus nuclei (see e.g. Samwer et al., 2013) I am having serious doubts.

Our reply: All of the known actin regulatory proteins we tested were localised to nuclei by immunofluorescence, using well characterised antibodies. As we are working with purified nuclei this allows us to eliminate cytoplasmic staining, which would obviously be much stronger and thus obscure any or most of the nuclear staining. We think it is highly unlikely that all the antibodies we use are non-specific, and indeed we can see very specific staining. As we mention above, we felt that the Samwer et al paper is not relevant as it looked at oocyte nuclei, which are structurally completely different. More relevant is the Khoudoli et al study (cited), where Arp2/3 was largely present on chromatin; among others, we could also cite Yoo et al., 2007, JBC.

Indeed, the website of the manufacturer of the AB (AbCam) shows a clean cytoplasmic staining. The authors apparently miss the specific (cytoplasmic!) signal, because they look only at nuclei.

Our reply: The Abcam website shows somatic cells, which have a cytoplasm. This is exactly why we isolated nuclei from egg extracts, otherwise we would indeed be unlikely to be able to see specific nuclear staining due to the more intense “background” from cytoplasm.

4. Statement 'We ... show that nuclear actin filaments form in early G1'. This is obviously an artifact of the detection methods. The expression of NLS-fused actin-binders (Lifeact or the anti actin chromobody) will necessarily cause nuclear import of actin and thus severly alter the nucleocytoplasmic distribution of actin.

Our reply: As mentioned above, we have stained nuclear actin filaments with phalloidin in cells not expressing the chromobody probe (Fig 2I). Incidentally, Robert Grosse independently developed (and published) the same tool, and also has shown that cells not expressing any probes have nuclear actin filaments that can be visualised with phalloidin. It seems highly unlikely that any actin bound to an antibody and then transported into the nucleus would subsequently be able to polymerise, or that an antibody would radically alter the nuclear actin concentration. If it did have this ability, then we would observe nuclear actin filaments in the entire cell cycle. It is partly the recent development of such nuclear-specific probes that have moved the nuclear actin field forward (Baarlink et al. 2012, Plessner et al., 2015).

We had indeed found that NLS-Lifeact binding peptide does disrupt actin dynamics, showing long nuclear cables, probably by preventing depolymerisation of the G1 filaments. We do not, however, think it likely that either tool provokes de novo actin filament formation. More likely Lifeact prevents depolymerisation. The antibody does not do this, since the filaments depolymerise, as we show, and the cells divide and proliferate without any alteration in their cell cycle length.

Such bias can be avoided by first fixing the cells and then staining acti-filaments with fluorescent phalloidin. It has been known for decades that nuclei of undisturbed cells are phalloidin-negative. This applies also to S-phase.Another way of looking at the issue in an unbiased manner would be to

expressed the chromobody of LifeAct without the NLS. Both actin-sensors would actually be small enough to enter nuclei by passive diffusion.

Our reply: As mentioned above, we performed exactly this experiment. We used glutaraldehyde fixation and confocal microscopy and the results are shown in Figure 2I. We indeed show nuclear filaments without any ectopic expression of probes. When using actin sensors without NLS, the cytoplasmic signal masks the nuclear signal, and such transient actin filaments are missed.

5. Statement: 'treatments that stabilize actin filaments abrogate nuclear transport'. This does not make any sense, and from my own experience I can tell that nuclear import works just perfectly fine in the presence of phalloidin (the most potent filament-stabilizing drug).

Our reply: Perhaps this was not the most perfectly worded phrase. What we meant was that the treatments that we tested which stabilise filaments also abrogated nuclear transport and DNA replication. It is not surprising that the nuclear transport works perfectly fine in the presence of phalloidin, since phalloidin is not cell permeable. We present a detailed disclosure of careful experimental results in several systems, and we cannot accept their dismissal on the basis of results that cannot be cited.

6. The authors state that staining of fluorescently labelled DNAse I (meant to detect actin) perfectly overlaps with the DNA staining. Are the authors able to exclude that the probe they used actually binds DNA?

Our reply: In our sentence (line 110) we merely state that DNAse 1, which has high affinity for G-actin (indeed, it is often used as an actin probe), mainly labels chromatin. We did not say that DNase I staining perfectly overlaps with DNA staining. Precisely because we cannot, as the reviewer asks, exclude that it binds DNA. Nevertheless, the literature on the use of DNase I specificity for G-actin is extensive (e.g. Zechel et al., 1980 Eur J Biochem; Kabsch et al., 1985 EMBO J).

7. If the authors want to make the point that nuclear actin filaments would be required for DNA-replication
then they should test this without messing with cytoplasmic actin structures. In fact, this experiment is very simple and requires just the addition of Xpo6 to the nuclear assembly reactions. It the authors' conclusion is correct, then one would expect a complete block of replication at sub-micromolar concentrations. Given however that these are conditions found in somatic cells, I doubt that nuclear exclusion of actin would compromise replication.

Our reply: At no point in this manuscript have we tried to make this point, since we do not believe it to be true. Thus, we would not expect adding Xpo6 to a nuclear assembly reaction to have any effect on DNA replication. We also feel that implying that we are “messing with cytoplasmic actin structures” is a misrepresentation of our work.

8. The authors claim that nuclear formin activity would be required for DNA replication. Which formin is this supposed to be and what is the evidence for such claim? The authors fail to recognize that a requirement cannot be proven by ectopic over-expression.

Our reply: We did not ectopically overexpress formins, we used the DAD fragment of mDia2 to activate the endogenous activity (by relieving each formin molecule from the autoinhibitory cis-interaction of DID-DAD domains) specifically in the nucleus. This unambiguously affects the activity of mDia2 (Baarlink et al., 2012).

Since formin inhibitors block onset of S-phase in somatic cells but this may be indirect, we can only study this in the Xenopus system (so far virtually everything found to be required for DNA replication in eukaryotes is conserved between species). Two completely different formin inhibitors with different chemical structures prevent DNA replication in nucleoplasmic extracts, (Figure 9D and EV5E). Although we have identified nuclear Xenopus homologues of mDia formins, as we report in this paper, we do not yet have tools to deplete them. We have tried specific depletion of Xenopus formins from the nucleus using available antibodies against mammalian formins, but we could not achieve a reasonable level of depletion. This will be addressed in future studies, but is outside the scope of this paper.

9. Figure 3: The finding that CytD has the same effect as Jasplakinolide, namely inducing nuclear actin polymerization is somewhat unexpected.
My interpretation of the authors' explanation is that CytD exclusively acts on cytoplasmic actin filaments, giving rise to a high level of monomeric actin that can enter assembled nuclei. What would prevent CytD from entering nuclei? And why do the authors add such a high concentration of the drug (400 micromolar)? Given that Cytochalasins are sparsely soluble in water this seems to be an inappropriate concentration. Why was latrunculin A not active at all, even at 100 µM? I am sorry to say, but such inconsistent pharmacology does not support the authors' conclusions.

Our reply: In lines 364-372 of our discussion we provide an explanation of the pharmacology and the doses of drugs that are required to elicit effects in Xenopus egg extracts. Latrunculin was active, in contrast to the reviewer's claim, as it reversed the nuclear actin polymerisation provoked by CytD. It did not, however, block replication at these concentrations, providing evidence that polymeric actin is NOT required for DNA replication (see also point 7).

We do not believe that CytD exclusively acts on cytoplasmic actin. The cytoplasmic actin cytoskeleton is not at all disrupted as actin filaments are conspicuously formed. I also do not see what would prevent CytD from entering the nucleus. Besides, cytochalasins are NOT actin depolymerising drugs, they bind the barbed end of filaments preventing further polymerisation and depolymerisation. Their effects on cellular actin have been very well characterised (for example, see John Cooper's review on effects of cytochalasins on actin, 1987, J Cell Biol 105, 1473-8, and Manfred Schliwa, 1982, J Cell Biol 92, 79-91; to quote, "Cytochalasin D treatment severely disrupts network organization, increases the number of actin filament ends, and leads to the formation of filamentous aggregates or foci composed mainly of actin filaments").

10. Figure EV4A: The small differences in band intensities do not support the conclusions given in the main text.

Our reply: Our main conclusion from EV4A is that most NUPs were present. We merely factually report the variations that we observed without ascribing any conclusion to them. Our conclusions are derived from other experiments: 3D-SIM and electron microscopy.

11. Figure 7A: The western blot provided does not support the conclusion that CytD treatment increases binding of importins to FG Nups. The 'CD lane' is similar to the 'Ctrl lane'.

Our reply: We disagree, the figure shows that importin-NUP binding is clearly increased.

12. Figures 7B, C, F: How specific is the postulated interaction between actin and FG Nups (Figure 7B) or Ran (Figures 7C, 7F) in the presence of CytD? Already the input lanes contain elevated levels of actin. Furthermore, the Mock control for this specific condition is missing in all three experiments. This is a concern, since IPs were performed in PBS, i.e. under conditions that might favour actin (re-) polymerisation. It might be really hard to distinguish between specific binding and unspecific sticking of precipitated filamentous actin to the beads.

Our reply: IPs were not performed in PBS, they were performed in the extract. Actin is not especially abundant in the nuclei but indeed, as the reviewer points out, might stick non-specifically to beads, as might any protein. This is why we do mock IPs. The point of the IPs was not to analyse actin-NUP or actin-Ran interactions at all. If we had wanted to do this, the suggestion of the reviewer, to do a mock IP with CD, would be a good one. The point was to look at NUP-importin and importin-cargo interactions in 7B, and Ran-importin and importin-cargo interactions in 7C. In 7F, the point was to test whether active Ran was increased. Actin in 7F effectively serves as a loading control. The only experiments where we tested whether Ran could directly bind actin were 7D and 7E.

13. Figure 7D, E: I have serious doubts that the interaction between actin and Ran is specific and would occur under conditions found in the cytoplasm. As the authors show, actin also binds to GST control alone. It is known, however, that actin is part of a functional export complex that in addition to Ran depends on the presence of exportin 6 and profilin. In light of this a binary interaction between Ran and actin seems highly unlikely.

Indeed, this would be first 'cargo' that shows direct interaction with Ran in the absence of its transport receptor. The authors do not provide any evidence for that, but rather show a low affinity and probably unspecific interaction between two proteins.

Our reply: We do not make claims from this experiment that direct Ran-actin interactions do occur, simply that they can occur. If this had not worked, we could have ruled out a direct binding of actin to Ran. And we show that there is an interaction between Ran and actin in the cytoplasm in Figure 7C.

13. While it is interesting that both SMIFH2 and CytD affect nuclear transport, the experiments presented in Figure 7 illustrating that monomeric actin itself inhibits nuclear transport by binding to Ran are not convincing. There might be other targets and/or other factors, released upon actin depolymerization, that need to be identified. Alternatively, especially at the high concentrations used in the XEE system the drugs might have side effects, even on NPC function. Overall, the mechanistic details as to how NPC assembly and nuclear transport are affected remain unsolved to me.

Our reply: In these conditions, cytochalasin D does not depolymerise actin: as we mention above, it prevents depolymerisation. Of course, there are mechanistic details left to be worked out, and we do not claim to have solved them all in this paper. I also refer the reviewer to our earlier comments on drug concentrations. Besides, we have controlled for the specificity of action of drugs. For example, we can rescue CytD effects with latrunculins or cofilin, as shown in Figure 4H, EV3B, and specific agents show synergy, eg, CytD + JPK, CytD + gelsolin (EV3A).

14. Figure 9A - C: The experiment is really difficult to understand, also because the rational behind it does not make much sense. Are all factors required for DNA replication shuttling proteins that would enrich in nuclei upon CRM1 inhibition? If yes, then leptomycin B treatment might indeed temporary uncouple replication from the necessity of having ongoing nuclear transport. In this case, nuclei should also replicate in the presence of WGA. Has this been tested?

Our reply: We apologise for not having explained the experiment clearly enough, but it is quite simple. Since previous experiments showed that CytD or SMIFH2 prevent nuclear transport, and that the essential replication factor PCNA is lost from the nuclei rather rapidly due to loss of the dynamic equilibrium between import and export, the results on replication thus far could potentially all be explained by loss of nuclear transport. The rationale behind this experiment was to rescue PCNA concentrations (and those of anything else that might have been lost) to see if the actin drugs would no longer prevent replication. They still do, and we show that CDK and PCNA cannot load onto chromatin in this case. Adding WGA will of course prevent import of PCNA and everything else, and we show in Figure 8 that ongoing nuclear transport is required for DNA replication, although its effects might indeed be lessened by inhibiting export with CRM1. But such an experiment would probably not tell us anything other than sufficient net nuclear import is required for DNA replication, which we already know. It would tell us nothing about the role of actin dynamics or formins.

1st Editorial Decision 05 April 2017

Thank you for your patience during our additional considerations of your tentative point-by-point response to the referee comments on your manuscript on nuclear actin roles in DNA replication. I have now received feedback from an expert editorial advisor (familiar with the Xenopus egg extract system and its use in the study of cell cycle-related nuclear events), who has him/herself read the paper as well as the referee reports and your tentative responses. In light of their feedback and our own assessment of your responses, we decided that we would like to consider a revised manuscript further for publication in The EMBO Journal.

For such a revision, please do carefully address all the points as proposed in your response letter. It will be particularly important to diligently address the technical issues raised by the replication expert, referee 1 (who was unfortunately not available at this stage to comment on your revision plans), given the primary focus of this work on DNA replication. Referee 2 would be largely satisfied with your revision plans, especially for making the manuscript more easily accessible (a

point mirrored by our advisor, see below), and otherwise emphasizes the importance of distinguishing the Xenopus extract and oocyte systems in order to avoid potential further misunderstanding. In addition, our editorial advisor made a number of points, copied below for your information, which would be very important to take into consideration as well when revising the manuscript. At the same time, the advisor does not see the extent of the criticisms of referee 3 justified and generally supports further consideration of this work.

We usually allow three months as standard revision time, and it is our policy that any related/competing work published during the revision period will have no negative impact on our final assessment of your revised study. In the present case, I would be happy to discuss the possibility of an extension beyond this three-months deadline, in case it should be helpful for you to comprehensively address the issues raised. In any case, I would appreciate if you could keep us updated on the progress of your revision and the status of any competing work that you may be aware of. Further information regarding preparation and submission of revised manuscripts can be found below and in our Guide to Authors.

Thank you again for the opportunity to consider this work, and for cooperating in this extensive pre-decision dialog. I look forward to your revision.

ARBITRATING EDITORIAL ADVISOR COMMENTS:

I read the paper and I do agree with reviewer 2 that it is quite difficult to read and follow. The authors should really make a large effort to make it easier to understand and select carefully the data that are essential, making it very clear when they go from one system to another and explaining the differences. In particular it would be essential to explain well the specifics of the Xenopus egg extract (which is in S phase and does not have G1 nor G2).

Some experimental data are missing, which is annoying. I could not find how they monitor DNA replication in egg extract, or how they purify the nuclei.

It would help very much if they could provide images of nuclei assembled in egg extract over time. In particular it would be really helpful to see the nuclei after purification and incubation in a new extract during the transfer experiments. The issue of the morphology and integrity of these nuclei is important and has also been raised by the reviewers.

Some experiments are not well represented in the figures or not quantified. For example the results shown in Figure 5A are based on single images of one selected sperm nucleus per condition.

Overall I found that the comments from reviewer 3 were overly too negative and not based on objective criticism but rather emotional. I think that with a careful revision the paper could be reconsidered.

1st Revision - authors' response 06 July 2017

Thank you for allowing us the opportunity to respond to all reviewers' comments. Reviewers 1 and 2 read our paper carefully and made some sound and constructive points. As you are aware, we feel that reviewer 3 misunderstood our paper and his/her review contained a number of important flaws. We greatly appreciate that you asked an additional advisor to add his/her comments, and we are pleased that this person concurred with us. We have now extensively revised the paper in accordance with all reviewers' comments. As most reviewers found the paper complex and difficult to read, especially given the frequent switching between the two systems (*Xenopus* egg extracts and human somatic cells) we have also rewritten it more or less completely, and reordered the paper so that there is only a single switch between systems. We feel that this is a significantly improved paper, and hope that you now find it acceptable for publication. Enclosed is a detailed point-by-point reply. We look forward to your decision [point-by-point response inserted in the following pages].

Reply to reviewers; referee #1

We reply to all reviewers below; we refer to the additional advisor as referee #4.

Referee #1

This paper describes a potential role for actin dynamics in nuclear transport and DNA replication. Although the conclusions are interesting, the following issues, some of which involve major technical concerns, would have to be addressed (listed in the order they come up in the paper).

Our reply: we appreciate that this reviewer has carefully read the paper and finds the conclusions interesting, and that he/she provides constructive criticism to address technical issues in order to improve the paper. We are confident that we have answered these technical concerns.

1. In Figure 3A, there should be a loading control, given that the drugs added might affect nuclear morphology/integrity which could indirectly affect recovery of soluble and insoluble proteins.

Our reply: We replaced figure 3A with data from a new experiment, in which we additionally show MCM6 and nucleoporin NUP62 (new figure 3F). Former figure 3A has now been moved to figure EV3A. Equal numbers of nuclei were loaded in each lane, which was stated in the methods section. We have now added a statement to this effect in the figure legends. It is the only appropriate way to load soluble/insoluble samples from nuclei purified from Xenopus egg extracts, because (the reviewer is correct) some of the drugs affect nuclear morphology as they affect nuclear transport, and it is therefore not possible that one can load equal numbers of nuclei *and* have equal protein amounts. We have shown however that the drugs do not affect nuclear integrity (former Figure 6A, B and EV4B, E; corresponding to new figure 5B, C, and EV4B-D) so this is not an issue.

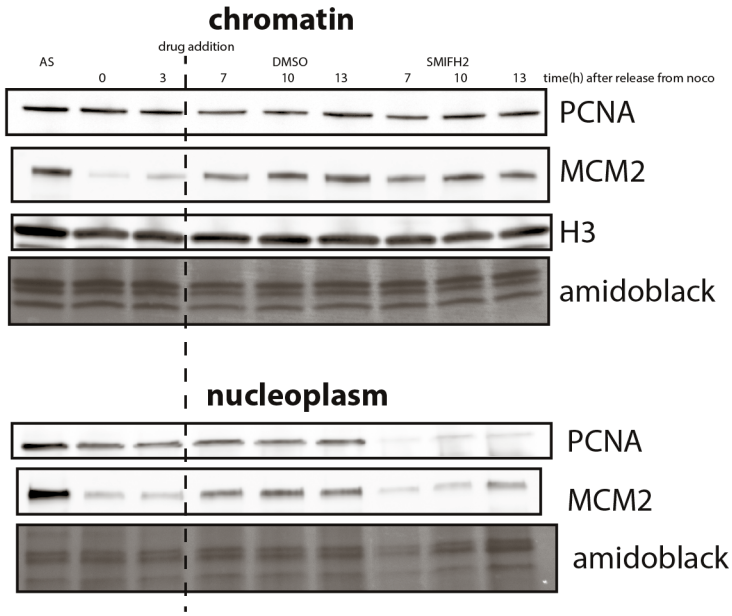
2. There is no reference to Figure 3B in the Results section.

Our reply: yes, there was, lines 136/137 in the original manuscript. We now refer to the equivalent figure (3D) twice, in lines 231 and 236.

3. In EV2C, the control lane showing how much PCNA would normally load in the absence of SMIFH2 is overloaded, as evidenced by the loading control. This undermines the conclusion drawn.

Our reply: It is true that there are some slight variations in loading. In the chromatin samples, the 13h control lane was more loaded than with SMIFH2, but it appeared that the differences in PCNA loading between these lanes were more than the difference in loading. We repeated this experiment again and while there was a very clear decrease in nucleoplasmic PCNA (and indeed MCM2) upon SMIFH treatment, consistent with reduced nuclear transport, there were no detectable differences in PCNA presence in the chromatin fraction (see image 1). Since the original data was not essential to the message and we have been asked to streamline the paper, we

have now removed this figure from the manuscript. This does not undermine in any way the conclusion drawn from the FACS analysis, and which shows complete block of the S-phase entry.



Authors' reply image 1: Upon SMIFH2 treatment, PCNA is decreased in the nucleoplasmic fraction but its levels in the chromatin fraction do not change.

4. Is the effect of SMIFH2 on S phase entry not explained by its inhibition of transcription (Figure 4B)?

Our reply: This has been re-written (lines 171-178). It is indeed very likely that the effect on S-phase entry in somatic cells is due to inhibition of transcription, which is why we analysed it. We performed additional experiments using DAD mutants of mDia to address effects of altering formin activity independently of SMIFH2. This had no global effect on transcription but did cause an increase in the S-phase cell population. Together with the effects of SMIFH2 on cells already in S-phase, the results imply that deregulating formin activity impedes S-phase progression. The data was shown graphically in original 4C and is now shown in EV1F, along with the images; as controls, we used untransfected cells in the same field. But the potential confounding effects of transcription was the main reason why we did most experiments in *Xenopus*, where the transcription is silenced.

5. No reference is made to the dnDiaNLS construct shown in Figure 4D.
Our reply: We apologise for this oversight. The dnDiaNLS construct did cause a slight increase in the fraction of EdU positive cells, but this difference from the controls (DiaDAD and untransfected) was not quite statistically significant (p=0.08). This has now been mentioned (the corresponding figure is now 1F; lines 193-4 in the text). Since the Dia.LG.NLS construct also causes an increase, it suggests that interfering with nuclear formin activity in either way impedes S-phase. However, overexpression of heterologous constructs and dominant negative constructs does

not allow much molecular insight, so we did not dwell on this. The most important point is that the cytoplasmic DiaDAD construct does not have any effect. We have also renamed the constructs “Nuc-DAD” and “Cyt-DAD” to make it easier to read.

6. Why does the R62D mutant decrease EdU incorporation (Figure 4E) but not increase the S phase population (Figure 4D)?

Our reply: the WT and R62D mutants had little effect on the percentage of EdU positive cells, unlike the S14C and G15S mutants. R62D was not statistically significantly different from WT. Taken together, it is clear that effects of WT and R62D mutant were negligible, compared to obvious effects of S14C and G15S. The text has been altered accordingly.

7. Line 169: gamm-H2AX staining provides evidence of DNA damage, but not necessarily dsDNA breaks.

Our reply: Technically the reviewer is correct as it has been reported that UV damage, which induces pyrimidine dimers, can give rise to gamma-H2A.X staining. However, it is likely that the latter is due to dsDNA breaks that arise indirectly during S-phase from the primary damage. Nevertheless, the text has been amended.

8. Line 174: the authors need to explain what is gelsolin and why is it used.

Our reply: gelsolin is a specific actin-binding protein that binds to the barbed end of filaments, arresting their dynamics, as well as severing filaments. Thus, it could be expected that it would have synergistic effects with agents like jasplakinolide, a peptide that stabilizes actin filaments. Text has been inserted to this effect (lines 478-481).

9. Figures EV3C and D should indicate the drug being added.

Our reply: for EV3C, we apologise for the legend that was obliterated by the graph, we hadn’t noticed this. It was the protein MICAL2 that was added. We will rectify this. For EV3D the legend already indicates the drug “2.4”, although the reviewer might have overlooked the definition in line 177 of the text and not noticed the figure legend, line 1177. We have added “Inh 2.4” in the figure to make it clearer.

10. Line 187: since PCNA is generally considered to mark replication sites, and actin appears to interact with PCNA, it is unclear why the authors conclude that actin is not present at replication sites. Is the actin-PCNA interaction replication-dependent? To address this, the authors could block initiation with a CDK inhibitor and repeat the experiment. As is, the data don't add anything and should be removed.

Our reply: we stated in the lines 186 and 187 that unlike PCNA, actin does not interact with either RPA or MCMs, which would be expected if it was present at replication forks, for instance. But we accept that the data of actin-PCNA interaction by PLA, which probably reflects a different pool of PCNA (e.g., Stoimenov &

Helleday, Biochem Soc Trans. 2009) did not add much and have been removed. However, we have shown PCNA – actin interaction from within nuclei in fig 6, and added data showing that CytD increases interactions with Importin beta and Nups.

11. Contrary to the authors conclusion that NUP staining is disorganized (line 199), Figure 5C indicates it is greatly reduced. Also, it appears that a highly non-representative portion of the nucleus in 5C (top right panel) was shown in the inset that is devoid of NUP staining.

Our reply: Original figure EV4A (now figure EV4F), which is a Western blot with equal nuclei loaded, shows that loading of some NUPs on chromatin nuclei is indeed somewhat reduced, whereas other NUPs show similar loading. We indeed showed a non-representative portion of the nucleus in 5C inset in the SMIFH2 sample to highlight an area where NUP staining was strongly reduced, but we highlighted this: "Upon SMIFH2 treatment, nucleoporin (NUP) staining was disorganised, with dense NUP clusters between NUP-free regions" (lines 198-199).
Now, we have inserted an additional experiment with wide-field microscopy, showing both lamina and NUPs (new Fig 5D), which was indicative that the NUP staining was not only generally reduced, but it was also differently organised. This prompted us to use the high-resolution microscopy to examine the NUP organisation more in detail. Thus, in our new figures, we have presented the 3D-SIM experiment but now with different and larger insets, again showing less homogenous distribution of NUP staining upon formin inhibition

12. These papers should be cited and discussed:

J Cell Sci. 2004 May 15;117(Pt 12):2481-90. Epub 2004 May 5.
Actin- and protein-4.1-containing filaments link nuclear pore complexes to subnuclear organelles in Xenopus oocyte nuclei.
Kiseleva E1, Drummond SP, Goldberg MW, Rutherford SA, Allen TD, Wilson KL.

J Cell Sci. 1992 Jan;101 (Pt 1):43-53.
DNA replication in cell-free extracts from Xenopus eggs is prevented by disrupting nuclear envelope function.
Cox LS1.

Our reply: we have now cited and discussed these two relevant references.

13. Imp and CD conditions in Figure 7A appear to be overloaded (compare NUP62 blot), undermining the claim that CD promotes importin-NUP interaction. Were the two sections of the blot exposed equally?

Our reply: The two sections of the blot were indeed exposed equally and came from the same gel, we have now mentioned this. Whereas NUP62 was hardly changed in Imp or CD compared to control, in controls there was no detectable importin (only background staining) but it was easily detectable in both Importazole and CD

conditions. It is, however, difficult to be extremely quantitative for interactions with IP-western blots. We have changed the text, which now reads: *Neither treatment decreased signal of importin-β, showing that NUP-importin-β binding is not inhibited, and if anything was increased, suggesting that NUP release onto chromatin might be less efficient (Fig 6A).* (The corresponding figure is now 6A).

14. Figure 7C lacks Ran loading control.

Our reply: Ran was covalently coupled to the beads used in the pulldown so is not an appropriate control. Equal volumes of beads were used, as evidenced by the fact that RCC1 and importin-beta are pulled down to similar extents in the three experimental samples (and not in the control, for which obviously no loading control is possible). We have amended the legend to explain this point.

15. Although cytochalasin D and SMIFH2 both inhibit nuclear transport (Figure 6A), they appear to do so by different mechanisms (Figure 7). Some attempt should be made to unify the observations of these two actin modulators.

Our reply: formins and CytD do not have quite the same effects on actin dynamics, either in cells or in egg extracts. In *Xenopus*, CytD increases nuclear actin levels, which binds directly to Ran-NUP-importin complexes, apparently hindering a productive interaction. Formin inhibition does not increase nuclear actin, and indeed generally causes reduced nucleoplasmic staining for many proteins. Explaining the result of Fig 6B, we indicate a possible defect in interaction between the cargo and importins in the presence of SMIFH2 (given that NUPs retain the same interaction with importin, but binding of the cargo, both PCNA and TPX2, is greatly reduced). Formins also have actin-independent mechanisms, which we relate in the discussion. Determining all the molecular details of formin action is outside the scope of this paper, but we have discussed these points.

16. The authors should cite appropriate previous papers showing that WGA inhibits replication in egg extracts.

Our reply: We have now done so, as we have cited the Cox et al. paper (point 12).

17. From Figure 8A, the authors conclude that once nuclei have formed, replication can proceed without further NPC formation. However, a critical control is missing, which is to show that in this experiment, NUP depletion was complete (which can be shown by placing sperm chromatin directly in this extract). Otherwise, it may be that the residual replication seen in WGA-bpdelta condition reflects residual de novo NPC formation.

Our reply: We already did the requested experiment; in Figure EV5B (now EV5C) we have shown that sperm chromatin does not replicate in the WGA-depleted extracts, which was part of the same experiment. We have now also included a western blot (new figure EV5B) of the depletion.

Conversely, it may be that the requirement for continued nuclear transport is due to

nuclear disruption during the transfer experiment (this caveat affects all the nuclear transfer experiments shown). It would be better to do the experiment without transfer and to simply titrate away the Geminin with recombinant Cdt1.

Our reply: this is not the case since we have shown controls (mock depletions and control conditions in all experiments), which replicate efficiently. It is impossible to do the experiment of letting the nuclei form properly before exposing them to conditions without NUPs by just titrating away geminin with Cdt1 (which does not work anyway, we have tried it) since the extract has to be depleted of NUPs – in which case the nuclei would not form properly. Nuclear transfers are the only way to perform the experiment.

To further demonstrate that nuclear transfer does not disrupt the nuclear integrity, we have performed dextran exlusion assays, comparing to detergent treatment (Triton X100), which is shown in new figure EV5D.

18. Why does the second condition in Figure 8B not replicate better? Also, the logic underlying this triple transfer experiment is not explained well. Is the purpose of this experiment to address whether SIMULTANEOUS nuclear transport and formin activity are required for replication?

Our reply: we apologise for not having well enough explained this experiment, which has now been entirely re-written, but the reviewer correctly understood. This was done to assess whether the roles of formins and nuclear transport are distinct or related. If they were distinct then the reciprocal transfers between SMIFH and WGA extracts should replicate; if they are involved in the same mechanism, they should not. Obviously the nuclei have to form in the first place, so this necessitates a double nuclear transfer. We should also mention that double nuclear transfer experiments are technically very challenging to achieve efficient replication and we are one of the few labs to do this. Therefore it cannot be expected to replicate to close to 100%. Thus, the single transfer from Geminin -> Ctl replicates at close to 100% (figure 8C), but geminin -> Ctl -> Ctl in the double transfer (triple extract condition) only reaches 40%. Some other labs use “% of control” to express the efficiency of replication for their experimental samples; we do not do this, we always quantify the replication in absolute terms.

19. The failure of the PAWGA condition to replicate (Figure EV5D) might again be due to nuclear disruption during transfer?

Our reply: again, this is not the case as the controls replicated fine. However, to streamline the paper, we removed some experiments that were not essential to the message of the paper, and this experiment is no longer present in the revised manuscript.

Also, why do the authors use this obscure CDK inhibitor? Roscovotine is much more widely used.

Our reply: Purvalanol A is hardly an obscure CDK inhibitor (it was first published in

Gray et al., Science, 1998)! We now cite the original paper. Roscovitine is more widely used since it has been around even longer and is used in clinical trials, but is less potent and less specific than purvalanol A. We have extensively characterised puvalanol A in kinetics terms against CDK1 and CDK2, the two main CDKs that promote origin firing in Xenopus egg extracts in a paper we cite (Echalier et al., Chem Biol., 2012).

20. How do the authors know that in Figure 8C, nuclear transfer does not disrupt the nuclear envelope. If this is the case, all the CDK activity would leak out, imposing a requirement for new nuclear transport in the second extract to re-accumulate CDK activity.

Our reply: again, we showed the controls, which replicate to virtually 100%. If the transfer disrupted the nuclear envelope, the control would not replicate. And we have now shown a dextran exclusion assay in new figure EV5D to demonstrate that nuclear transfers do not disrupt the nuclear envelope.

21. Why does SMIFH2 inhibit DNA replication in 9D but barely at all in EV5E? The result in Figure 9E does not explain the total block in replication shown in Figure 9D.

Our reply: We explained in the methods that unless otherwise stated, SMIFH2 is used at 500μM to achieve a complete block. These were not the same concentrations that were used in EV5E (now EV5G). In this experiment SMIFH2 is used at 200μM, as compound 2.4 is not very soluble and we could not achieve higher concentrations, so we compared it to SMIFH2 at the same concentration. We have added text to this effect. Incidentally, this shows that the SMIFH2 effects are dose-dependent. The main text has also been rewritten.

There must be a step that is inhibited downstream of Cdc45 loading. The authors should check GINS, RPA, polymerase etc to determine where the block occurs.

Our reply: we agree with the reviewer, these were good suggestions. However, we felt that our chromatin preparations from NPE might not have been clean enough and the baseline for these proteins might have been too high. Thus, LK spent two weeks in Puck Knipscheer's lab in Utrecht learning NPE preparation. We optimised chromatin preparations, and our experiments now show that although low levels of Cdc45, RPA and PCNA were initially loaded onto chromatin, they were rapidly lost. This mirrors the unloading of the same proteins in low speed extracts in SMIFH2 (Fig 4D). We have also previously published (Krasinska et al., 2009) that the same proteins can be loaded on to chromatin in high-speed extracts, *ie* in the absence of a functional nucleus, and therfore have no absolute requirement for nuclear function for their chromatin association – but simply to achieve normal levels. We now cite this earlier work. Finally, since formin inhibition clearly has drastic effects on chromatin loading of replication proteins, we have tested whether it also affects replication of single stranded DNA in high-speed extracts: a model for replication elongation. New figure EV5H shows that formin inhibition also considerably slows elongation.

22. The discussion was not very informative, as it contained almost no discussion of how actin/formin might regulate replication.

Our reply: This has been almost entirely re-written and should now be much improved.

Referee #2

Parisis et al. describe a novel role for actin and actin-assembling formins in DNA replication. The onset for the project is a mass spec study from Xenopus nuclear extracts, which identifies several actin-regulators in replicating nuclei. In their studies, the authors use both Xenopus egg extracts (XEE) and somatic mammalian cells. The XEE system is transcriptionally silent, which allows the authors to uncouple the potential role of actin in gene expression from its role in replication. Moreover, the XEE system permits elegant sequential inhibition experiments, which would not be possible in a whole cell model. The authors show that in mammalian cells, nuclear actin displays cell cycle-dependent dynamics, with actin filament formation at the early G1 phase of the cell cycle. By using actin-binding drugs, actin-regulators and formin-manipulation, the authors then show that nuclear actin dynamics (and formin activity) are required for DNA replication. Their studies further point to a role in both nucleo-cytoplasmic transport, which is critical for DNA replication, as well as in a subsequent steps of chromatin loading of replication factors and initiation of replication. Mechanistically actin seems to interact with Ran GTPase, with implications on cargo-release during nucleo-cytoplasmic transport.

Overall, this study is very interesting and reports a completely novel function for actin dynamics and formin activity in DNA replication. The use of two very different experimental systems supports the generality of the findings, and the XEE system permits very elegant experiments, such as the transfer of nuclei between differentially treated extracts. The experiments are carefully done, and link between actin/formin activity and efficient DNA replication is obvious. Also plausible mechanistic explanation regarding the nucleo-cytoplasmic transport is provided. This study thus broadens the functional implications of nuclear actin and its regulators, and is therefore very important to the field.

Our reply: We are glad that the reviewer perfectly understood the paper and its significance in the field and is convinced by our experimental demonstrations.

The major problem with the manuscript at the moment is that it is very difficult to read and follow, due to the huge amount of data and juggling the two different experimental systems. I suggest that the authors simplify the figures, by carefully evaluating which experiments are absolutely necessary to prove their point. In addition, the figures should be labeled clearly so that it is immediately evident, which system (XEE or mammalian cells) is used. For example in figure 5, it is impossible to tell from the figure and figure legend, which system is used in each panel without reading the text. The introduction lacks intro to the replication process, which would make the manuscript easier to follow for readers not specialists in this topic.

Our reply: These are good suggestions. We agree that the switching and juggling between two different systems would probably confuse the reader. Thus, we have re-ordered the paper so that there is only a single change: we start in somatic human cells, and then move to *Xenopus* to study transcription-independent roles of actin dynamics and formins. Second, it has also been almost entirely rewritten to improven the understanding of the experimental logic. We have, as requested, improved the introduction for non-specialists in replication. Third, we have eliminated quite a number of experiments that were not central to the story, and moved some experiments to extended view figures rather than keeping them in main figures. The main figures therefore gain in simplicity and impact (*e.g.* Fig 4).

One confusing fact is that some treatments, especially the formin inhibitor SMIFH2, seem to operate differently in the two systems. In XEE, SMIFH2 does not seem to affect nuclear actin amounts or polymerization, but in mammalian cells it seems to stabilize the nuclear filaments. So in one case, one would be looking at formin-inhibition that is not affecting actin, and in the other case, an indirect effect of stabilizing nuclear actin filaments?

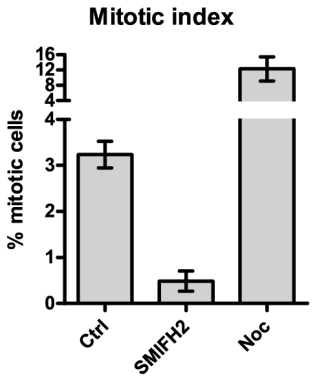
Our reply: Again, this is more or less exactly what we believe. We do think that SMIFH2 affects nuclear actin dynamics in XEE but since there are no actin filaments in these nuclei, they cannot be stabilised; this has been better discussed. As regards SMIFH2 response, XEE nuclei are similar to S-phase somatic nuclei. The apparent differences between somatic G1 nuclei and XEE nuclei in the intrinsic nature of nuclear actin, and hence the effects of SMIFH2, is likely related to their functional differences. Somatic nuclei in G1 are formed from post-mitotic chromatin, and are transcriptionally competent, whereas XEE nuclei are formed de novo from the virtually naked chromatin of sperm, and replicate but do not transcribe. The transient G1 nuclear actin filaments of somatic cells might well be a way of sequestering monomeric nuclear actin to allow transcriptional reactivation. This has been added to the discussion.

What effect does the other formin-inhibitor, 2.4, have on actin in these two systems?

Our reply: this is a good suggestion, we have done these experiments. In *Xenopus*, inhibitor 2.4 caused similar effects on nuclear actin levels to Cytochalasin D and jasplakinolide, increasing both soluble and insoluble actin, but it did not cause nuclear filament formation (new Fig 3E, F). However, 2.4 is rather insoluble and cell impermeable and cannot be used in living cells (this was published in the original paper, Gauvin et al., Biochemistry, 2009, which we cite; and was also mentioned in the Baarlink et al., Science paper, also cited; anyway we verified using chromobody-expressing cells, and found that it had no effect on nuclear actin dynamics).

The interpretation of the results from SMIFH2-treated mammalian cells is problematic, since SMIFH2 most likely affects also cytoplasmic actin. For example in 5B, SMIFH2 treatment is shown to have an effect on nuclear morphology. Did the cells divide during the experiment?

Our reply: we completely agree; this is why we did not analyse SMIFH2 much further in mammalian cells, especially since we found that it abolishes transcription which confounds a clean analysis of its effects on DNA replication. At no point in the manuscript did we attribute the observed effects of inhibitors exclusively to the nuclear actin. In the experiment in original figure 5B, the treatment was for 4 hours only and then the cells were fixed. However, from other experiments shown we know that cells treated with SMIFH indeed do not divide, which is shown in Movies EV3 and EV4, and has now been mentioned explicity. We provide data below (but have not included it in the paper) that confirm that formin inhibition in cells does not allow progression into M-phase.



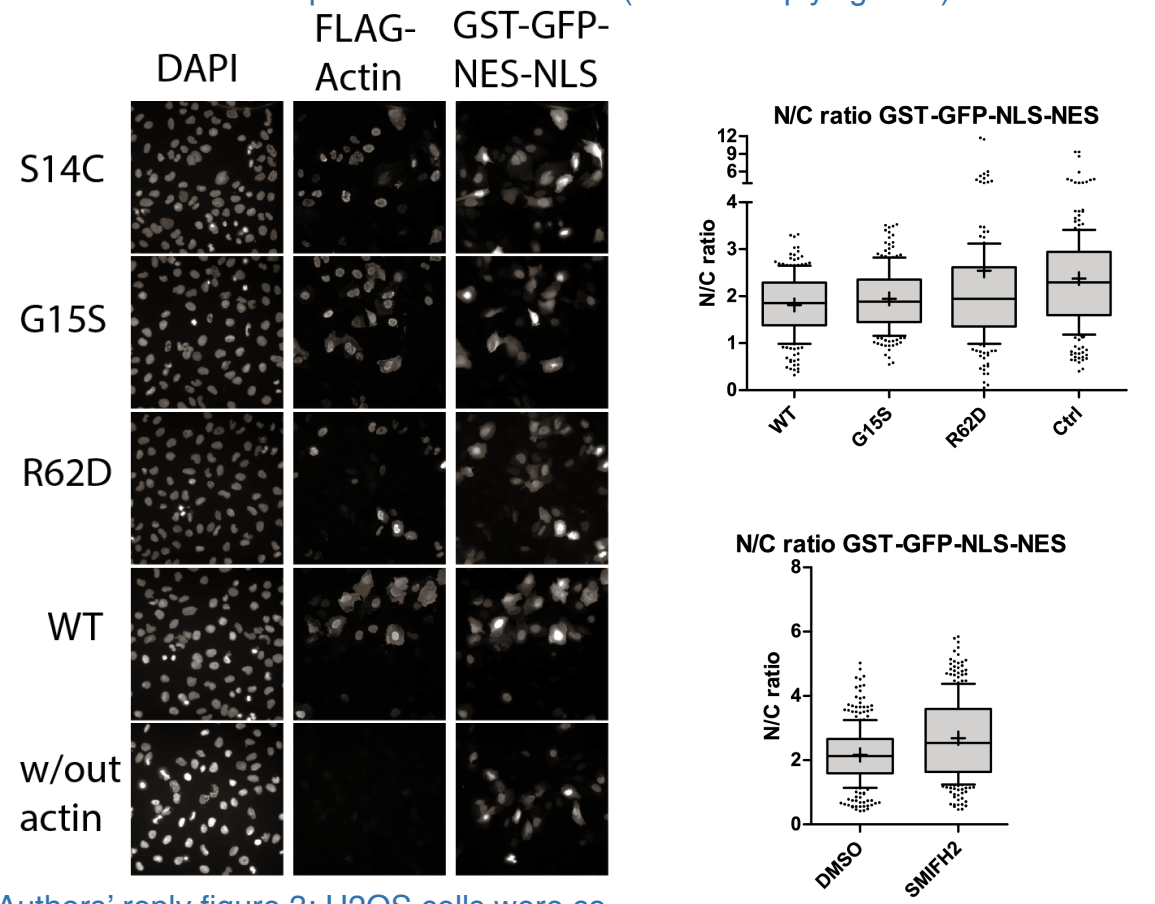
Authors' reply image 2: SMIFH2 treatment eliminates mitotic onset. U2OS treated with DMSO or SMIFH2 or nocodazole (for positive control) for 6h when cells were fixed with PFA and DNA was stained with DAPI. Mitotic cells were counted, n=2, >5000 cells per condition, mean±SD.

The finding that actin dynamics plays a role in nucleo-cytoplasmic shuttling by regulating Ran-cargo release is very interesting, but the experiments in mammalian cells are not very convincing. In addition (or instead of) of looking at an endogenous shuttling protein, which can be regulated by cell morphology as pointed out by the authors, the authors should look at an engineered construct (like they use in XEE), such as NLS-GFP-GST-NES.

Our reply: To obtain clear and quantifiable results, we needed an inducible system of nuclear transport. Originally we acquired and tested Rebecca Heald's ionomycin-inducible GFP-NFAT nuclear translocation system, but this did not work well at all. The ionomycin inducibility of the translocation was not obvious, it was also very leaky, and importazole did not inhibit it. This was why we looked at NFkB, which for us had the advantage of being a highly physiologically relevant nuclear transfer of an endogenous protein, and cytokine treatment bypasses any effects of cytoplasmic actin dynamics on signalling. Besides, this also allowed us to study the effect on nuclear transport in non-transformed cells. It should be noted that in this experiment we used 1h treatment with SMIFH2, and at this timepoint the nuclear morphology was not altered significantly (as was the case after 4h treatment in the original figure 5B).

Nevertheless, we tried the experiment suggested by the reviewer, since it was possible that arresting nuclear import of this construct would result in loss from the nuclei, if it shuttles as expected. We obtained the original pC3 RevNES-GST-GFP-SV40 NLS construct (Knauer et al., Traffic, 2005) from Roland Stauber's lab and measured the nuclear:cytoplasmic ratio in transfected cells. However, this was variably cytoplasmic and nuclear in different cells. In the original paper, only

leptomycin B treatment to inhibit export caused nuclear accumulation, but we could not do this for obvious reasons. Furthermore, in this paper, drugs that were previously reported to affect nucleo-cytoplasmic transport had no effect on the nuclear accumulation. Thus, we were not particularly surprised to find that we did not see variations in nucleocytoplasmic shuttling of this construct between the mutants nor with SMIFH2. We present the data here (authors' reply figure 3):



Authors' reply figure 3: U2OS cells were co-transfected with the GST-GFP-NES-NLS construct and NLS-tagged actin mutants, and after 30 hours cells were fixed and stained. For SMIFH2 treatment, transfected cells were treated for 90 minutes. For quantification, nuclear fluorescence was determined in the area stained by DAPI, and an expanded ring around this was used to measure cytoplasmic fluorescence. Untransfected cells were used to establish thresholds. N = 2, 500 cells / condition, whiskers in box plots show 10-90 percentiles.

As mentioned above, SMIFH2 likely also affects cytoplasmic actin. If the SMIFH2 functions here by stabilizing nuclear actin filaments, does expression of NLS-G15S/S14C actin mutants also impair nucleo-cytoplasmic shuttling?

Our reply: We indeed tried using the S14C and R62D mutants on NFkB nuclear translocation in HeLa cells and did not observe any effect of the mutants; we did not include this data since at the moment we cannot say whether this is due to insufficient strength of the effects of the mutants compared to chemical blocks (which could in part be due to technical issues, such as negative feedback on import of the actin mutants), or whether it is cytoplasmic actin that controls nuclear transport.

Note, however, that CytD did not inhibit NFkB translocation in cells at the concentrations we used (original figure 6, now Fig 2C, D). Since nuclear transport is very highly conserved, we felt that XEE were best suited for these experiments.

The actin filament formation in early G1 is very interesting, but it is not clear how this fits with the rest of the story.

Our reply: We understand why the reviewer would say that, but it is important since it allows us to better understand the effects of the drugs on actin dynamics, and the apparent differences between *Xenopus* and somatic cells. The S-phase nuclei, whether in somatic cells or Xenopus, do not form actin filaments when treated with SMIFH2. SMIFH2 probably only changes the dynamics of existing filaments, as shown in the Rizvi et al paper, which we cite. However, we agree that it is not central to the story and have removed this from the abstract.

In the discussion, the authors speculate that "deregulated nuclear actin dynamics, rather than an increase in nuclear actin levels or filament formation per se, prevents DNA replication". What is actually meant by this?

Our reply: we apologise for the rather terse discussion. This, in particular, was not a well-worded sentence. The discussion, and, indeed, most of the paper, has been completely rewritten. We showed, for example, that jasplakinolide, or addition of purified Arp2/3 complex and GST-WASP, cause a similar increase in nuclear actin levels to cytochalasin D, but does not induce formation of filaments nor block replication, whereas Arp2/3 complex and formin inhibition block do not promote filament formation either but do block DNA replication. The common denominator is that all these agents disrupt actin dynamics: i.e. the polymerisation/depolymerisation equilibrium. Rebecca Heald's lab claimed in 2003 that latrunculins, which prevent actin polymerisation, block DNA replication, although the data were not shown in their paper. We could not reproduce this with the concentrations of latrunculins that they used (in fact we found rather the opposite, that we could rescue the block caused by cytochalasin D). We do, however, find a block with higher concentrations of latrunculins. But not to complicate the story further, we have not included this data. We also removed data using the CK666 Arp2/3 inhibitor and its inactive analogue, that also block DNA replication, since we have not further studied the possible interplay between the Arp2/3 complex and formins. The data that we do show could be perceived as being consistent with the possibility that nuclear actin filaments abolish replication, since under no circumstances in which we see strong nuclear actin filament formation do we see efficient DNA replication. But we think it is likely that filaments simply sequester actin monomers that might promote DNA replication, directly or indirectly.

In Serebryannyy et al, 2016, it was speculated that persistent nuclear actin filaments decrease nuclear actin monomer levels, and this causes the transcription defect in their system. Do the authors speculate that something like this is taking place also in their system?

Our reply: Yes; see above.

Reply to reviewers; referee #2

Minor points
SMIFH2 is abbreviated differently (SF or FH) in different figures.
In EV3C, Mical-2 text below the x-axis is not visible.

Our reply: Thanks for pointing out these minor labelling and formatting errors, we have corrected them.

Referee #3

(Report for Author)
The authors of this study look into connections between DNA replication and the actin cytoskeleton. The make the claims that nuclear actin filaments form in G1 of human cells, that stabilisation of nuclear actin filaments blocks nuclear transport and DNA replication, that actin directly binds Ran-importin complexes and is required for cargo release, and that nuclear forming activity is required for DNA replication.

Unfortunately, however, these claims are neither plausible nor are they supported by conclusive evidence. Instead, the study is an example of wishful thinking and inadequate experimental methods.

Our reply: We are very surprised by these statements, which strongly contrast with those of reviewers 1 and 2. For a long time, almost nobody believed in nuclear actin in somatic cells due to the difficulties in visualising it with the available technologies and the overwhelming amount of actin in the cytoplasm. But nuclear actin has since been purified multiple times as a component of a variety of transcription complexes, chromatin remodeling complexes, etc; both import and export transporters have been identified; numerous imaging probes have verified its existence (including phalloidin staining). The key is to look for the nuclear actin filaments at the right time (20-30 seconds after serum stimulation, during cell attachment (both from R. Grosse lab), and upon cytokinesis in the small daughter cells that are normally ignored during immunofluorescence experiments (this study). These transient polymerisation events would not be visible without imaging probes that do not strongly disrupt actin dynamics. We (and the Grosse lab) validated with phalloidin that the filaments we see are indeed actin.

Specific points:

Introduction: it is remarkable that the author introduce the issue of nucleocytoplasmic actin distribution without a single mentioning of exportin 6 (Xpo6), which excludes actin from nuclei in somatic cells.

Our reply: This comment is not justified since we had cited the Stuven et al., 2003 paper on Xpo6 in the introduction. We are well aware that Xpo6 exports actin from the nucleus of many cell types, but not in the giant nuclei (GV, or germinal vesicles) of *Xenopus* oocytes, which do not express it. But we cannot agree that Xpo6

expression means that there is no actin in the nucleus of somatic cells, since there is now overwhelming evidence to the contrary.

It is also remarkable that the authors did not mention that an unbiased phalloidin stain gives a bright cytoplasmic but no nuclear signal.

Our reply: We did not omit this. We stated in lines 63-65 in the text: “Physiological nuclear actin polymerisation remains poorly characterised due to difficulties in staining nuclear actin with phalloidin”. Indeed, this is probably why it was thought not to exist for so long. We cannot say that there is no nuclear signal because we can detect it in G1 cells using glutaraldehyde fixation with confocal microscopy, which we showed in Figure 2I (this data is now in Fig 1B).

The authors also fail to introduce that Xenopus oocytes are special in that they lack Xpo6 and thus do not exclude actin from their nuclei. Xpo6 reaches normal cellular levels not before stage 7 of embryonic development (see figure 4, Bohnsack et al. (2006), doi: 10.1038/ncb1357). It is thus not surprising that nuclei reconstituted from Xenopus egg extract are still compromised in their ability to exclude actin.

Our reply: we are not working on *Xenopus* oocytes. We stated this explicitly in lines 65-69, where we point out the unusual structure of oocyte germinal vesicles and the presence of actin filaments. Xpo6 is abundant in *Xenopus* eggs (and therefore also in the egg extracts), as shown in the Figures 3 and 4 of the same paper that this reviewer refers to. Independently, there is overwhelming experimental evidence for nuclear actin in the literature.

In fact, the lab of Rebecca Heald reported earlier that nuclei assembled in XEE contain nuclear actin (Krauss et al, 2003). Furthermore, Krauss et al. showed that latrunculin A interfered with nuclear assembly and DNA replication. It is unclear why this paper was cited only for a methodological detail.

Our reply: We are very familiar with this paper. However, not only was there not even a beginning of an analysis of any mechanisms, and nuclear assembly and transport was not tested, no data on DNA replication was presented (it was mentioned as “data not shown”). Besides, only a single approach was used to manipulate actin (adding 0.1mM latrunculin A). We could not find any inhibitory effect of this concentration of latrunculin A – rather the opposite, in fact. Nevertheless, we of course cited this study.

The authors also fail to mention that a comprehensive study for identifying nuclear actin-binding proteins has been published before (Samwer et al., 2013, doi: 10.1038/emboj.2013.108).

Our reply: We felt that the Samwer et al. article was not very relevant to our study. This paper identified a number of actin binding proteins in Xenopus oocyte nuclei, which we are not working on. In contrast, we studied the entire nuclear structural proteome in the replication-competent nuclei assembled in egg extracts. We do cite a much more closely related study, that of Khoudoli et al., 2008.

2. Figure 1: The authors intend to identify intranuclear actin-binding proteins by a proteomics analysis of in vitro assembled nuclei.

Our reply: This is a misrepresentation of our experiment, which was not done to identify intranuclear actin binding proteins, but to assess possible cell cycle regulation of nuclear assembly (original version, lines 92-97). As mentioned above, we report a very complete proteomics dataset of nuclear assembly in the presence or absence of CDK activity.

Although it is not detailed in the methods, I guess, the nuclei were simply isolated by centrifugation.

Our reply: The nuclei were isolated by differential sedimentation through a 24% sucrose cushion, as explained in the methods section for the nuclear transfer experiments (original version, lines 553-559).

Analyzing such pellet is, however, rather problematic: How do the authors distinguish proteins that are enclosed by nuclear envelope (true intra-nuclear proteins) from actin structures that associates from the outside or indeed simply polymerised actin? Without rigorous controls, such analysis is meaningless.

Our reply: We confirmed localisation of actin and actin regulators directly by imaging, including 3D imaging, as shown in original figure 2, now Fig 3. Contamination by cytoplasm was negligible in both proteomics data (we could state the numbers in the text) and western blotting for tubulin (original Figure 2B, now 3C). Furthermore, this figure and the original figure 3A (now EV3A) as well as the new Fig 3C, show that actin was present in nucleoplasm (supernatant of lysed nuclei) and is thus not cytoplasmic. Besides, actin was not present in chromatin preparations in the absence of added sperm DNA (original figure 4J, now Fig 4B), and we now show that the same is true for Arp3 and cortactin (new Fig 3B). Therefore, it is not simply insoluble cytoplasmic actin.

A similar concern applies to Figure 2B; 3A; 4J; EV3F; 7A, B, C, F; EV4A etc. In these experiments, the authors should have thoroughly tested if their fractionation procedures are suited to cleanly (!) discriminate between nuclear and cytoplasmic actin pools.

Our reply: In Figure 2B we showed WB for tubulin to control for cytoplasmic contamination; there was none. In Figure 3 we show that trace labeling to image nuclei for actin in different conditions reproduces exactly the effects of drugs on WB for nuclear actin. We could also show immunofluorescence using the antibody 2G5 that recognises both nuclear and cytoplasmic actin. We also showed proximity ligation assay for endogenous actin (original EV3G); this has been removed for sake of space. In origianl figures EV3F and 7A we did not look at actin at all. In original Figure 7B we performed immunoprecipitations from lysed nuclei with NUPs, so it is not possible to discrimate any actin that could associate with NUPs on the cytoplasmic side of the envelope from the nuclear side. In original Figures 7C and 7F

we performed Ran or active Ran pulldowns and also found actin. Original Figure 2A (now 3A) shows evidence for nuclear actin regulators by direct immunofluorescence of purified nuclei. Thus, taken together, there is ample evidence for actin and actin regulators in nuclei in *Xenopus* egg extracts.

3. Figure 2A: What are the specificity controls for antibodies and IF-conditions used? Given that the authors suggest the Arp2/3 complex showed nuclear accumulation, while others clearly found that this complex is well excluded from Xenopus nuclei (see e.g. Samwer et al., 2013) I am having serious doubts.

Our reply: All of the known actin regulatory proteins we tested were localised to nuclei by immunofluorescence, using well characterised antibodies. As we are working with purified nuclei this allows us to eliminate cytoplasmic staining, which would obviously be much stronger and thus obscure any or most of the nuclear staining. We think it is highly unlikely that all the antibodies we use are non-specific, and indeed we can see very specific staining. As we mention above, we felt that the Samwer et al paper is not relevant as it looked at oocyte nuclei, which are structurally completely different. More relevant is the Khoudoli et al study (cited), where Arp2/3 was largely present on chromatin.

Indeed, the website of the manufacturer of the AB (AbCam) shows a clean cytoplasmic staining. The authors apparently miss the specific (cytoplasmic!) signal, because they look only at nuclei.

Our reply: The Abcam website shows somatic cells, which have a cytoplasm. We are not interested in looking at cytoplasmic staining. This is exactly why we isolated nuclei from egg extracts, otherwise we would indeed be unlikely to be able to see specific nuclear staining due to the more abundant protein present in the cytoplasm and thus more intense signal from cytoplasm.

4. Statement 'We ... show that nuclear actin filaments form in early G1'. This is obviously an artifact of the detection methods. The expression of NLS-fused actin-binders (Lifeact or the anti actin chromobody) will necessarily cause nuclear import of actin and thus severly alter the nucleocytoplasmic distribution of actin.

Our reply: As mentioned above, we stained nuclear actin filaments with phalloidin in cells not expressing the chromobody probe (original Fig 2I, now figure 1B). Robert Grosse independently developed (and published) the same tool, and also has shown that cells not expressing any probes have nuclear actin filaments that can be visualised with phalloidin. It is partly the recent development of such nuclear-specific probes that have moved the nuclear actin field forward (Baarlink et al. 2012, Plessner et al., 2015). It seems highly unlikely that any actin bound to an antibody and then transported into the nucleus would subsequently be able to polymerise, or that an antibody would radically alter the nuclear actin concentration. If it did have this ability, then we would observe nuclear actin filaments in the entire cell cycle.

We had indeed found that NLS-Lifeact binding peptide does disrupt actin dynamics, showing long nuclear cables, probably by preventing depolymerisation of the G1

filaments. We do not, however, think it likely that either tool provokes de novo actin filament formation. More likely Lifeact prevents depolymerisation. The antibody does not do this, since the filaments depolymerise, as we show, and the cells divide and proliferate without any alteration in their cell cycle length. To avoid confusion between the two tools, and knowing that Lifeact is not just a tool staining actin filaments but also perturbs actin dynamics, we have removed the Lifeact data from the revised version of the paper.

Such bias can be avoided by first fixing the cells and then staining acti-filaments with fluorescent phalloidin. It has been known for decades that nuclei of undisturbed cells are phalloidin-negative. This applies also to S-phase.Another way of looking at the issue in an unbiased manner would be to expressed the chromobody of LifeAct without the NLS. Both actin-sensors would actually be small enough to enter nuclei by passive diffusion.

Our reply: As mentioned above, we performed exactly this experiment. We used glutaraldehyde fixation and confocal microscopy and the results were shown in Figure 2I, now in Fig 1B. We indeed show nuclear filaments without any ectopic expression of probes. When using actin sensors without NLS, the cytoplasmic signal masks the nuclear signal, and such transient actin filaments are missed.

5. Statement: 'treatments that stabilize actin filaments abrogate nuclear transport'. This does not make any sense, and from my own experience I can tell that nuclear import works just perfectly fine in the presence of phalloidin (the most potent filament-stabilizing drug).

Our reply: Perhaps this was not the most perfectly worded phrase. What we meant was that the treatments that we tested which stabilise filaments also abrogated nuclear transport and DNA replication. It is not surprising that the nuclear transport works perfectly fine in the presence of phalloidin, since phalloidin is not cell permeable. We present a detailed disclosure of careful experimental results in several systems, and we cannot accept their dismissal on the basis of results that cannot be cited.

6. The authors state that staining of fluorescently labelled DNAse I (meant to detect actin) perfectly overlaps with the DNA staining. Are the authors able to exclude that the probe they used actually binds DNA?

Our reply: In our sentence (line 110) we merely state that DNAse 1, which has high affinity for G-actin (indeed, it is often used as an actin probe), mainly labels chromatin. We did not say that DNase I staining perfectly overlaps with DNA staining. Precisely because, as the reviewer suggests, we cannot exclude that it binds DNA. Nevertheless, the literature on the use of DNase I specificity for G-actin is extensive (e.g. Zechel et al., 1980 Eur J Biochem; Kabsch et al., 1985 EMBO J). But again, we have removed this data in the more streamlined revised version.

7. If the authors want to make the point that nuclear actin filaments would be required for DNA-replication

then they should test this without messing with cytoplasmic actin structures. In fact, this experiment is very simple and requires just the addition of Xpo6 to the nuclear assembly reactions. It the authors' conclusion is correct, then one would expect a complete block of replication at sub-micromolar concentrations. Given however that these are conditions found in somatic cells, I doubt that nuclear exclusion of actin would compromise replication.

Our reply: At no point in this manuscript have we tried to make this point, since we do not believe it to be true. Nowhere did we suggest that nuclear actin filaments are required for DNA replication. On the contrary, we showed that they are, and probably have to be, disassembled prior to DNA replication. Thus, we would not expect adding Xpo6 to a nuclear assembly reaction to have any effect on DNA replication. We also feel that implying that we are “messing with cytoplasmic actin structures” is a misrepresentation of our work.

8. The authors claim that nuclear formin activity would be required for DNA replication. Which formin is this supposed to be and what is the evidence for such claim? The authors fail to recognize that a requirement cannot be proven by ectopic over-expression.

Our reply: In human cells, we did not ectopically overexpress formins, we used the DAD fragment of mDia2 to activate the endogenous activity (by relieving each formin molecule from the autoinhibitory cis-interaction of DID-DAD domains) specifically in the nucleus. This unambiguously affects the activity of mDia2 (Baarlink et al., 2012).

Since formin inhibitors block onset of S-phase in somatic cells but this may be indirect, we can only study this in the Xenopus system (so far virtually everything found to be required for DNA replication in eukaryotes is conserved between species). Two completely different formin inhibitors with different chemical structures prevent DNA replication in nucleoplasmic extracts, as shown in original Figures 9D and EV5E (now 8D and EV5G, respectively). Although we have identified nuclear Xenopus homologues of diaphanous 1 (mDia1), diaphanous 3 (mDia2) and formin 2, as we report in this paper, we cannot exclude that other formins are present and we do not yet have tools to deplete them. We have tried specific depletion of Xenopus formins from the nucleus using available antibodies against mammalian formins, but we could not achieve a reasonable level of depletion. Inhibitor 2.4 is highly specific as it inhibits mDia1 and mDia2 but does not inhibit mDia3 (Gauvin et al), while SMIFH2 inhibits FH2-domains (which are present in all homologues that we found). Which formin is involved in these phenotypes will be addressed in future studies, but is outside the scope of this paper.

9. Figure 3: The finding that CytD has the same effect as Jasplakinolide, namely inducing nuclear actin polymerization is somewhat unexpected.
My interpretation of the authors' explanation is that CytD exclusively acts on cytoplasmic actin filaments, giving rise to a high level of monomeric actin that can enter assembled nuclei. What would prevent CytD from entering nuclei? And why do the authors add such a high concentration of the drug (400 micromolar)? Given that Cytochalasins are sparsely soluble in water this seems to be an inappropriate

concentration. Why was latrunculin A not active at all, even at 100 μM? I am sorry to say, but such inconsistent pharmacology does not support the authors' conclusions.

Our reply: In lines 364-372 of our discussion we provided an explanation of the pharmacology and the doses of drugs that are required to elicit effects in *Xenopus* egg extracts. Latrunculin was active, in contrast to the reviewer's claim, as it decreased the total and insoluble actin fractions in the nuclei and it reversed the nuclear actin polymerisation provoked by CytD. It did not, however, block replication at these concentrations, providing evidence that polymeric actin is NOT required for DNA replication (see also point 7).

We do not believe that CytD exclusively acts on cytoplasmic actin. The cytoplasmic actin cytoskeleton is not at all disrupted as actin filaments are conspicuously formed. I also do not see what would prevent CytD from entering the nucleus. Besides, cytochalasins are NOT actin depolymerising drugs, they bind the barbed end of filaments preventing further polymerisation and depolymerisation. Their effects on cellular actin have been very well characterised (for example, see John Cooper's review on effects of cytochalasins on actin, 1987, J Cell Biol 105, 1473-8, and Manfred Schliwa, 1982, J Cell Biol 92, 79-91; to quote, "Cytochalasin D treatment severely disrupts network organization, increases the number of actin filament ends, and leads to the formation of filamentous aggregates or foci composed mainly of actin filaments").

10. Figure EV4A: The small differences in band intensities do not support the conclusions given in the main text.

Our reply: Our main conclusion from original figure EV4A (now fig EV4E) is that most NUPs were present. We merely factually reported the variations that we observed without ascribing any conclusion to them. Our conclusions are derived from other experiments: 3D-SIM and electron microscopy.

11. Figure 7A: The western blot provided does not support the conclusion that CytD treatment increases binding of importins to FG Nups. The 'CD lane' is similar to the 'Ctrl lane'.

Our reply: We disagree, the figure (now 6A) clearly shows that importin-NUP binding is increased.

12. Figures 7B, C, F: How specific is the postulated interaction between actin and FG Nups (Figure 7B) or Ran (Figures 7C, 7F) in the presence of CytD? Already the input lanes contain elevated levels of actin. Furthermore, the Mock control for this specific condition is missing in all three experiments. This is a concern, since IPs were performed in PBS, i.e. under conditions that might favour actin (re-) polymerisation. It might be really hard to distinguish between specific binding and unspecific sticking of precipitated filamentous actin to the beads.

Our reply: IPs were not performed in PBS, they were performed in the extract. Actin is not especially abundant in the nuclei but indeed, as the reviewer points out, might stick non-specifically to beads, as might any protein. This is why we do mock IPs. The point of the experiments the reviewer refers to was not to analyse actin-NUP or actin-Ran interactions, but to look at NUP-importin and importin-cargo interactions in 7B (now 6B), and Ran-importin and importin-cargo interactions in 7C (now 6C). In 7F (now 6D), the point was to test whether active Ran was increased. Actin in 7F (6D) effectively serves as a loading control. The only experiments where we tested whether Ran could directly bind actin were 7D and 7E (in the revised version we have only retained the latter, now 6E).
To analyse actin interactions in nuclei, the suggestion of the reviewer, to do a mock IP with CD, would be a good one. We now show equivalent data using pulldowns with biotinylated Lifeact, an actin binding protein, from nucleoplasm (new figure 6F). In the absence of Lifeact peptide, no actin, NUPs, PCNA or importins are pulled down, showing that this is specific, while CytD strongly increases pulldowns of all these proteins.

13. Figure 7D, E: I have serious doubts that the interaction between actin and Ran is specific and would occur under conditions found in the cytoplasm. As the authors show, actin also binds to GST control alone. It is known, however, that actin is part of a functional export complex that in addition to Ran depends on the presence of exportin 6 and profilin. In light of this a binary interaction between Ran and actin seems highly unlikely.
Indeed, this would be first 'cargo' that shows direct interaction with Ran in the absence of its transport receptor. The authors do not provide any evidence for that, but rather show a low affinity and probably unspecific interaction between two proteins.

Our reply: We do not understand why the reviewer questions whether Ran-actin interactions would occur in the cytoplasm; Ran is a predominantly nuclear protein involved in controlling nuclear transport. The important point that we show is that Ran-actin interactions occur in the nucleus. Yes, there was a background actin band in GST pulldowns in original figure 7D, but this was far lower than the amount bound to GST-Ran. This is real data! Actin does not strongly bind to GST alone, as shown in original figure 7E (now 6D). And we did not make claims from the experiment with purified recombinant proteins that direct Ran-actin interactions do occur, simply that they can occur. If this experiment had not worked, we could have ruled out a direct binding of actin to Ran.

While it is interesting that both SMIFH2 and CytD affect nuclear transport, the experiments presented in Figure 7 illustrating that monomeric actin itself inhibits nuclear transport by binding to Ran are not convincing. There might be other targets and/or other factors, released upon actin depolymerization, that need to be identified. Alternatively, especially at the high concentrations used in the XEE system the drugs might have side-effects, even on NPC function. Overall, the mechanistic details as to how NPC assembly and nuclear transport are affected remain unsolved to me.

Our reply: In these conditions, cytochalasin D does not depolymerise actin: as we

mention above, it prevents depolymerisation. Of course, there are mechanistic details left to be worked out, and we do not claim to have solved them all in this paper. I also refer the reviewer to our earlier comments on drug concentrations. Besides, we have controlled for the specificity of action of drugs. For example, we can rescue CytD effects with latrunculins or cofilin, as shown in Fig EV3B, C, and specific agents show synergy, *e.g.*, CytD + Jspk, CytD + gelsolin (EV3D).

14. Figure 9A - C: The experiment is really difficult to understand, also because the rational behind it does not make much sense. Are all factors required for DNA replication shuttling proteins that would enrich in nuclei upon CRM1 inhibition? If yes, then leptomycin B treatment might indeed temporary uncouple replication from the necessity of having ongoing nuclear transport. In this case, nuclei should also replicate in the presence of WGA. Has this been tested?

Our reply: We apologise for not having explained the experiment clearly enough, but it is quite simple. Since previous experiments showed that CytD or SMIFH2 prevent nuclear transport, and that the essential replication factor PCNA is lost from the nuclei rather due to loss of the dynamic equilibrium between import and export, the results on replication thus far could potentially all be explained by loss of nuclear transport. The rationale behind this experiment was to rescue PCNA concentrations (and those of anything else that might have been lost) to see if the actin drugs would now no longer prevent replication. They still do, and we show that CDK and PCNA cannot load onto chromatin in this case. Adding WGA will of course prevent import of PCNA and everything else, and we showed in Figure 8 (now figure 7) that ongoing nuclear transport is required for DNA replication, although its effects might indeed be lessened by inhibiting export with CRM1. But such an experiment would probably not tell us anything other than sufficient net nuclear import is required for DNA replication, which we already know. It would tell us nothing about the role of actin dynamics or formins.

Referee #4

I read the paper and I do agree with reviewer 2 that it is quite difficult to read and follow. The authors should really make a large effort to make it easier to understand and select carefully the data that are essential, making it very clear when they go from one system to another and explaining the differences. In particular it would be essential to explain well the specifics of the Xenopus egg extract (which is in S phase and does not have G1 nor G2).

Our reply: We also agree that the paper had become difficult to read due to its complexity. All sections of the paper have now been almost entirely rewritten, and the paper has been extensively reordered. We now only have a single switch from somatic cells to *Xenopus.* We have removed a fair amount of non-essential data, and added new experiments which answer the concerns of all reviewers. And we have explained the *Xenopus* system in more detail.

Some experimental data are missing, which is annoying. I could not find how they monitor DNA replication in egg extract, nor how they purify the nuclei.

Our reply: We understand that the reviewer is referring to the protocols rather than data. We originally cited the paper explaining the replication assay protocol; we still do this, but we also detail it in the methods section. The same goes for the nuclear purification.

It would help very much if they could provide images of nuclei assembled in egg extract over time. In particular it would be really helpful to see the nuclei after purification and incubation in a new extract during the transfer experiments. The issue of the morphology and integrity of these nuclei is important and has also been raised by the reviewers .

Our reply: We have done this in experiments shown in new figures, Appendix Fig S2, and Figure EV5D, respectively. These experiments show that nuclei do not assemble properly in CytD and SMIFH2 conditions (Appendix Fig S2) and that nuclei transferred from one extract to another are intact, as they still exclude Dextran-70. But we already knew that this was the case since transferred nuclei replicate well in control conditions in recipient extracts.

Some experiments are not well represented in the figures or not quantified. For example the results shown in Figure 5A are based on single images of one selected sperm nucleus per condition.

Our reply: In this and all experiments where we show single nuclei, they were of course representative; a single one was shown to better see the nuclear morphology (or staining, as appropriate). To reassure the reviewer, we now show wide-field images for these conditions in a new figure, EV4A, along with the percentage of normally formed nuclei for each condition.

Overall I found that the comments from reviewer 3 were overly too negative and not based on objective criticism but rather emotional.

Our reply: we agree completely with this comment.

I think that with a careful revision the paper could be reconsidered.

2nd Editorial Decision 09 August 2017

Thank you for submitting your revised manuscript for our consideration, and apologies for the somewhat delayed re-evaluation. We have now carefully gone through your response to the original comments and looked at the new experiments, and the revised version has also been assessed once more by one of the original reviewers. In combination with our earlier advisor consultation based on your previous tentative response letter, I am pleased to let you know that we consider all the key criticisms now satisfactorily addressed/clarified, and therefore shall be happy to accept the study for publication in The EMBO Journal. Before we shall be able to proceed, there are however several editorial issues that still will need to addressed.

I am therefore returning the manuscript to you for an additional round of minor revision, to allow you to upload the accordingly modified files. Once we will have received this final version, we should hopefully be able to swiftly proceed with formal acceptance and production of the manuscript.

REFEREE REPORT

Referee #2:

The authors have significantly restructured and rewritten the manuscript, and in the process responded to all my concerns.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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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 χ2 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?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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http://www.selectagents.gov/	List of Select Agents

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?	This study did not involve experiments with living animals. Thus, it was not necessary to define sample sizes in advance to ensure adequate statistical power.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Our study conforms to the guidelines for statistics in cell biology described in Vaux, D.L. (2014) Basic statistics in cell biology. Annu Rev Cell Dev Biol 30, 23-37. Thus, most conclusions were drawn from independent lines of experiment rather than inferential statistics. All experiments were performed at least twice, and in most cases many more; the large scale proteomics analysis was performed three times as biological replicates, and replicate analysis is shown in Appendix fig S1. Graphs without error bars are often presented in paired analysis where inter-group differences are important and reproducible, but inter-experimental variation across groups is large due to biological sensitivity of the assay - especially replication assays and nuclear transfer experiments. Here, representing the average and variance across experiments would obscure the intra-experimental difference between groups unless very large numbers of experiments were performed, which would be unfeasible for all experiments, although this has been done for key experiments (Figs 4A, C). We do not present all replicates of the other experiments to avoid cluttering the paper with essentially duplicate data. Western blots and microscopy images are also representative of multiple experiments. Where individual cells or nuclei are shown, this is to better see their morphology, but they are representative of either the vast majority or all the cells/nuclei seen in the experiment. Where statistical tests are shown we report the method in the figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For the parametric tests used the data fit the normal distribution. Statistical methods are described in a section of the Materials and Methods.
Is there an estimate of variation within each group of data?	Where multiple experiments are compiled to show statistics, error bars show the variance as standard deviation, as presented in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes; please see answer to q5 above and statistical section in the Materials and methods, lines 772-778

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 are described in detail, including catalogue numbers for commercial antibodies, in Materials and Methods, lines 571-587.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were from the ATCC and were verified on a weekly basis for mycoplasma contamination

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	At the end of the Materials and Methods section, we have provided the accession number for the proteomics data set (MSV000081201). This is currently available for download only with a password but will be released as public data upon acceptance of the paper.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	See above
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A