THE EMBO JOURNAL

Manuscript EMBO-2014-88057

Post-transcriptional regulation by NANOS is up-regulated and functionally significant in pRb deficient cells.

Wayne O. Miles, Michael Korenjak, Lyra M. Griffiths, Michael A. Dyer, Paolo Provero, Nicholas Dyson

Corresponding author: Nicholas Dyson, Massachusetts General Hospital/Harvard Medical School

Review timeline:

Submission date:	28 January 2014
Editorial Decision:	14 March 2014
Revision received:	10 June 2014
Editorial Decision:	02 July 2014
Revision received:	11 July 2014
Accepted:	15 July 2014

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.)

1st Editorial Decision 14 March 2014

Thank you again for submitting your manuscript to The EMBO Journal. I need to apologize for the undue delay in getting back to you with a decision, which was owed to one of the referees unfortunately being very late in sending their report. We have now finally received all three sets of comments, copied below for your information. As you will see, the referees express (to varying degrees) interest in your findings and conclusion, but also raise a number of major issues that would need to be satifactorily clarified and improved before eventual publication.

Given the overall interest and potential wider significance of the work, I would like to give you a chance to address these concerns in form of a revised version of the study. I should however point out that it is our policy to allow only a single round of major revision, making it important to carefully respond to all the individual points raised at this stage. In this respect, while it may not be required to experimentally follow up on some of the further-reaching mechanistic queries (e.g. ref 3's point 3 or ref 1's point 9), it will be essential to adequately address the various technical and experimental concerns, including those of potential inconsistencies or limited significance of some of the results. From an editorial point of view, I would also appreciate if you could carefully revise and proof-read the manuscript text prior to resubmission, including adjusting the reference citation format and author listings to EMBOJ guidelines, and making sure to equally include all relevant primary citations.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

Should you have any further questions regarding this decision or the referee reports, please do not hesitate to contact me. I look forward to your revision.

———

REFEREE REPORTS:

Referee #1:

In this manuscript Miles et al identified the Pumilio translational repressor complex (Pum) as a target of the E2F/pRB pathway in flies and in human cells. In the first part, the authors describe that NANOS1, a component of the Pum complex, is upregulated after pRB inactivation. Further experiments show that Pum genetically interacts with E2f2/ Rbf1 in wing development in Drosophila. In addition, the loss of Nanos causes synthetic lethality in different human and mouse cell lines deficient of pRb. In the second part, the authors identify Map2k3 as a target of NANOS1 in pRb deficient cells that is suggested to contribute to the synthetic lethal effect.

Although the findings are of potential interest, this study is in a somewhat preliminary stage that diminishes the validity of the conclusions. Additional experiments are necessary to securely establish the link between E2F/pRB, p53 and NANOS1 and its downstream target Map2k3. In addition, there are inconsistencies in some of the Figures that have to be resolved (see below).

Major points:

1) It is not clear how changes in gene expression profiles can give information about the role of Nanos1 in survival of pRb-deficient cells, as the Pum complex post-transcriptionally regulates gene function. In other words: What is the mechanism of transcriptional downregulation of PUM substrates (which do not contain E2F-motifs in their promoters) in retinoblastoma tumors vs. normal tissue. This has not been addressed.

2) The authors state that Pum1, Pum2 and NANOS1 are upregulated on protein level in pocket-protein depleted BJ cells. However in Supplemental Figure Fig. 3B, an increase in NANOS1 expression is not obvious. Furthermore, although Pum1 and Pum2 levels appear to be moderately increased, the proteins levels do not correlate well with the transcript levels shown in Fig. 3A.

3) In Figure 2B it should be explained in more detail how the phenotype was scored. The genetic interaction of some combinations is not obvious in the photomicrographs provided in the figure (e.g. e2f2 RNAi/ Nos RNAi). Also it not clear why some combinations produce strong phenotypes, but others show no or only a weak phenotype (e.g. pum-RNAi (36676) vs. pum3). In addition, the results from Rbf1 RNAi (shown in Fig. 3A) should be included in the table in Figure 3B. Furthermore, it should be specified which specific RNAi was used in the example photographs shown in Fig. 3A.

4) The shRNA mediated depletion of mouse Pum1, Pum2 and Nanos1 has to be validated by western blotting (Figure 3A).

5) All viability assays (Fig. 3A, Fig. 4A, Fig. 4C) have to be quantified as has been done for the experiment shown in Figure 3D. The statistical significance has to be demonstrated. This is important because in some cases, e.g. in the cell line NCI-H1666, the synthetic lethality is not obvious from the data provided.

6) In Figure 3A and in Supplemental Figure 7B exactly the same crystal violet stained dishes (or wells) are shown. The only difference is that in Figure 7B the triple knockout cells are included. To show the same data twice is unnecessary and misleading, because it implies an independent experiment. Furthermore, although the same data are shown, the labels are not consistent: In Figure 3A the second and third rows are labeled mNos1-3 and mNos1-4, respectively, while in Supplemental Figure 7B rows 2 and 3 are both labeled as mNos1-3.

Figure 4A, Supplemental Figures 9A and 9B show the exactly same data. The wells in the second row in Supplemental S9A are labeled as Corl-105. Supplemental Figure S9B shows the same pictures in last row, but here they are now labeled as NCI-H1734.

7) Fig. 3 C: The crystal violet data from the rescue assay shown in Supplemental Figure 8B should be included in Figure 3C, as they are also included in the quantification of the experiment shown in Figure 3D,

8) Figure 4A (last row of cells) and Figure 4C (top row): Although these data are presented as independent experiments, the pictures for the negative controls are identical (HCT166 cells transfected with scr control). Therefore, it is not clear whether these are independent experiments and whether a negative control was included in each case.

9) The authors suggest that p53-mediated apoptosis is the reason for the reduced cell number when Nanos1 is depleted in pRb deficient cells. However the mechanism for the cooperativity has not been investigated. Is it indeed due to apoptosis or due to reduced proliferation? This has to be addressed.

10) In Figure 6E, it is shown that treatment with a p38 inhibitor improves the viability of Nanos1/pRb1 deficient cells. The increase in viability is modest (from approximately 40% to 60 %) and the errors are high. Is the difference significant? Is p38 MAPK activity increased in Nanos1-depleted cells? Is this increase dependent on Map3K1 and/or Map2K3? It is not described in the manuscript which p38 inhibitor was used, how long the cells were treated with the inhibitor and what concentrations were used. Also, there is no indication that the inhibitor is working as expected. Knockdown of p38 or Map2K3/Map2K1 should be used to confirm the observation.

11) Are Map3k1 and Map2k3 also upregulated in HCT166 wildtype cells when Nanos1 is depleted?

12) To confirm that the regulation is post-transcriptional, it should be demonstrated that mRNA levels of Map3k1 and Map2k3 are unchanged when Nanos1 is depleted.

Additional points:

1) Supplementary Figure 2C: It is not clear how relative change in gene expression was calculated.

2) In the experiment shown in Figure 1D, Nanos2 expression remains unchanged after pocket proteins are depleted. However this is inconsistent with Supplemental Fig. 3A which shows a four to sevenfold upregulation of Nanos2 in p107, p130 and pRb depleted cells. How reproducible are these effects?

Referee #2:

The manuscript by Miles et al "Post-transcriptional regulation by NANOS is up-regulated and functionally significant in pRb deficient cells" convincingly reveals a novel mechanism that allows cells to tolerate the loss of the Rb tumor suppressor. Presented data are comprehensive, physiological, mechanistic and highly convincing. And the importance of NANOS (part of the Pumilio post-transcriptional repressor complex) regulation by Rb loss is shown in flies (in the wing), in normal mouse and human fibroblasts, and in cancer cells. Their demonstration that Pumilio or Nanos impairment in the wing disrupts the structure only in the context of Rb mutation (and vice versus) is particularly powerful. Their studies answer a long mystery: how Rb mutant cells can contribute relatively normally to tissues in mice and flies. The demonstration of this synthetic lethal interaction between Rb and Nanos also has potential for exploitation in the treatment of Rb deficient cancers that maintain p53 activity. Finally, they provide substantial mechanistic insight into how Rb (and the dREAM complex) normally regulates Pumilio complex components, and how Pumilio substrates (such as Map2K3 and Map3K1 mRNAs) are repressed in Rb deficient cells by Nanos upregulation, promoting the survival of these cells. Thus, their conclusions are well supported by their data, and this manuscript truly takes the story full circle.
Minor comments:
1) The western in Supplemental Fig 3B is not very convincing (unlike very convincing RT-PCR

analyses for the same genes), in part due to uneven loading.
2) In some ways, the data on human cancers in Suppl Fig 4 is glossed over in the Results section (and doesn't really do these interesting data justice).

Referee #3:

This manuscript from the Dyson lab reports an interesting and heretofore unappreciated, mechanistically direct connection between retinoblastoma protein function and regulation of Nanos gene expression, leading to altered function of the pumilio complex. This in turn, it is argued, specifically compromises derepression of certain target genes including two mapk pathway kinases that are involved in apoptosis induction. This then provides protection from what would otherwise be a cell lethal event upon Rb loss or pathway inactivation consequent to p16 disruption. This work continues the robust series of seminal observations that this lab has published resulting from careful comparisons of drosophila phenotypes resulting from manipulation of the much simpler RB/E2F system in that organism. This is then appropriately coupled with assays in genetically modified murine and human cells, both normal and tumor derived. The data shown here are in general robust, support the central conclusions drawn, and highlight the utility of genetic model systems, whether they be invertebrate, mouse, or human cells.

I believe this work will be of general interest to the cancer biology field, and of particular interest to the numerous labs attempting to understand and therapeutically exploit changes in this pathway in tumor cells. The work is of high potential impact and appropriate for EMBO journal. However, there are a few issues that arise as mentioned below that would improve the clarity of the study if addressed with additional text or, in some cases, with a simple additional experiment.

1. It is rather striking that deletion of p107 or p130 significantly deregulates nanos in BJ (human fibroblast) cells, but immortalized MEFs show nanos upregulation only when RB is lost. This bears comment. In particular, in the human case, it is worth considering whether the role of p107 and p130 as nanos suppressors implied by the data shown indicate that these RB family members must be compromised in tumorigenesis to allow the full value of nanos upregulation. Can the authors simply show if p107 (or p130) function (promoter binding?) is compromised upon RB loss in BJ cells, and/or in the tumors used - perhaps most relevant would be the state of p107 on nanos promoter in retinoblastoma tumors/cell lines.

2. In supplemental Figure 3B, I believe I see very little change in nanos protein in BJ cells with various RB family manipulations (yet pum changes are clear); this is in contrast to nanos message. This bears comment - do the authors believe changes in nanos protein are significant, or if not, how does its function changes in these cells?

3. The fact that p16 loss, as well as RB loss, is important as it implicates the nanos regulation and cell survival mechanism in a very large number of human tumors (although far from all, in particular subsets, as the authors point out). This should be explored more deeply to link p16 loss to the nanos deregulation seen with Rb family mutation. Here it is important to knockdown p16 in BJ cells and ask what level of nanos and pum family deregulation is seen. Also, this should be tested in HCT116, used to good effect to test the role of p53. In the latter case, one might predict that these cells, which are relatively normal except for p16 disruption, might be sensitive to restoration of RB activity through the use of cdk4/6 inhibitor. This is a simple experiment that would expand the impact of the work. Finally, the role of p16 makes situation in p107 and p130 3T3 cells even more interesting.. one might predict these cells would need to lose p16 and deregulate RB to become immortal, and yet no nanos upregulation is seen. What is the p16 status in these cells? Alternatively, both RB and p16 may be intact, and yet the cells are able to achieve immortality (without the need to suppress apoptosis through nanos upregulation) perhaps providing clues to what is going on in p16/RB sufficient tumors. At the very least, comment on these issues in the discussion would be useful.

4. MCF7 rb mut p53 wt don't die when nanos is reduced? Supplementary figure 9. Since this is the only exception to the general rule of synthetic lethality of RB mutant;p53 wt genotype with nanos reduction, it bears comment. Perhaps it's enough to speculate that p53 can be deregulated in ways other than loss or mutation.

1st Revision - authors' response 10 June 2014

Referee #1:

Referee 1 gave a positive review of the manuscript and their comments helped us improve the overall quality of the paper by improving the quantification and statistically analysis of our data. In addition, to address the reviewers' comments, we conducted an in-depth analysis of how NANOS1 and p38 are involved in the post-transcriptional regulatory network important for preventing apoptosis of pRb-deficient cells.

We addressed the specific points listed by this reviewer as described below:

1) To address the reviewer's comments on how PUM contributes to the lower transcript levels seen in Retinoblastoma tumors, we analyzed how PUM contributes to the mRNA stability of PRE and non-PRE containing mRNAs. Using Y79 Retinoblastoma cell lines, we measured the mRNA stability of transcripts in cells (using Actinomycin D) depleted of PUM or scrambled control sequences. As shown in Supplementary Figure 13, transcripts containing PRE's (MAP3K1 and MAP2K3) showed a significant increase in mRNA stability in cells lacking PUM function compared to non-PRE controls (E2F4). These findings suggest that the PUM complex post-transcriptionally down-regulates the levels of PRE-containing transcripts by inducing mRNA instability and turnover.

2) We have tested our results from Supp Fig 3B in another human cell line (Retina Precursor cells (RPEs)) and in agreement with our work from BJ cells, we find elevated NANOS1 protein levels upon pocket protein depletion; in accordance with its transcriptional up-regulation (Sup Fig 3C). In addition, we also find higher protein levels of both PUM1 and PUM2. Previous work has identified NANOS as an important stabilizer of the PUM complex and our data suggests that elevated NANOS1 levels also indirectly stabilize both PUM proteins as part of the PUM-NANOS complex.

3) Included a detailed description of how the genetic interaction experiments were conducted and scored, as well as providing information of the various strengths of the RNAi lines and mutations within the methods. We have also included quantification of the RBF1 RNAi genetic interaction in Figure 3B.

4) Western blots using the antibodies specific to the human homologs of mouse Pum2 and Nanos1 were used to validate the depletion of these proteins by shRNA (Supp Fig 9B). The human Pum1 antibody produced very high background when used on MEF protein extract, making interpretation difficult and was therefore not included.

5) We have quantified and statistically analyzed the viability assays in Figure 3A, 4A and 4C; these have been included in Supp Fig 9E, 11A and 11C, respectively.

6) We have removed duplicated experimental data from Figure 3A and Supp Fig 7B and modified the incorrectly labeled row 2 and 3. In addition, we have removed Supp Fig 9A and 9B as the data is already present in the main body of the manuscript and corrected the mislabeled well.

7) Included the rescue data from Supp Fig 8 in Figure 3C and included the quantification of Figure 3D.

8) Replaced the duplicated image of HCT116 Scr control crystal violet stain in Figure 4C.

9) To address the reviewers' comments, we tested the cellular consequence of NANOS1 depletion on cell number using siRNAs (targeting NANOS1), in Y79 Retinoblastoma and NCI-H1666 lung cancer cell lines. As shown in Supp Fig 12, depletion of NANOS1 using siRNA in either Y79 or NCI-H1666 cells leads to a reduction in cell number. These findings support our model that NANOS1 functions to suppress apoptosis in pRb-deficient cells.

10) To address the reviewers comments, we statistically analyzed the increase in viability of HCT116 cells depleted of NANOS1, upon treatment with the p38 inhibitor and do not find the

rescue statistically significant. In addition as shown in Supp Fig 14, we find elevated MAP2K3 and MAP3K1 activity in NANOS1 shRNA treated cells (Supp Fig 14B). Depletion of MAP3K1 is insufficient to rescue the reduced viability of HCT116 cells upon NANOS1 loss (Supp Fig 14F). We have tested an additional p38 inhibitor, which produced highly similar results to the data included in the paper (Supp Fig 14F). A detailed description of the experimental procedures, including p38 time course and concentrations have been added the methods section.

11) Western blots of MAP3K1 and MAP2K3 from HCT116 cells ("wild-type" containing p53) are included in Supp Fig 14B.

12) RT-PCR results of MAP3K1 and MAP2K3 from HCT116 cells depleted for NANOS1 are included as Supp Fig 14C.

Additional points:
1) A detailed description of how the relative changes in gene expression has been included in the material and methods.

2) We have repeated the RT-PCR for the BJ cells presented in Figure 1D and Supp Fig 3A and find very little difference in NANOS2 expression levels upon pocket protein depletion.

Referee #2:

This review of our manuscript helped to improve the paper by asking us to provide a greater focus and discussion on the human cancer data.

We addressed the minor points listed by this reviewer as described below:

Minor comments:
1) We have tested our results from Supp Fig 3B in another human cell line (Retina Precursor cells (RPEs)) and in agreement with our work from BJ cells, we find elevated NANOS1 protein levels upon pocket protein depletion; in accordance with its transcriptional up-regulation (Sup Fig 3C). In addition, we also find higher protein levels of both PUM1 and PUM2. Previous work has identified NANOS as an important stabilizer of the PUM complex and our data suggests that elevated NANOS1 levels also indirectly stabilize both PUM proteins as part of the PUM-NANOS complex.

2) Additional references to the human cancer data in Supp Fig 4 have been included in the results section.

Referee #3:

Referee 3 gave a positive review of our manuscript and enhanced our work by asking us to test whether DREAM regulation of NANOS1 is direct and to investigate how upstream regulators of pRb (CDK4/6 and p16) affect NANOS1 expression.
We addressed the specific points listed by this reviewer as described below:

1. We carried out ChIP experiments using antibodies specific for the DREAM components E2F4, p107 and p130 in both BJ cells and Y79 Retinoblastoma cells. As shown in Supp Fig 4A, depletion of pRb from BJ cells diminishes the levels of E2F4 and p107 binding to the NANOS1 promoter. In support of these results, we find that the DREAM complex (E2F4, p130 and p107) is not bound to the NANOS1 promoter in Y79 Retinoblastoma cells, which have homozygous mutations in the Rb1 gene (Supp Fig 4B). These findings suggest that pRb loss compromises DREAM recruitment to the NANOS1 promoter.

2. We have tested our results from Supp Fig 3B in another human cell line (Retina Precursor cells (RPEs)) and in agreement with our work from BJ cells, we find elevated NANOS1 protein levels upon pocket protein depletion; in accordance with its transcriptional up-regulation (Sup Fig 3C). In addition, we also find higher protein levels of both PUM1 and PUM2. Previous work has identified NANOS as an important stabilizer of the PUM complex and our data suggests that elevated NANOS1 levels also indirectly stabilize both PUM proteins as part of the PUM-NANOS complex.

3. To address the reviewers' comments we depleted p16 from BJ cells and found higher expression levels for NANOS1, NANOS3, PUM1 and PUM2 but not NANOS2 (Supp Fig 7A). We have additionally tested the sensitivity of HCT116 cells to the CDK4/6 inhibitor (PD0332991) (Supp Fig 7B) and measured the affect of the inhibitor on the levels of NANOS and PUM. Our results demonstrate that re-activation of pRb by the CDK4/6 inhibitor is sufficient to repress NANOS expression levels (Supp Fig 7C).We have also analyzed the p16 status of the 3T3 cells used in Figure 3 and find that only p107 -/- MEFs lack p16 expression (Supp Fig 10B).

4. We have included a section in the discussion outlining MCF7 cells as the general exception to the pRb mut and p53 wild-type rule and point out that this cell line contains a number of mutations within MAP kinases that are closely related to MAP3K1 and MAP2K3. We suggest that these may contribute to the failure of these cells to undergo apoptosis upon NANOS1 depletion.

2nd Editorial Decision 02 July 2014

Thank you again for submitting your revised manuscript for our consideration. It has now been reviewed once more by two of the original referees, whose comments are copied below. Both reviewers consider the paper generally improved in response to the original comments, however referee 1 does retain several concerns regarding the experimental support for some of the conclusions, as well as presentational issues. Following discussions with the other referee, and given the multiple lines of evidence in support for the key conclusions, I feel that these remaining points should all be addressable by text changes and figure modifications, without the need for generating additional experimental data. However, please make sure to carefully revise both text and figures (and to check for congruency between these parts) before resubmitting a final version of the manuscript. With regard to the claims referee 1 considers unjustified, I think toning down and qualifying the conclusions so they do not overreach the data would be important (e.g. not claiming that MAPK activity modulation has been shown directly, or referring to reduced clonogenic survival instead of increased apoptosis that has not been measured directly).

When resubmitting a re-revised manuscript, please also address the following two editorial points:

- to make the title slightly more compelling (and to avoid redundant use of 'regulation'/'regulated'), I would propose altering it to
"Post-transcriptional gene expression control by NANOS is up-regulated and functionally important in pRb-deficient cells"

- in order to make the primary data behind the often pixelated and/or solarized bands on the blot panels more directly represented and accessible, I would kindly ask you to include figure source data for the gels, blots and autoradiographs in both the main and the supplementary figures. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the the main and supplementary figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and they would be linked as such to the respective figures in the online publication of your article.

I am thus returning the study to you for one additional, final round of revision, in order to clarify these points, hoping you will be able to re-submit a final version of the manuscript as early as possible - we should then hopefully be in a position to accept the manuscript and swiftly proceed with its production for publication in The EMBO Journal. Should you have any further questions in this regard, please do not hesitate to get back to me.

———

REFEREE REPORTS:

Referee #1:

The authors have addressed some of the comments raised.
However, there are still some points that have to be clarified:

1) Figure 2:
a) Results from Rbf crosses still not included in Figure 2B.
b) The explanation of how the phenotype was scored should be included in the figure legend.
c) E2F2 RNAi/ Pum RNA; mip120 RNAi /Pum RNAi and mip130 RNAi/ Pum RNAi: Phenotype is scored as +++ (severely blistered and deformed wings) but this is not obvious, especially in comparison to e.g. mip120 RNA/ Brat RNAi which appears to be more severe (but is scored as ++). Higher magnifications may be helpful. Arrows or arrowheads would also be useful to indicate the abnormalities.

2) Figure 3A/ Suppl. Figure 9C,E: Quantification of cell staining of triple negative cells is still missing.

3) Supplementary Figure 9C: Wells of wild-type cells duplicated from Figure 3A. This should be at least indicated in the Figure Legend.

4) Figure 3B: Results from WERI Retinoblastoma cells described in text (page 11) and Figure Legend but data missing in Figure 3B.

5) Figure 3B: "mock" not defined in text or Figure Legend.

6) Figure 4A and Suppl. Figure 11A: Quantification and statistics is only shown for cell lines labeled as sensitive. "Non-sensitive" cells should also be quantified. This is important as some cell lines labeled as "non-sensitive" such as Saos-2, T24 and RPE may also be affected by Nanos1 depletion (especially when compared to the sensitive cell line NCI-1563).

7) Figure 4A: Have the authors confirmed the knockdown of Nanos1 in the sensitive and non-sensitive cell lines in parallel to the viability assays?

8) Whether p38 activity is increased in HCT116 cells depleted of Nanos1 (Figure 6) has still not been addressed (e.g. by blotting with a phospho-specific p38 antibody).

9) Whether the synthetic lethality of pRB and Nanos inactivation is due to apoptosis has not been addressed (this could be done e.g. by Annexin V staining).

10) Figure 6E: According to the authors the increase in viability of cells treated with the p38 inhibitor is not statistically significant. Yet the experiment is still shown and described in the manuscript as if it was significant. If the result is not statistically significant, do not show the data.

In summary, how can the authors conclude that "MAP kinase activity is of similar importance as E2F1-induced cell death" (page 16) if:
a) increased MAP kinase activation has not been demonstrated
b) it has not been shown that the reduction in cell number is due to apoptosis
c) the inhibition of MAP kinase activity produces a minor effect that is not significant.

Referee #2:

This manuscript convincingly reveals a novel mechanism that allows cells to tolerate the loss of the Rb tumor suppressor. Using both fly and mammalian models, they nicely show that components of the NANOS/PUM post-transcriptional repressor complex are upregulated in Rb deficient cells, leading to suppression of the post-transcriptional expression of key cell death effectors like

MAP2K3 and MAP3K1. It is the sum of their many different avenues of investigation that make a very compelling case for their conclusions. The supplementation of new experimental data with bioinformatics analyses of large gene expression data sets for human tumors increases the generality of their discoveries.

This paper provides insight into an old mystery: how do cells tolerate loss of Rb? Their data provide at least one mechanism: by upregulation of NANOS/PUM and the consequent suppression of pro-apoptotic programs.

Minor points (which can easily be addressed by text changes):

1) They seem to have mixed up referencing of their Supplemental Figures in the Results section. For example, "E2F4, p107 and p130, confirmed that all three proteins bind directly to the promoter of NANOS1 (NOS1) in human fibroblasts (BJ cells) (Supp Fig 4A)." Shown in Supp Fig 5A. In addition, "Knock-down of p16 in BJ cells stimulated the expression of the entire PUM complex except NANOS2 (Supp Figure 7A)." Shown in Supp Fig 4A.

2) For Supp Fig 12, they state that "depletion of NANOS1 resulted in reduced cell number from both cell lines after 5 days, suggesting NANOS1 functions to prevent the apoptosis of pRb-deficient cells", but this result could just as easily be explained by growth inhibition. To claim "apoptosis", you actually need to measure it (and even just showing cell death isn't sufficient, as there are many ways to die). General phrasing like "inhibited cell expansion" is more appropriate.

2nd Revision - authors' response 11 July 2014

Referee #1:

We thank the reviewer for reading the manuscript so carefully and have made the following revisions to address his/her comments.

1) Figure 2:
a) Results from Rbf crosses still not included in Figure 2B.

We have added a column to Figure 2B that summarizes the results of the Rbf1 crosses

b) The explanation of how the phenotype was scored should be included in the figure legend.

A description of the scoring system used to assess the phenotypes has been added to the legend for Figure 2

c) E2F2 RNAi/ Pum RNA; mip120 RNAi /Pum RNAi and mip130 RNAi/ Pum RNAi: Phenotype is scored as +++ (severely blistered and deformed wings) but this is not obvious, especially in comparison to e.g. mip120 RNA/ Brat RNAi which appears to be more severe (but is scored as ++). Higher magnifications may be helpful. Arrows or arrowheads would also be useful to indicate the abnormalities.

Representative images of the various genotypes are shown in Figure 2A. We added the Table in Figure 2B because some changes in wing morphology are not always evident in photographs of the flattened wings. This is especially true with wing blisters, and particularly the case when the blistering is minor (scored as ++). In addition, the penetrance of the defects in a population of flies varies in different genotypes. The table gives our overall assessment of the severity and the penetrance of the defects in the different genotypes. We've included this table for the very reason that the reviewer has pointed out (that the extent and frequency of blistering is not always clear in a photograph of a single wing).

2) Figure 3A/ Suppl. Figure 9C,E: Quantification of cell staining of triple negative cells is still missing.

We have added the quantification of triple negative cells to Supplemental Figure 9.

3) Supplementary Figure 9C: Wells of wild-type cells duplicated from Figure 3A. This should be at least indicated in the Figure Legend.

These samples were all part of the same experiment. In the legend to Supplemental Figure 9 we have added a statement that the controls in Supplemental Figure 9 are a duplicate of Figure 3A.

4) Figure 3B: Results from WERI Retinoblastoma cells described in text (page 11) and Figure Legend but data missing in Figure 3B.

Similar results were obtained using both Y79 and WERI cells. The reviewer correctly pointed out that only the results with Y79 data are shown in the Figure. To eliminate any potential confusion we have removed the reference to experiments with WERI cells from the text.

5) Figure 3B: "mock" not defined in text or Figure Legend.

We have clarified this in both the text and figure legend.

6) Figure 4A and Suppl. Figure 11A: Quantification and statistics is only shown for cell lines labeled as sensitive. "Non-sensitive" cells should also be quantified. This is important as some cell lines labeled as "non-sensitive" such as Saos-2, T24 and RPE may also be affected by Nanos1 depletion (especially when compared to the sensitive cell line NCI-1563).
We have added quantification of the non-sensitive cells to Supplemental Figure 11.

7) Figure 4A: Have the authors confirmed the knockdown of Nanos1 in the sensitive and non-sensitive cell lines in parallel to the viability assays?

Yes. Efficient knockdown of Nanos 1 is illustrated in Figure 6 and Supp Fig 14.

8) Whether p38 activity is increased in HCT116 cells depleted of Nanos1 (Figure 6) has still not been addressed (e.g. by blotting with a phospho-specific p38 antibody).
We have added a western blot to Figure 6E showing the elevated levels phospho-p38 upon NANOS1 depletion.

9) Whether the synthetic lethality of pRB and Nanos inactivation is due to apoptosis has not been addressed (this could be done e.g. by Annexin V staining).

We have changed the text when we describe the experiments showing the combined effect of pRB and nanos inactivation and now point out that these conditions reduce the number of cells, rather than saying that they induce apoptosis.

10) Figure 6E: According to the authors the increase in viability of cells treated with the p38 inhibitor is not statistically significant. Yet the experiment is still shown and described in the manuscript as if it was significant. If the result is not statistically significant, do not show the data. In summary, how can the authors conclude that "MAP kinase activity is of similar importance as E2F1-induced cell death" (page 16) if:
a) increased MAP kinase activation has not been demonstrated
b) It has not been shown that the reduction in cell number is due to apoptosis
c) The inhibition of MAP kinase activity produces a minor effect that is not significant.

Our reason for including this data was to show that the effect of p38 inhibitors on the viability of cells depleted of pRb and Nanos was comparable to the effects of depleting individual E2F proteins. However we appreciate the reviewer's point that, since the changes are not strong enough to be statistically significant, there is no point in including this data. We have deleted the data from the figure and the discussion of the effects from the text. In the revised text we point out that pRb

inactivation likely activates multiple stress responses, and that the effects of NANOS1 and PUM on the MAP kinases, are only one component of a broad program of regulation that impacts the viability of pRB-deficient cells. To specifically address point 10a, we added Western blots to Figure 6E that demonstrate the elevated activation of p38 following depletion of NANOS1.

Referee #2:

This manuscript convincingly reveals a novel mechanism that allows cells to tolerate the loss of the Rb tumor suppressor. Using both fly and mammalian models, they nicely show that components of the NANOS/PUM post-transcriptional repressor complex are upregulated in Rb deficient cells, leading to suppression of the post-transcriptional expression of key cell death effectors like MAP2K3 and MAP3K1. It is the sum of their many different avenues of investigation that make a very compelling case for their conclusions. The supplementation of new experimental data with bioinformatics analyses of large gene expression data sets for human tumors increases the generality of their discoveries.

This paper provides insight into an old mystery: how do cells tolerate loss of Rb? Their data provide at least one mechanism: by upregulation of NANOS/PUM and the consequent suppression of pro-apoptotic programs.

Minor points (which can easily be addressed by text changes):

1) They seem to have mixed up referencing of their Supplemental Figures in the Results section. For example, "E2F4, p107 and p130, confirmed that all three proteins bind directly to the promoter of NANOS1 (NOS1) in human fibroblasts (BJ cells) (Supp Fig 4A)." Shown in Supp Fig 5A. In addition, "Knock-down of p16 in BJ cells stimulated the expression of the entire PUM complex except NANOS2 (Supp Figure 7A)." Shown in Supp Fig 4A.

We have corrected this mistake. We apologize for the error.

2) For Supp Fig 12, they state that "depletion of NANOS1 resulted in reduced cell number from both cell lines after 5 days, suggesting NANOS1 functions to prevent the apoptosis of pRb-deficient cells", but this result could just as easily be explained by growth inhibition. To claim "apoptosis", you actually need to measure it (and even just showing cell death isn't sufficient, as there are many ways to die). General phrasing like "inhibited cell expansion" is more appropriate.

We have changed the text, as recommended, to be more precise on this point.

