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RB regulates pancreas development by stabilizing Pdx-1

Yong-chul Kim, So Yoon Kim, Hariom Yadav, William Niedermyer, Jose Manuel Mellado-Gil, Anil Kumar Kamaraju, Sushil Rane

Corresponding author: Sushil Rane, NIDDK, NIH

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 06 July 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the long delay in getting back to you with a decision. We sent your manuscript to three referees, and - despite numerous reminders - are still awaiting the final report. Since at this stage it is not clear whether this will come in, and in order to save you from a further loss of time, we have decided to take a decision based on the two reports we currently have to hand. Of course, should the third report come in, we will send it on to you.

As you will see, both referees express significant interest in your analysis of the interaction between Pdx1 and Rb, but both also raise serious concern with the study in its current format. The most significant criticism concerns the physiological significance of this interaction: you show some data looking at the pancreas phenotype of Rb knockouts, but no evidence is provided that these phenotypes result from the destabilisation of Pdx1, as opposed to the well known functions of Rb in regulating E2F activity. Minimally, it would be essential to examine Pdx1 levels in the Rb knockout pancreas. Related to this, referee 2 highlights the need to provide better evidence that Rb phosphorylation induces its interaction with Pdx1, while affinity for E2F is lost. A second major area of concern, highlighted primarily by referee 1, concerns the conclusiveness of the data showing that Rb promotes Pdx1 stability; this is a critical point and needs to be shown definitively.

Given the overall interest expressed by the referees, as well as their constructive suggestions as to how to extend the study, we would like to give you the opportunity to revise your manuscript according to their comments. However, I would stress that we would only consider a revised version if you are able to address the major concerns highlighted above, to the satisfaction of both referees. I realise that this will entail a significant amount of work, and that the outcome of these experiments is uncertain. Consequently, if you prefer to take your manuscript elsewhere at this point, or if you are unable to resolve the criticisms raised, please let me know. I should add that it is EMBO Journal

policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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. Given the amount of work involved, I can see that the three-month deadline may be insufficient; if so, please let me know and we should be able to accommodate an extension - up to a maximum of six months.

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

This manuscript addresses a potentially highly interesting observation in which the pancreatic homeodomain transcription factor Pdx1 is shown to physically interact with the critical cell cycle regulator RB. Much of the study focuses on defining the interaction motifs and providing evidence to suggest that the interaction may stabilize Pdx1 protein. Finally, an attempt at in vivo corroboration is made with the examination of pancreas development in Rb-/- embryos in which the proliferation of Pdx1+ pancreatic progenitors is found to be reduced.

While the initial observation of Pdx1-RB interaction could be important, the study falls short both biochemically and especially in terms of establishing in vivo relevance.

Specific comments:

1. In Figure 1, which form of Rb is felt to be interacting with Pdx1? Under or hyper phosphorylated forms? If Pdx1 associates with the Hyperphospho form of RB in contrast to E2f association with the Under phospho form, then how are they able to compete with one another for binding to RB as shown in Figure 1e?

2. In Figure 1f, the inference from the data presented is that both A and B domains are required for the interaction of Rb with Pdx1, yet the only mutant that is not generated and studied is the A-B mutant. This mutant must be generated and its ability to interact with Pdx1 shown. Similarly, the ability of a full length Rb mutant lacking the A-B domains to interact with Pdx1 should be investigated, with the expectation based on the authors interpretation that interaction will be abolished.

3. A) Quantitation of Western blot signals should not be selective. Loading controls should be quantitated and all expression levels and interactions should be quantitated in parallel. For example, in Figure 2C, tubulin. IN Figure 2d, Pdx1. In Figure 2e, Pdx1. Figure 3a, Pdx1. Figure 3b, Pdx1. What is the point of showing a markedly overexposed blot of IgG signal in Figure 2e?
b) For proper quantitation, the data from at least 3 independent experiments should be quantitated and presented in graphical format. What measures have the authors undertaken to ensure linearity of the Western blot signals? How is significance (statistical) being determined?

4. Given the overriding theme of the manuscript and the interpretation that the Pdx1 and RB interaction is influencing Pdx1 stability, it is critical that Pdx1 protein stability be directly studied by examining Pdx1 protein half-life in the critical settings of mutagenesis that influences the protein-protein interaction, with gain and loss of Rb function both in cells and in vivo and in the setting of Cdk4 gain and loss of function (both genetic and pharmacologic). The ubiquitination studies are a step in the right direction but do not prove that Pdx1 stability is correspondingly affected.

5. In general, the mutagenesis of phosphorylated Serines on Rb and of the RIM motif of Pdx1 do not support the authors conclusion that these residues mediate the physical interaction. Rather, the data support an effect of these mutations on the expression level of Rb and Pdx1 respectively, which in turn affects the strength of the co-immunoprecipitation signal, as would be predicted (see Figures 2c, 2d, 3a, 3b). Less partner, less signal and vice versa. Thus, the reduced expression level and stability of the Pdx1 and Rb mutants complicates the interpretation of the co-IP experiment. One specific example: the Pdx1 L195P mutant maintains ~80% of Rb interaction (3b), yet has the most dramatically reduced expression level/stability (3e). This suggests that this site may be critical to maintaining Pdx1 stability via an RB-independent mechanism. These coIP experiments should be performed with equal starting amounts of Pdx1 and Rb respectively.

The authors do get at this point on page 7 "It is plausible that the reduced affinity between RB and Pdx-1 mutants is, at least in part, attributable to the reduced expression of the mutant Pdx-1 proteins", and again on page 8 "It is interesting that although it interacts weakly with RB, the Pdx-1 M mutant gets stabilized by RB which is suggestive of alternate points of contact between RB and Pdx-1." In fact, this is the likely interpretation and would be clarified by uniform quantitation of the Western blot signals in Figures (see point 3 above). Thus, the physical interaction motifs for both sides of this interaction may not yet have been identified in the current study. Any revision must definitively map the interaction motifs.

6. The effect of glucose on Pdx1 expression in the current study (Figure 3i, j, k) is at odds with published literature in the same cell line in which Pdx1 half-life is acutely stabilized under high glucose conditions and destabilized under low glucose conditions in the same beta cell line (Humphrey et al). The discrepant findings could be a consequence of the non-physiological design of the current experiments in which Min6 cells are completely starved of glucose for 24 hours before being rechallenged with different levels of glucose. Given the experimental design then, it is no surprise that the ability of Rb to associated with Pdx1 target genes would correlate with the expression level of Pdx1, so these data do not specifically add support for the model. It is also possible that this is a consequence of working with tumor cell lines in culture that may drift in phenotype over time and in different labs. This highlights the need to corroborate these effects of glucose in primary islets. Further, the glucose regulation of RB is incomplete- how does glucose regulate Rb levels? Is Rb transcript different in Min6 cells under different glucose conditions? Is RB itself being turned over in high glucose conditions?

7. In general, the critical aspects of the biochemical model as interpreted by the authors lack validation in the Rb null model. The finding of reduced proliferation of Pdx1+ progenitors can easily be understood from known molecular roles of RB and could likely be independent of RB-Pdx1 physical association. Experiments addressing Pdx1 expression level and stability in RB-/- pancreas in vivo and in the explant system should be performed. Although Pdx1 target genes are evaluated, Pdx1 transcript and protein levels must also be examined. If the pancreas development phenotype of RB deficiency cannot be definitively linked to its interaction with Pdx1, then those findings do not belong in this manuscript.

8. The x-axis labels are missing for Figure 4c.

Additional points:
1. The title is misleading as it is never demonstrated that Pdx-1 is destabilized in the Rb knockout embryos.

2. Why are the different GST fusion constructs not appearing to migrate according to their size (Figure 1f)? The pRb 379-612 does not migrate as the similarly sized 612-928 construct.

3. In figure 3g, Pdx-1 wild type and mutant are not significantly ubiquitinated yet when Rb is added

there is significant laddering. Doesn't this indicate that Rb is inducing Pdx-1 ubiquitination and not protecting it as they authors claim?

4. Does interaction with RB enhance Pdx1 transactivation (aid in the recruitment of coactivators, alter histone modifications at Pdx1 target sites?)

5. An error bar is missing in figure 5d.

6. Figure 1b, need to show the Pdx1 IP blot

7. Figure 2 (b) Need to show Pdx1 IP blot. Would also be nice to see ppRB blots of Pdx1 IP- that is- is one of the phospho-RB forms preferentially interacting with Pdx1?

8. Figure 3 (k) Need to show the Pdx1 IP blot

Referee #3 (Remarks to the Author):

The authors show that Rb can associate with Pdx1 and influence expression of Pdx1-dependent genes, and thus pancreas formation, in a positive fashion. Pdx1 uses a motif similar to that used by E2f to interact with Rb, and the two proteins can compete for binding to RB, yet, remarkably, Pdx1 is suggested to bind most strongly to phospho Rb, whereas the same phosphorylation prevents E2F association.

In addition, the data suggest that one consequence of RB association with Pdx1 is stabilization of Pdx1 (although nonbinding mutants of pdx1 are still stabilized by RB), and RB null pancreas in the mouse is small and Rb knockdown limits proliferation based on cell staining, suggesting key functional roles for RB/Pdx1 complex binding to Pdx1 regulated promoters.

The data presented are very interesting as far as they go, and most important is the concept that phosphoRb binds and regulates Pdx1 while simultaneously losing affinity for E2F. However, the evidence for this aspect of the model, which is the most unique and globally important component of this work, is lacking, and should be much more rigorously tested. In addition, the role of RB in pancreas cell survival should be tested in concert with the proliferation studies in the final figure to allow the reader to assess the source of the reduction in gene expression and proliferating cells. With these changes, this could be a seminal work.

1) As mentioned above, a favoritism of Pdx1 for phospho Rb stands in contrast to the affinity of E2F for hypophosphorylated RB, and is at the core of the authors' model in figure 5. However the evidence to support this is thin, essentially based on RB migration in figure 1 and some mutant binding experiments that are compromised by changes in RB level that may disguise a lack of change in affinity. Most remarkable about this possibility is that the two target proteins use arguably highly similar motifs to interact with RB, so the difference in regulation by phosphorylation is fascinating, but puzzling. The following experiments should be performed to rigorously test this model:
a) Blot with phosphoRB specific antibodies in Pdx1 (or tagged Pdx1) IPs. Which forms of RB are favored in this approach? The model predicts an enrichment for S780.

b) IP with phosphoRb antibodies and blot for Pdx1. Does this enrich Pdx1 co-Ip vs. ip with an antibody that pulls down all or only hypophosph. RB?

c) most compelling would be ChIP with phosphoRB antibodies to complement the above IPs.

d) phosphatase-treat RB-containing lysates prior to IP with Pdx1, and use E2F as a control. Does this decrease Pdx1 binding but increase E2F binding? Note that this may reveal that some phosphorylation is required for association of both proteins, as has been suggested by some in the literature. Would a D780 mutant differentially effect Pdx1 and E2F binding in this case?

e) Use readily-available purified GST RB small pocket and/or large pocket with and without in vitro phosphorylation by cdk4 to determine how this alters Pdx1 and E2F binding in vitro.

2) The "lack of proliferation" in RB knockdown pancreas and decrease in gene expression could be a result of proliferation control per se, or could be the result of cell death. It would be straightforward to assay for apoptotic cell death in these knockdown cultures, and such death would be consistent with that seen in a number of other models of RB loss in tissues. This is important to know to determine if the loss of proliferation is a possible result of cell loss, or truly a cell cycle effect.

3) if Rb predominantly controls Pdx1 through stability, can enforced overexpression of Pdx1 increase gene expression, proliferation, and promoter binding in the cell-based assays used here?

4) In the domain mapping experiments in figure 1, why was the "small pocket" (A spacer B only) domain omitted? Would this potentially demonstrate differences in binding between Pdx1 and E2F, vs. the large pocket domain?

1st Revision - authors' response 30 December 2010

Response to Referees' Comments:

Referee #2 (Remarks to the Author):

1. In Figure 1, which form of Rb is felt to be interacting with Pdx1? Under or hyper phosphorylated forms? If Pdx1 associates with the Hyperphospho form of RB in contrast to E2f association with the Under phospho form, then how are they able to compete with one another for binding to RB as shown in Figure 1e?

Response: We have conducted additional experiments to address this concern.

First, we examined interaction of Pdx-1 with phospho-forms of RB in MIN6 cells using antibodies that detect RB phosphorylated on either serine 780 or on serines 807/811. Results from these experiments (Fig. 2E) show that Pdx-1 interacts with RB phosphorylated on Serine 780, but not on Serine 807/811. This data is in agreement with the site-directed mutagenesis data shown in Fig. 2F, that shows evidence of reduced Pdx1 interaction with RB carrying a mutant Serine 780, but a strong interaction with RB mutated on phospho-807/811.

Also, we show that Pdx-1 immunoprecipitated from wild type or Cdk4R24C islets binds the slower migrating phosphorylated form of RB (Fig. 2D), but Pdx-1 does not interact with underphosphorylated forms of RB (Fig. S2).

In addition to the data in islets and MIN6 cells, we evaluated RB/Pdx1 interaction in another beta cell line, -HC9. Similar to that observed in the islets and MIN6 cells we observed that Pdx-1 associated with the slower migrating phosphorylated form of RB, and not with the underphosphorylated forms of RB (Fig. S1).

Further, in the experiments assaying the effects of glucose levels on RB/Pdx-1 interaction, Pdx-1 preferentially associated with the slower migrating phosphorylated form of RB, and not with the underphosphorylated forms of RB (Fig. 5F, G).

Together, with results obtained from the co-immunoprecipitation experiments conducted with the Cdk4 inhibitor (Fig. 2B), we conclude that Cdk4-mediated phosphorylation promotes Pdx-1 interaction. We have now added this new information in the revised manuscript (Page 6, Lines 20 onwards till Page 7; Page 10, lines 12-14).

Regarding the competition, it has been shown that E2Fs associate with RB using the RIM domain as well as another marked-box region (O'Connor & Hearing, 1994). In addition, E2F association involves multiple binding regions on RB and E2Fs (Helin et al, 1992; Qin et al, 1992; Shan et al,

1996). We show that the RIM domain of Pdx-1 is involved with interaction with RB. However, we also find that mutations in several core amino acid residues of the RIM domain do not result in complete disruption of RB/Pdx-1 association. This is similar to E2F as well where RIM domain mutant E2F is able to associate weakly with RB. Together, these observations suggest that regions in addition to the RIM domain modulate RB/Pdx-1 interaction, similar to that documented for the RB-E2F interactions (Dick & Dyson, 2003). Indeed, after the first reports of the RB-E2F interactions via the RIM domain (Helin et al, 1992), only more recently it was shown that RB has an alternate binding region for E2F (Dick & Dyson, 2003). Thus, RB interacts with E2Fs via at least two distinct domains (Dick & Dyson, 2003). It is plausible that the RB-Pdx1 interaction also utilizes multiple binding regions, both on Pdx-1 and on RB. It is known that sequential phosphorylation of RB leads to dissolution of many of its interactions (with HDACs and E2Fs;(Harbour et al, 1999)). It is plausible that disruption of these interactions creates available binding sites for other proteins (like Pdx-1), with affinity for the phosphorylated forms of RB. This is now part of the discussion in the revised paper (Page 15, Lines 3 onwards; Page 16, lines 1-10).

2. In Figure 1f, the inference from the data presented is that both A and B domains are required for the interaction of Rb with Pdx1, yet the only mutant that is not generated and studied is the A-B mutant. This mutant must be generated and its ability to interact with Pdx1 shown. The ability of a full length Rb mutant lacking the A-B domains to interact with Pdx1 should be investigated, with the expectation based on the authors interpretation that interaction will be abolished.

Response: We have now included this data in the revised manuscript (Fig. 1F). Initially, during the first submission, we had problems expressing GST-RB (A+B). We subsequently obtained this mutant from the laboratories of William Kaelin (USA) and Sybille Mittnacht (UK). We have now conducted the suggested experiment. We find that Pdx-1 interacts well with the RB "large pocket" (A+B+C domain). However, Pdx-1 fails to bind the "small pocket" of RB (A+B) that lacks the C-domain. Also, Pdx-1 fails to associate with the C-domain (that lacks A+B domain). Moreover, we also observe that Pdx-1 associates weakly with the RB large pocket" containing a mutation in the critical CDK4-phosphorylatable serine residue #780 (Fig. 2F). Together, these results suggest that the A+B domains (small pocket), and the C-domain, individually, are necessary but not sufficient for Pdx-1 interaction. Instead, Pdx-1 requires the entire "large pocket" of RB for complete binding. This is analogous to that seen with E2F which also requires the RB "large pocket" for full association with RB (Qin et al, 1992). We have included this information in the revision (Page 6, Lines 12-17; Page 15, Lines 3-14).

3. Quantitation of Western blot signals should not be selective.

Response: Each western blot in this study has been repeated multiple times. We have diligently strived to keep cell lysis methods, protein isolation methods, and downstream assays consistent throughout the study to allow accurate comparison. Also, different exposures are taken from western blot auto-radiograms to ensure linearity of the signal. We use equal amounts of proteins in reactions, and use appropriate loading controls as shown. Our hope is that the rigor of the scientific integrity is evident in the data included in this manuscript.

4. Given the overriding theme of the manuscript and the interpretation that the Pdx1 and RB interaction is influencing Pdx1 stability, it is critical that Pdx1 protein stability be directly studied by examining Pdx1 protein half-life in the critical settings of mutagenesis.

Response: We have now performed a set of experiments that addresses this concern.

First, we examined half-life of Pdx-1 in Cdk4-wild type and Cdk4-R24C/R24C islets. As seen in Fig. 4E, we observe that Pdx-1 exhibits a longer half-life in Cdk4R/R islets compared to that observed in wild-type islets.

Second, we examined half-life of Pdx1 in normal wild type islets in the presence or absence of a CDK4-inhibitor. As seen in Fig. 4D, we observe that Pdx-1 exhibits a reduced half-life in islets incubated with the CDK4 inhibitor.

Third, we examined half-life of Pdx-1 in normal wild-type islets infected with or without

lentiviruses that express RB shRNA. As seen in Fig. 4C, we observe that Pdx-1 exhibits a reduced half-life in islets infected with RB shRNA.

These observations, taken together with the (i) reduced Pdx1 levels in MIN6 cells and islets incubated with RB siRNA (Fig. 4A) and RB-shRNA (Fig. 4B), (ii) RB-knockout embryonic pancreas (Fig. 7F), (iii) the increased Pdx1 levels in Cdk4R24C/R24C islets (Fig. 2C), (iv) the ubiquitination data (Fig. 4G) and, (v) studies with restoration of RB expression to RB-null cells (Fig. 4F, G), suggest that Pdx1 half-life and stability is regulated, at least in part, by the Cdk4-RB pathway.

We have now added this new information in the revised manuscript (Page 8, Lines 14 onwards till Page 9).

Recently, the Stoffers group elucidated that the Pcif1/SPOP protein interacts with Pdx1 and that the Pcif1/SPOP-Cu3 complex targets Pdx-1 protein for ubiquitination and proteasomal degradation (Claiborn et al, 2010). It remains to be seen whether the RB/Pdx-1 interaction and the effects on Pdx-1 stability, that we report here, modify the ability of Pcif1/SPOP-Cul3 complexes to target Pdx-1.

We have added this new information in the revised manuscript (Page 17, Lines 11-15).

5. In general, the mutagenesis of phosphorylated Serines on Rb and of the RIM motif of Pdx1 do not support the authors conclusion that these residues mediate the physical interaction. Rather, the data support an effect of these mutations on the expression level of Rb and Pdx1 respectively, which in turn affects the strength of the co-immunoprecipitation signal, as would be predicted (see Figures 2c, 2d, 3a, 3b). Less partner, less signal and vice versa. Thus, the reduced expression level and stability of the Pdx1 and Rb mutants complicates the interpretation of the co-IP experiment. These coIP experiments should be performed with equal starting amounts of Pdx1 and Rb respectively. The authors do get at this point on page 7 "It is plausible that the reduced affinity between RB and Pdx-1 mutants is, at least in part, attributable to the reduced expression of the mutant Pdx-1 proteins", and again on page 8 "It is interesting that although it interacts weakly with RB, the Pdx-1 M mutant gets stabilized by RB which is suggestive of alternate points of contact between RB and Pdx-1." Thus, the physical interaction motifs for both sides of this interaction may not yet have been identified in the current study. Any revision must definitively map the interaction motifs.

Response: We attempted to use equal amounts of Pdx-1 mutants in the co-immunoprecipitation assays. As seen in Fig. 3a and 3b, the levels of Pdx-1 proteins are equal yet we observe a lower RB binding in the Pdx-1 RIM mutants. However, we agree that the protein levels may influence the co-immunoprecipitation assays and have mentioned this very point in the paper (Page 8, Lines 3-6).

Please also see response to Point#1 above regarding the mapping of the interaction motifs.

6. The effect of glucose on Pdx1 expression in the current study (Figure 3i, j, k) is at odds with published literature in the same cell line in which Pdx1 half-life is acutely stabilized under high glucose conditions and destabilized under low glucose conditions in the same beta cell line (Humphrey et al). Further, the glucose regulation of RB is incomplete- how does glucose regulate Rb levels? Is Rb transcript different in Min6 cells under different glucose conditions? Is RB itself being turned over in high glucose conditions?

Response: We show that glucose levels regulate the ability of RB to occupy the promoter of Pdx-1 and of its target genes. We expanded the prior glucose regulation experiment to include (i) more time points, (ii) low and high glucose conditions, and (iii) short (2 hour) and long (24 hour) incubation conditions (Please see Fig. 5). We show that glucose regulates the RB/Pdx-1 interaction, with high glucose able to dissociate the RB/Pdx-1 complex. Further, we find that high glucose level promotes Pdx-1 ubiquitination and also reduces levels of Pdx-1 and RB proteins. We also show the regulation of RB levels by glucose.
As the reviewer correctly points out, the effect of glucose on Pdx-1 in our study is at odds with published literature in the same MIN6 cell line in which Pdx-1 half-life is acutely stabilized under high glucose conditions and destabilized under low glucose conditions (Humphrey et al, 2010). We

suggest that the different findings could be a consequence of the different experimental design of the experiments. We examined the half-life of endogenous Pdx-1 and its glucose-dependent interaction with endogenous RB using MIN6 cells without using an overexpression system. The study by Humphrey et al monitored the effects of GSK and AKT kinases on exogenous Pdx-1 levels in MIN6 cells transfected with Cytomegalovirus promoter-driven expression vectors encoding HA-tagged wild type and mutant Pdx-1. Further, our glucose-stimulation condition also differed from the study by Humphrey et al, where we starved MIN6 cells in glucose-free media for 24 hours, primarily to ensure that MIN6 cells are in a synchronized cell cycle state in consideration of the role of RB and Cdk4 in cell cycle progression. In addition, the purpose of pre-incubating the cells under glucose starvation conditions was to optimize the opportunity to observe the effects of glucose addition on Pdx-1 levels, and RB/Pdx-1 interactions. This method was also used by others in MIN6 cells (Martinez et al., Diabetes 55, 1581-91).
This new information is on Page 9, Line 19 onwards; Page 16, Line 11 onwards.

7. In general, the critical aspects of the biochemical model as interpreted by the authors lack validation in the Rb null model. The finding of reduced proliferation of Pdx1+ progenitors can easily be understood from known molecular roles of RB and could likely be independent of RB-Pdx1 physical association.

Response: We have now conducted the suggested experiment that examined the relative Pdx-1 protein levels in the RB knockout pancreas. This data (Fig. 7F) shows reduction in Pdx-1 protein levels due to RB deficiency. In addition, we have examined the level of apoptosis in the RB knockout pancreas. Based on the data shown, we interpret that RB deficiency results in increased apoptosis in the RB knockout embryonic pancreas (Fig. 7D, E). These observations, taken together with the reduced proliferation of Pdx-1+ pancreas progenitors (Fig. 7A, C), allows us to conclude that the reduced pancreas size in the RB knockout embryonic pancreas is due to a combination of limited proliferation of pancreas progenitors and increased apoptosis. It is unclear whether the two processes, defective proliferation and increased apoptosis, are both due to the destabilized Pdx-1. It is plausible that the increased apoptosis could be a result of a general cell cycle role played by RB. We have now added this new information in the revised manuscript (Page 12, Lines 6-15; Page 18, Lines 16-25).

8. The x-axis labels are missing for Figure 4c.

Response: We have edited the figure (new Fig. 6C)

Additional points:

1. The title is misleading as it is never demonstrated that Pdx-1 is destabilized in the Rb knockout embryos.

Response: We hope that the data on Pdx1 protein levels in the RB knockout embryonic pancreas addresses this concern.

2. Why are the different GST fusion constructs not appearing to migrate according to their size (Figure 1f)? The pRb 379-612 does not migrate as the similarly sized 612-928 construct.

Response: We believe that the GST fusion constructs are migrating as expected based on their predicted sizes (as indicated by asterisks).

3. In figure 3g, Pdx-1 wild type and mutant are not significantly ubiquitinated yet when Rb is added there is significant laddering. Doesn't this indicate that Rb is inducing Pdx-1 ubiquitination and not protecting it as they authors claim?

Response: We believe that absence of RB allows ubiquitination and subsequent degradation of the ubiquitinated Pdx1 protein. However, when RB is present, Pdx-1, although ubiquitinated, does not get degraded in the proteasomal system. This is similar to adding a proteasomal inhibitor to the assay, when detectable ubiquitinated forms are observed. We infer that RB interaction protects Pdx-1 from getting degraded, although it may not necessarily protect it from getting ubiquitinated. We have included this in the discussion on Pages 17, Lines 7-15.

4. Does interaction with RB enhance Pdx1 transactivation?

Response: Insulin is a bona-fide Pdx1 target gene. We observe that RB, but not phospho-mutant RB, enhances the activity of the luciferase linked insulin reporter in MIN6 cells. Please see supplementary Fig. S4.

5. An error bar is missing in figure 5d.

We have edited the figure (new Fig. 7H)

6. Figure 1b, need to show the Pdx1 IP blot

It is shown in the revised figure.

7. Figure 2 (b) Need to show Pdx1 IP blot. Would also be nice to see ppRB blots of Pdx1 IP; is one of the phospho-RB forms preferentially interacting with Pdx1?
It is shown in the revised figure. Also, see new Figure 2E.

8. Figure 3 (k) Need to show the Pdx1 IP blot

It is shown in the revised figure (new Fig. 5C, D)

Referee #3:

1) As mentioned above, a favoritism of Pdx1 for phospho Rb stands in contrast to the affinity of E2F for hypophosphorylated RB, and is at the core of the authors' model in figure 5. However the evidence to support this is thin, essentially based on RB migration in figure 1 and some mutant binding experiments that are compromised by changes in RB level that may disguise a lack of change in affinity. Most remarkable about this possibility is that the two target proteins use arguably highly similar motifs to interact with RB, so the difference in regulation by phosphorylation is fascinating, but puzzling. Experiments should be performed to rigorously test this model.

Response: We have conducted additional experiments to address this concern.

First, we examined interaction of Pdx-1 with phospho-forms of RB in MIN6 cells using antibodies that detect RB phosphorylated on either serine 780 or on serines 807/811. Results from these experiments (Fig. 2E) show that Pdx-1 interacts with RB phosphorylated on Serine 780, but not on Serine 807/811. This data is in agreement with the site-directed mutagenesis data shown in Fig. 2F, that shows evidence of reduced Pdx1 interaction with RB carrying a mutant Serine 780, but a strong interaction with RB mutated on phospho-807/811.

Also, we show that Pdx-1 immunoprecipitated from wild type or Cdk4R24C islets binds the slower migrating phosphorylated form of RB (Fig. 2D), but Pdx-1 does not interact with underphosphorylated forms of RB (Fig. S2).

In addition to the data in islets and MIN6 cells, we evaluated RB/Pdx1 interaction in another beta cell line, -HC9. Similar to that observed in the islets and MIN6 cells we observed that Pdx-1 associated with the slower migrating phosphorylated form of RB, and not with the underphosphorylated forms of RB (Fig. S1).

Further, in the experiments assaying the effects of glucose levels on RB/Pdx-1 interaction, Pdx-1 preferentially associated with the slower migrating phosphorylated form of RB, and not with the underphosphorylated forms of RB (Fig. 5F, G).

We performed the phosphatase-treatment experiment as suggested by the reviewer. The results were inconclusive. We observed that phosphatase treatment retained Pdx-1 and E2F binding to RB. Our interpretation being that either the phosphatase treatment did not work optimally or that some degree of dephosphorylation is required to regulate Pdx-1/E2F association with RB (as correctly alluded to

by the reviewer). As these results were largely inconclusive, we have not included them in the revision.

Together, with results obtained from the co-immunoprecipitation experiments conducted with the Cdk4 inhibitor (Fig. 2B), we conclude that Cdk4-mediated phosphorylation promotes Pdx-1 interaction. We have now added this new information in the revised manuscript (Page 6, Lines 20 onwards till Page 7; Page 10, lines 12-14).

2) The "lack of proliferation" in RB knockdown pancreas and decrease in gene expression could be a result of proliferation control per se, or could be the result of cell death. It would be straightforward to assay for apoptotic cell death in these knockdown cultures, and such death would be consistent with that seen in a number of other models of RB loss in tissues. This is important to know to determine if the loss of proliferation is a possible result of cell loss, or truly a cell cycle effect.

Response: We have now conducted the suggested experiment that examined the relative Pdx-1 protein levels in the RB knockout pancreas. This data (Fig. 7F) shows reduction in Pdx-1 protein levels due to RB deficiency. In addition, we have examined the level of apoptosis in the RB knockout pancreas. Based on the data shown, we interpret that RB deficiency results in increased apoptosis in the RB knockout embryonic pancreas (Fig. 7D, E). These observations, taken together with the reduced proliferation of Pdx-1+ pancreas progenitors (Fig. 7A, C), allows us to conclude that the reduced pancreas size in the RB knockout embryonic pancreas is due to a combination of limited proliferation of pancreas progenitors and increased apoptosis. It is unclear whether the two processes, defective proliferation and increased apoptosis, are both due to the destabilized Pdx-1. It is plausible that the increased apoptosis could be a result of a general cell cycle role played by RB. We have now added this new information in the revised manuscript (Page 12, Lines 6-15; Page 18, Lines 16-25).

3) if Rb predominantly controls Pdx1 through stability, can enforced overexpression of Pdx1 increase gene expression, proliferation, and promoter binding in the cell-based assays used here?

Response: This is a very good point that will require studies where RB levels and RB function (via a CDK4 activation/inactivation) are altered in cells, and preferably in vivo. We are unable to address this larger question in this present manuscript.

4) In the domain mapping experiments in figure 1, why was the "small pocket" (A spacer B only) domain omitted? Would this potentially demonstrate differences in binding between Pdx1 and E2F, vs. the large pocket domain?

Response: We have now included this data in the revised manuscript (Fig. 1F). Initially, during the first submission, we had problems expressing GST-RB (A+B). We subsequently obtained this mutant from the laboratories of William Kaelin (USA) and Sybille Mittnacht (UK). We have now conducted the suggested experiment. We find that Pdx-1 interacts well with the RB "large pocket" (A+B+C domain). However, Pdx-1 fails to bind the "small pocket" of RB (A+B) that lacks the C-domain. Also, Pdx-1 fails to associate with the C-domain (that lacks A+B domain). Moreover, we also observe that Pdx-1 associates weakly with the RB large pocket" containing a mutation in the critical CDK4-phosphorylatable serine residue #780 (Fig. 2F). Together, these results suggest that the A+B domains (small pocket), and the C-domain, individually, are necessary but not sufficient for Pdx-1 interaction. Instead, Pdx-1 requires the entire "large pocket" of RB for complete binding. This is analogous to that seen with E2F which also requires the RB "large pocket" for full association with RB (Qin et al, 1992). We have included this information in the revision (Page 6, Lines 12-17; Page 15, Lines 3-14).

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Pre-acceptance letter 03 February 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74771R. Please let me first apologise for the delay in getting back to you with a decision on this: unfortunately, it took quite some time for referee 3 to provide us with his/her comments. In addition, referee 2 was unable to re-review the manuscript, but we asked referee 3 to comment on your responses to referee 2's concerns as well.

As you will see from his/her comments below, referee 3 is happy with your revision, and is now fully supportive of publication in EMBOJ without further changes. I am therefore pleased to be able to tell you that we will be able to accept your manuscript for publication here. However, there are just a couple of issues from the editorial side that I need to ask you to deal with first.

- You give fold changes as quantifications of your Western Blots in most cases, but there is no indication as to whether this is simply the fold change from that one experiment, or whether it represents an average over several experiments. A clear statement in the Figure Legends would be necessary here.
- Similarly, while you give the error bars and statistical significance on your various graphs, you do not state the number of replicates done - again, this needs to be explicit in the figure legends.
- We now routinely require Author Contributions and Conflict of Interest statements for all accepted manuscripts - please can you include these (below the Acknowledgements section)?
- I'm not entirely happy with the title: I find it tautological, and currently it implies that the stabilising of Pdx1 and the precluding proteasomal degradation are independent functions of Rb. I would propose the following alternatives:
"RB regulates pancreas development by protecting Pdx1 against proteasomal degradation"
or simply
"RB regulates pancreas development by stabilising Pdx1"
Please can you let me know whether you would be happy with one of these alternatives, and change the title accordingly. Some other variation on the theme would also be fine...

If you can make these various changes in the text file, and send us a new version, we can upload this

in place of the previous file. Once we have this, we should then be able to accept your manuscript for publication without further delay.

Many thanks and best wishes,

Editor
The EMBO Journal

Referee 3 comments:

The significant new data provided greatly strengthen this manuscript, and I believe it is now suitable for publication. The findings will be of high interest to the RB field, and, of course, to those interested in pancreas development. The general interest is also likely to be quite strong. Thus, while cell death following RB loss may be the fundamental cause of reduced size of the pancreas, the proliferative effects of reduced Pdx1 in RB null cells certainly could contribute, as argued by the authors.
Whether Pdx1 levels alone could fix this defect in the absence of RB remains to be determined, and indeed addressing this would be a lot to ask in the context of this already-extensive work. Finally, the interaction data are now quite nice and demonstrate how different RB binding factors can share domain interactions yet be quite different in their association with RB.

