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CAVIN-3 regulates circadian period length and PER:CRY protein abundance and interactions

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Please note that this manuscript was originally submitted to the EMBO Journal where it was peer-reviewed and revised. It was then transferred to EMBO reports with the original referees comments attached. (Please see below)

Original referee comments – EMBO Journal

Ms #**EMBOJ-2012-80652**
Referee #1 Review

Schneider et al. identify CAVIN-3, a cavelolae associated protein, as a novel PER2 interacting protein that is required for normal circadian dynamics. They use mass spectrometry to screen for proteins present in immunocomplexes with PER2 that has been ectopically overexpressed in a murine fibroblast cell line. In addition to the usual (known) suspects, CAVIN-1 and CAVIN-3 were among the PER2 interaction partners, which attracted the attention of the authors, since those are reported (and later also shown by the authors) to be primarily cytosolic proteins. The authors confirmed that CAVIN-3 can interact with PER2 when overexpressed in cell culture. They then go

on and try to investigate a potential effect of CAVIN-3 on PER2 protein properties. The use a mammalian two-hybrid system to study the interaction between PER2 and one of its established interaction partners, CRY2. Interestingly, the signal that is used to report interaction is substantially and robustly increased, when CAVIN-3 is co-expressed (or reduced when CAVIN-3 is downregulated). Surprisingly, however, at the same time also the abundance of the overexpressed hybrid proteins are altered in an unexpected direction: when CAVIN-3 is overexpressed PER2 and CRY2 hybrid protein levels are reduced in spite of an increased reporter signal. Next, the authors show that altering CAVIN-3 gene dosage shortens (when downregulated) and lengthens (when overexpressed) the circadian period in oscillating fibroblasts, which indicates an important role for CAVIN-3 in the core circadian clock.
This is an interesting study, which provides evidence that CAVIN-3 - a novel PER2 interacting protein - is important for circadian rhythmicity. While the experiments presented are well performed and mostly carefully conducted, the mechanism of CAVIN-3 action remains largely elusive despite the fact that the authors claim that the binding of PER2 and CRY2 is strengthened. This claim might be true but need at least some independent verification, since also alternative explanations are possible.

Here are my concerns more specifically:
1. Does the interaction of CAVIN-3 and PER2 also occur in vivo? The authors should use their polyclonal serum to show that endogenous CAVIN-3 interacts with endogenous PER2.
2. Mammalian two-hybrid studies: In my opinion, the fact that the bioluminescence signal increases upon co-expression of CAVIN-3 does not provide strong evidence to conclude that the affinity of the PER2-CRY2 interaction is increased. Is relatively more CRY2 immunoprecipitated with PER2 when CAVIN-3 is overexpressed? Or is there any other independent way to show that the binding is stronger? Isn't it possible that the signal is higher because the subcellular localization of the hybrid complex is altered towards a more nuclear localization? I realize that NLS have been added to the hybrid proteins to ensure (a better) nuclear localization. However, by doing so the authors cannot investigate the role of CAVIN-3 on this process, which might be conceivably influenced by a cytosolic PER2 interaction partner. Wouldn't the hybrid proteins be localized in the nucleus anyway, since PER2 has its own NLS and CRY is also always nuclear?
3. It is interesting that an alteration of CAVIN-3 gene dosage changes the abundance of PER2 and CRY2 when expressed as hybrid proteins. Is this also true for the endogenous proteins? It may well be that this property of CAVIN-3 is causing the circadian phenotype (and not the suggested increase in affinity of the PER2-CRY2 complex).
4. RNAi studies: It is important to show that the endogenous transcripts or proteins are downregulated, since this downregulation is claimed to cause the circadian phenotype. It is not sufficient to show that the RNAi constructs are in principle able to downregulate (with ectopically expressed proteins) - we want to know whether they did it in the cells (Figs. 3D, 3F, S7F). This is especially important, since here the circadian phenotype does not seem to be correlated with the downregulation efficiency of the overexpressed proteins (Fig.S8): hp6 and hp8 show similar phenotypes but substantially different downregulation. What is the phenotype of hp5 that nicely downregulates? In addition, the appropriate control is a mutated, scrambled or irrelevant hairpin construct (not an empty vector). The authors did this control (Fig.S8C), but apparently without repetition (no error bars). However, it is important to assess, how much of the phenotype is attributed to engaging the endogenous RNAi processing machinery per se and how much comes from downregulating endogenous CAVIN-3.

Minor points:
- Fig. 1B: how do the authors explain this dramatic induction of PER2 protein by serum shock? This is probably not correlated with increase of the corresponding mRNA, correct?
- Supplementary Fig. S8B: Panels apparently mislabeled - this is the overexpression, not hp3.
- Supplementary Fig. S7D: Error bar of hp8?
- Supplementary Fig. S7F: what is hp1-8?
- Please provide a list of all identified peptides and associated proteins of the mass spectrometry experiment in a supplementary table.
- Fig. 6 doesn't add to the understanding of CAVIN-3 function and should be removed from the main text.

Ms #**EMBOJ-2012-80652**
Referee #2 Review

The authors present convincing evidence that cavin-3 stabilizes the interaction of PERIOD and CRYPTOCHROME. All experiments are of high technical quality and fully support the conclusions drawn by the authors, there are no additional experiments required. The authors interpret their data with great care. The data are of high general interest.

Minor points:

Figure 1B. Please explain why expression of PER2-TAP and TAP-luciferase, respectively, under control of the CMV promoter is dependent on a serum shock?

Figure 4F: the effect of the synchronization protocol on circadian period length is descriptive and does not contribute to paper.

Ms #**EMBOJ-2012-80652**
Referee #3 Review

In this manuscript, the authors implicate the cytoplasmic protein CAVIN-3 as a novel PER2-interacting partner, and describe the potential circadian function of CAVIN-3. The authors present intriguing observations that may suggest how CAVIN-3 controls the activity of the circadian clock's inhibitory complex, PER:CRY. Apparently the circadian function of CAVIN-3 is independent of its conventional role as a component in the plasma membrane structures known as caveolae. It is well established that posttranslational regulation of PER:CRY is essential for the circadian clock's core transcriptional feedback loop: the cytoplasmic accumulation of PER:CRY and/or nuclear translocation of PER:CRY must be delayed so that their inhibitory activity occurs at the appropriate time. Thus, the cytoplasmic interaction between PER:CRY and CAVIN-3 could be critical for the circadian clockwork. However, the presented data are not very convincing and there are no mechanistic studies to address how CAVIN-3 may be involved in the clock mechanism. In fact, the authors did not propose any molecular mechanism for how CAVIN-3 could affect the clock through regulation of PER:CRY. The findings are potentially very interesting, but they are too premature to be considered for publication in EMBO. My major concern is that CAVIN-3 was identified under artificial conditions, suggesting that the interaction may not be biologically relevant: CAVIN-3 may only interact with PER when PER is highly overexpressed. The interaction has to be thoroughly verified and characterized in vivo using the clock antibodies available to the authors. I have several other concerns, described below.

1. In fig 1, the level of coimmunoprecipitated CRY seems to be higher than the overexpressed bait protein, PER2-TAP. The CRY must be endogenous and would be less abundant than the overexpressed PER-TAP. Further, the interaction can not be preserved 100% throughout the purification process. This result needs to be explained. The authors used the constitutive CMV promoter to drive the expression of PER2-TAP. Why is there no protein at 0 and 1 hr after serum shock in fig 1B? In fig 1D and 1E, a more relevant negative control would be IP of extracts from CAVIN-3-transfected cells with anti-Flag antibody, since the anti-Flag antibody could pull down CAVIN-3 in the absence of PER2.

2. The authors claim that CAVIN-3 does not oscillate, but it seems to oscillate as much as PER2 in NIH 3T3 cells. Even non-specific bands seem to be oscillating relative to the constitutive control U2AF65. PER2 oscillations in Fig2D are not consistent with those in fig 1B, which showed a dramatic difference between 0 and 4 hours after serum shock. I am wondering why ICC has been done on exogenous CAVIN-3 when antibody to endogenous CAVIN-3 is available. The authors should show the interaction with endogenous proteins since the antibodies against the endogenous proteins are available. Time-course studies in vivo, whether in cells or mouse tissues, should be performed as well, as these would be very informative towards a potential clock role of CAVIN-3.

3. I could not understand the rationale and significance of the mammalian two-hybrid data presented in fig 3, unless this is validated in vivo. CAVIN-3 may up- or down-regulate levels/activity of

PER:CRY in the cytoplasm, as the authors showed, but how does this translate to a role in the in vivo circadian clock? How does this result in the altered periods shown in fig 4? There is a big gap between fig 3 and fig 4. I do not understand how data in fig 3 could lead to those in fig 4.

In summary, although the authors present potentially interesting findings and may have identified a new regulatory mechanism for the circadian clock, they fail to provide any mechanistic insights from their observations. This work is not publishable in EMBO at this time. However, the manuscript could be significantly improved if the authors provide more in vivo validation and mechanistic studies.

Ms #**EMBOJ-2012-80652R**
Referee #1 Review

The authors did a considerable effort to improve their manuscript in response to my and the other Reviewers' comments.
I think they did an excellent job and the ms is now sufficiently improved to qualify for publication, because:
(i) engogenous PER2-CAVIN-3 interaction is now shown (using a strategy already applied by Maier et al., G&D 2009)
(ii) they provide evidence for an involvment of a kinase binding site on CAVIN-3 for both the period effect and the effect in the M2H-assay - an indication that these two phenotypes are linked.
(iii) discussion/interpretation/title are now appropriately careful

Ms #**EMBOJ-2012-80652R**
Referee #3 Review

The authors made a great effort to improve the manuscript, but my concerns have not been generally addressed. I still do not see mechanistic insights into how CAVIN3 can contribute to the timekeeping mechanism. Does it enhance PER:CRY complex formation in the cytoplasm, which may result in earlier nuclear entry of the complex? The authors have all the means to manipulate CAVIN3 expression in vivo using lentiviral vectors and shRNAs and measure circadian rhythms using an endogenous reporter such as the endogenous Per2-Luc reporter. Further, the authors have good antibodies to endogenous CAVIN3 and PER2. I do not understand why these antibodies cannot be used to detect the endogenous proteins and their interactions in CAVIN3 overexpressed and knockdown cells. Contrary to the authors' claim, anti-PER2 antibody immunoblots seem very clean (Fig 1B, D, S12A). The authors should complement their in vitro reporter and two-hybrid assays-which are complicated and error-prone-with quantitative in vivo coimmunoprecipitation assays and endogenous immunoblots. The following are other concerns.

1. Fig 1F is missing a control. PER2-LUC may be more prone to non-specific precipitation than CMV-LUC, which can cause higher background, since PER2-LUC is a much larger protein. The authors need to show that the high levels of luciferase are due to specific coprecipitation by the anti-CAVIN3 antibody.

2. In Fig 2, why can't the authors measure circadian rhythms from the endogenous Per2 promoter using the Per2-Luc cells after CAVIN3 is modulated? Readers would be more convinced if the period changes are observed from an endogenous reporter with more robust peaks rather than artificial promoters with 2-3 cycles.

3. The authors suggest that CAVIN3 destabilizes individual PER2 and CRY but increases stability of the PER2:CRY complex. I can't envision how this mechanism can significantly affect the period of the clock because CAVIN3's function in the clock would make a futile cycle by promoting two reactions in the opposite direction.

In summary, the in vitro nature of most of the data and lack of convincing mechanistic insights make this reviewer hesitate to recommend this manuscript for publication in EMBO.

Transfer - authors' comments – Initial correspondence 23 August 2012

Dear editors,

As suggested by the editor of EMBO journal, we would like to transfer our manuscript to EMBO reports for publication.

We expect we can provide a revised version with the requested additional experiments within 2 to 3 weeks.

1st Revision - authors' response 24 September 2012

Response to Reviewers.

Referee #1:
We thank the Referee for his interest in our study and for his valuable comments and suggestions, which allowed us to significantly improve our manuscript. We hope that we have been able to satisfy most of Referee #1's concerns in our new, revised version.

Point-to-point response:

Major points

1. Does the interaction of CAVIN-3 and PER2 also occur in vivo? The authors should use their polyclonal serum to show that endogenous CAVIN-3 interacts with endogenous PER2.

We have now added data on the endogenous PER2:CAVIN-3 interaction as **Figure 1F**.
Since our polyclonal anti-PER2 serum gave inacceptable background signal in the western blots from cell extracts, we have developed a new strategy to demonstrate the specific IP of endogenous CAVIN-3 and PER2:
We made use of *Per2::Luciferase* knock-in mice that were developed by Joseph Takahashi's lab (Yoo et al, 2004) and that are widely used in the circadian field as a circadian reporter mouse strain. Briefly, the expression of the PER2::Luciferase fusion protein is controlled by the endogenous *Per2* locus and encodes a fully functional PER2 protein, as shown in numerous studies. From these animals, we grew primary tail fibroblasts, prepared cell extracts, and precipitated endogenous CAVIN-3 using our polyclonal antiserum (or pre-immune serum for the control-IP). We then measured PER2::Luciferase co-IP by determining co-precipitated luciferase activity. In three independent experiments we have thus observed that the IP with anti-CAVIN-3 antibodies led to a >20-fold higher luciferase activity in the immunoprecipitate as compared to the pre-immune serum control IP.
As a control cell line for non-specific luciferase precipitation in the IP, we performed the identical experiment using NIH3T3 cells stably expressing luciferase under the control of the CMV promoter. From extracts of these cells, anti-CAVIN-3 antibodies precipitated a similar amount of luciferase activity (less than 1.5-fold difference) than did the pre-immune serum. We thus concluded that the observed co-purification of luciferase activity in the *Per2::Luciferase* samples was not due to cross-reactivity of the antibodies with luciferase protein, but indeed reflected the endogenous CAVIN-3:PER2::Luc interaction.
Finally, we also controlled for the level of CAVIN-3 expression in the two cell lines because a considerably higher CAVIN-3 expression in the *Per2::Luciferase* primary cells would have compromised the interpretability of the observed enrichment. As shown in **Supplementary Fig. 3**,

CAVIN-3 is in fact expressed at lower levels in the primary cells as compared to NIH3T3 cells, ruling out an unspecific enrichment that is merely due to higher CAVIN-3 expression in the *Per2::Luciferase* cells.
We hope that these new data, together with the data on the tagged proteins that we already presented in the first version of the manuscript, provide strong evidence that CAVIN-3 is indeed a novel *bona fide* interaction partner of PER2.

2. Mammalian two-hybrid studies: In my opinion, the fact that the bioluminescence signal increases upon co-expression of CAVIN-3 does not provide strong evidence to conclude that the affinity of the PER2-CRY2 interaction is increased. Is relatively more CRY2 immunoprecipitated with PER2 when CAVIN-3 is overexpressed? Or is there any other independent way to show that the binding is stronger? Isn't it possible that the signal is higher because the subcellular localization of the hybrid complex is altered towards a more nuclear localization? I realize that NLS have been added to the hybrid proteins to ensure (a better) nuclear localization. However, by doing so the authors cannot investigate the role of CAVIN-3 on this process, which might be conceivably influenced by a cytosolic PER2 interaction partner. Wouldn't the hybrid proteins be localized in the nucleus anyway, since PER2 has its own NLS and CRY is also always nuclear?

We agree with the reviewer that the evidence from the two-hybrid data presented in the first version of the manuscript was not sufficient to permit the conclusion that "CAVIN-3 stabilizes Period:Cryptochrome interactions". In the new version of the manuscript, we are now more careful with our interpretation of the two-hybrid experiments, we have added more data to address different mechanistic possibilities, and we discuss alternative explanations in more detail. We have also changed the title of the manuscript to *"CAVIN-3 regulates circadian period length and PER:CRY protein abundance and interactions"* in order to avoid a potential overstatement.
In the following paragraphs, we have tried to provide hopefully adequate responses to the questions posed by the reviewer.

Different possibilities are conceivable to explain the CAVIN-3-mediated increase in PER:CRY two-hybrid signal:

(1) After all the cause could be an increased interaction between the two proteins, either (1a) by a direct mechanism, i.e. by engaging into a tertiary PER:CRY:CAVIN3 complex. Since the two-hybrid signal is generated by interactions in the nucleus and since CAVIN-3 is primarily cytoplasmic, this scenario is perhaps rather unlikely. Stabilization could also occur (1b) more indirectly, with CAVIN-3 acting on PER:CRY complex components in the cytoplasm by a mechanism that improves their interaction even upon nuclear import of PER:CRY. Such a mechanism could for example involve posttranslational modifications on PER and/or CRY proteins.

(2) The higher two-hybrid signal could be a mere consequence of CAVIN-3's effect on PER and CRY protein levels. CAVIN-3 overexpression and knockdown led to a decrease and increase, respectively, of PER and CRY abundance (see also the referee's next question below) and it is imaginable that under certain conditions low levels of PER and CRY actually yield a stronger two hybrid signal. This could for example be the case when excess PER and CRY titrate away an endogenous factor that could then become limiting for productive transcription of the two-hybrid reporter.

(3) Conceivably, CAVIN-3 may be involved in titrating away an (unknown) inhibitory protein that would otherwise reduce the activity of the PER:CRY complex in the two-hybrid system. In a related model, this could also involve mechanisms such as the one suggested by the reviewer, i.e. that CAVIN-3 influences the nuclear localization of the two-hybrid partner proteins.

We have now undertaken considerable efforts to distinguish between these possibilities and we discuss alternative interpretations of the data with more caution.
With regard to model (1), we have made several attempts to assess whether relatively more CRY2 is precipitated with PER2 when CAVIN-3 is overexpressed, as suggested by the reviewer. However, given that CAVIN-3 overexpression also had strong effects on the abundance of PER2 and CRY2, these experiments turned out to be rather complicated to interpret. With absolute quantities of the proteins changing strongly in the inputs and IPs, the calculation of relative IP efficiencies from

western blots was thus highly unreliable and we have not included these experiments in the manuscript.
However, we have now addressed alternative models by new experiments. To simulate the scenario (2) according to which it is only the CAVIN-3-mediated changes in PER and CRY abundance that alter the two-hybrid signal, we have titrated the two-hybrid plasmids to 5-fold lower and 2-fold higher levels. As shown in **Supplementary Fig. 13**, we did not observe a general sensitivity of the two-hybrid assay to BD-PER2 and AD-CRY2 quantities. Moreover, CAVIN-3 was able to increase the two-hybrid signal over the whole range of the titration. We concluded from this experiment that the increased two-hybrid signal observed upon CAVIN-3 overexpression was unlikely to reflect a simple secondary effect of changes in PER2 and CRY2 abundance, thus arguing against model (2).
Admittedly, we cannot rigorously rule out model (3) according to which CAVIN-3 titrates away an (unknown) inhibitory factor of the PER2:CRY2 two-hybrid assay. By contrast, we find it less probable that a CAVIN-3-mediated shift of cytoplasmic PER2 and CRY2 to the nucleus was responsible for the increased reporter levels. PER and CRY proteins are already predominantly nuclear, and the AD and BD tags carry additional nuclear localization signals. We thus deem it unlikely that the very strong effect CAVIN-3 has on the PER:CRY two-hybrid signal, would be caused by shifting the subfraction of previously cytoplasmic protein to the nucleus.
Finally, we have added new data that could point to the molecular mechanism underlying the activity of CAVIN-3 in the two-hybrid assay and in the circadian clock in general. CAVIN-3 was previously identified as an interacting protein for PKCδ and was subsequently proposed to serve as an adapter protein for protein kinase recruitment. We have now created and functionally tested a CAVIN-3-mutant protein, in which two serines and a threonine that had been reported to be important for kinase interaction, were mutated to alanine **(Supplementary Fig. 14)**. Interestingly, in contrast to wild-type CAVIN-3, transfection of this construct no longer caused the gain-of-function phenotype in the two-hybrid assay (**Fig. 5F**), nor did it increase the circadian period length (**Fig. 5E**). These results would suggest that CAVIN-3 requires its known kinase-binding site and that a (yet to be characterized) kinase might be involved in the underlying mechanism.

3. It is interesting that an alteration of CAVIN-3 gene dosage changes the abundance of PER2 and CRY2 when expressed as hybrid proteins. Is this also true for the endogenous proteins? It may well be that this property of CAVIN-3 is causing the circadian phenotype (and not the suggested increase in affinity of the PER2-CRY2 complex).

In the first version of our manuscript, we had shown that CAVIN-3 gene dosage changed the abundance of BD-PER2 and AD-CRY2 proteins, but an analysis of endogenous PER2 levels was missing. In **Supplementary Fig. 12** we now show that CAVIN-3 overexpression also affects endogenous PER2 levels, which are ca. 2-fold lower.
As mentioned in the answer to point 2, we deem it unlikely that the effect of CAVIN-3 on the two-hybrid signal is just a consequence of reduced PER and CRY quantities, since titration of the two-hybrid expression plasmids to lower levels did not mimic the effect of CAVIN-3 co-expression. Our data rather support a model according to which there are qualitative differences of the PER:CRY complex (e.g. post-translational modifications) that engender increased two-hybrid signals. Conceptually, however, the reduction in PER and CRY quantities that we observe upon CAVIN-3 co-expression may be a consequence of the proposed qualitative changes.
With regard to the effect of CAVIN-3 on the free-running circadian period length, the effects on PER and CRY levels may very well be of importance, as it is well established that altered PER2 stability and expression levels influence the circadian period length (Godinho et al, 2007). In the circadian reporter assays, we are unfortunately unable to differentiate between an effect of PER and CRY quantities and the proposed qualitative changes within the complex. However, the fact that CAVIN-3-mut, carrying point mutations in the PKC binding domain, no longer promotes the gain-of-function phenotype in either the two-hybrid assay or in the circadian reporter assay, is suggestive of a common molecular basis.

4. RNAi studies: It is important to show that the endogenous transcripts or proteins are downregulated, since this downregulation is claimed to cause the circadian phenotype. It is not sufficient to show that the RNAi constructs are in principle able to downregulate (with ectopically expressed proteins) - we want to know whether they did it in the cells (Figs. 3D, 3F, S7F). This is especially important, since here the circadian phenotype does not seem to be

correlated with the downregulation efficiency of the overexpressed proteins (Fig.S8): hp6 and hp8 show similar phenotypes but substantially different downregulation. What is the phenotype of hp5 that nicely downregulates? In addition, the appropriate control is a mutated, scrambled or irrelevant hairpin construct (not an empty vector). The authors did this control (Fig.S8C), but apparently without repetition (no error bars). However, it is important to assess, how much of the phenotype is attributed to engaging the endogenous RNAi processing machinery per se and how much comes from downregulating endogenous CAVIN-3.

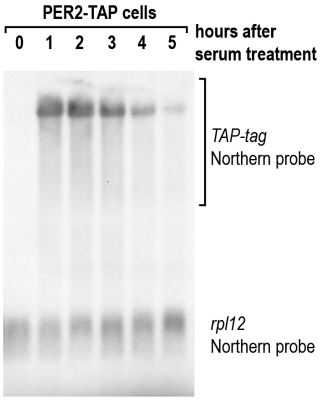
We agree with the reviewer that monitoring endogenous *Cavin-3* mRNA or protein levels are by far the better experiment to assess knockdown efficiencies and we have added such data as **Fig. 3F**. Since transfection efficiencies in our NIH3T3 cells are fairly low (ca. 20%), we FACS-sorted transfected cells on the GFP that is also expressed from the shRNA plasmids, and we then measured endogenous *Cavin-3* mRNA levels by qPCR. This is a fairly tedious procedure that requires large amounts of cells and we have performed it for two distinct *Cavin-3*-directed shRNA constructs (hp1 and hp7). Compared to the control construct, these shRNAs thus downregulate endogenous *Cavin-3* by around 80% (**Fig. 3F**). In view of this qualitatively better data, we have removed the experiments using ectopically expressed proteins from the manuscript.
We agree that an irrelevant hp is the better control in the loss-of-function experiments. We have repeated this control several times and are showing the data in **Supplementary Fig. 6**. The irrelevant hp-treated cells thus display a marginally shorter period length as compared to the empty vector. With regard to the irrelevant hp, the three hairpins hp1, hp4 and hp7, show a statistically significant ($p<0.05$) period shortening. Several other hps shown in **Supplementary Fig. 6** have a tendency towards a short period as well.

Minor points:

5. Fig. 1B: how do the authors explain this dramatic induction of PER2 protein by serum shock? This is probably not correlated with increase of the corresponding mRNA, correct?

This result was a surprise to us as well and we were initially excited by the possibility that there might be a serum-responsive element localized within the *Per2* cDNA. However, a number of follow-up studies then showed that stably transfected transgenes using our TAP-tag vector were frequently serum-responsive even in the absence of any *Per2* sequence. This strong serum induction actually also already occurred on the mRNA level, as shown in the Northern blot in the accompanying figure.
Interestingly, similar observations have been made previously. Brightwell et al. for example found that (1) the expression of CMV-driven transgenes was strongly affected by the cell cycle (essentially silent in G0/G1, i.e. in a confluent dish of cells) and (2) the expression was strongly induced up to 10-fold within 2 hours of serum exposure (Brightwell et al, 1997). It would thus appear that in NIH3T3 cells, stably transfected transgenes driven from the viral CMV promoter are subject to silencing and re-activation upon serum treatment; the CMV promoter may thus not be as constitutive as expected after all.
In our TAP-tag purifications, cells were indeed also grown to confluence in order to obtain the maximum amount of cell extract for the experiment, and then subjected to the 50% horse serum shock to synchronize cellular oscillators. We think that it is rather coincidental that the PER2-TAP induction kinetics are very similar to those of endogenous PER2.

Northern blot showing *Per2-TAP* mRNA induction. Total RNAs extracted from PER2-TAP expressing cells submitted to serum treatment (0, 1, 2, 3, 4, 5 hours) were analyzed by Northern blot with a probe recognizing the tag sequence. A probe directed against the ribosomal protein of the large subunit 12 (*rpl12*) served as a loading control.



We have now added the following sentences on pages 5/6 of the manuscript: *"Surprisingly, PER2-TAP induction, driven by the CMV promoter, followed similar kinetics as endogenous PER2 protein (Fig. 1B). This effect was most likely due to the CMV-promoter's silencing and immediate early-like reactivation that has been previously reported to occur in fibroblasts (Brightwell et al, 1997)."*

6. Supplementary Fig. S8B: Panels apparently mislabeled - this is the overexpression, not hp3.

We would like to apologize for this mistake, which we have now corrected (now: **Supplementary Fig. 7**).

7. Supplementary Fig. S7D: Error bar of hp8?

In the former Supplementary Fig. 7D (now: Supplementary Fig. 6), the error bar of the hp8 was indeed missing. We repeated the experiments and added the error bars and performed the statistical test of all hairpins against the irrelevant hairpin control, as already described under point 4., above.

8. Supplementary Fig. S7F: what is hp1-8?

hp 1-8 represented a pool of all the haripins used in the loss-of-function experiments, transfected together. The idea was to obtain the most complete knockdown possible by targeting *Cavin-3* mRNA at various siRNA binding sites. For the reasons mentioned in point 4., above, we have now removed this panel.

9. Please provide a list of all identified peptides and associated proteins of the mass spectrometry experiment in a supplementary table.

Protein identification by mass-spectrometry was performed back in 2005 by a lab in Sweden, which our collaborator (a post-doc, Thomas Köcher, co-author on the manuscript) left shortly after the collaboration; in spite of our efforts, the computer that contained the mass-spec raw data is unfortunately not recoverable.
We know that nowadays the standards for data presentation in the mass-spec field have changed and that protein selection merely according to the MASCOT score is less commonly used today. We thus realized already in the first version of the manuscript, that we would not be able to include other potential PER2-interacting proteins identified by mass-spectrometry, unless they were confirmed by solid biochemical co-IP data. However, we have not attempted such experiments for most of the candidates. Nevertheless, we feel that the body of biochemical and functional data we have on the PER2:CAVIN-3 interaction, will make up for the lack of the mass-spec raw data.
Apparently this is also seen similarly by other researchers in the field. We would like to point out that other co-purifications and mass-spec identifications of circadian clock interactors that were recently published in high-impact journals did not include such supplementary mass-spec information either. Robles et al. (Robles et al, 2010) or Duong et al. (Duong et al, 2011) thus neither present identified peptides nor a list of other purified proteins, which in our view presents no problem since the authors compellingly verify their findings by biochemical co-IP studies.

10. Fig. 6 doesn't add to the understanding of CAVIN-3 function and should be removed from the main text.

We have removed this figure as suggested by the reviewer.

Referee #2:
We thank the Referee for his interest in our study and for his comments and suggestions. We have accommodated the Referee's concerns in our new, revised version of the manuscript as described in the point-to-point response given below.

Minor points:

1. Figure 1B. Please explain why expression of PER2-TAP and TAP-luciferase, respectively, under control of the CMV promoter is dependent on a serum shock?

This point was also raised by Reviewer 1, and we here repeat our answer.
This result was a surprise to us as well and we were initially excited by the possibility that there might be a serum-responsive element localized within the *Per2* cDNA. However, a number of

follow-up studies then showed that stably transfected transgenes using our TAP-tag vector were frequently serum-responsive even in the absence of any *Per2* sequence.
Interestingly, similar observations have been made previously. Brightwell et al. for example found that (1) the expression of CMV-driven transgenes was strongly affected by the cell cycle (essentially silent in G0/G1, i.e. in a confluent dish of cells) and (2) the expression was strongly induced up to 10-fold within 2 hours of serum exposure (Brightwell et al, 1997). It would thus appear that in NIH3T3 cells, stably transfected transgenes driven from the viral CMV promoter are subject to silencing and re-activation upon serum treatment; the CMV promoter may thus not be as constitutive as expected after all.
In our TAP-tag purifications, cells were indeed also grown to confluence in order to obtain the maximum amount of cell extract for the experiment, and then subjected to the 50% horse serum shock to synchronize cellular oscillators. We think that it is rather coincidental that the PER2-TAP induction kinetics are very similar to those of endogenous PER2.
We have now added the following sentences on pages 5/6 of the manuscript: *"Surprisingly, PER2-TAP induction, driven by the CMV promoter, followed similar kinetics as endogenous PER2 protein (Fig. 1B). This effect was most likely due to the CMV-promoter's silencing and immediate early-like reactivation that has been previously reported to occur in fibroblasts (Brightwell et al, 1997)."*

2. Figure 4F: the effect of the synchronization protocol on circadian period length is descriptive and does not contribute to paper.

We agree that this finding is of lesser importance for the manuscript as a whole and have thus moved this data to the Supplemental Material **(Supplementary Fig. 7).** We nevertheless found it important not to remove the data completely as researchers in the field may find it useful when wanting to reproduce our observations on period length changes upon CAVIN-3 knockdown/overexpression.

Referee #3:
We would like to thank the Referee for his critical remarks to our study, as well as for his comments and suggestions. We have worked hard to address the main criticism that concerned the validity of the PER2:CAVIN-3 interaction also for the endogenous proteins (co-IPs in the first version of the manuscript had only used tagged proteins). The new data and our responses to Referee #3's other concerns are presented in the point-to-point response below. We thus hope that our revised version of the manuscript will now meet with the Referee's approval.

Major points:

1. My major concern is that CAVIN-3 was identified under artificial conditions, suggesting that the interaction may not be biologically relevant: CAVIN-3 may only interact with PER when PER is highly overexpressed. The interaction has to be thoroughly verified and characterized in vivo using the clock antibodies available to the authors.

Reviewer 1 had the same justified criticism and we here repeat the answer provided to this reviewer.

We have now added data on the endogenous PER2:CAVIN-3 interaction as **Figure 1F**.
Since our polyclonal anti-PER2 serum gave inacceptable background signal in the western blots from cell extracts, we have developed a new strategy to demonstrate the specific IP of endogenous CAVIN-3 and PER2:
We made use of *Per2::Luciferase* knock-in mice that were developed by Joseph Takahashi's lab (Yoo et al, 2004) and that are widely used in the circadian field as a circadian reporter mouse strain. Briefly, the expression of the PER2::Luciferase fusion protein is controlled by the endogenous *Per2* locus and encodes a fully functional PER2 protein, as shown in numerous studies. From these animals, we grew primary tail fibroblasts, prepared cell extracts, and precipitated endogenous CAVIN-3 using our polyclonal antiserum (or pre-immune serum for the control-IP). We then measured PER2::Luciferase co-IP by determining co-precipitated luciferase activity. In three independent experiments we have thus observed that the IP with anti-CAVIN-3 antibodies led to a

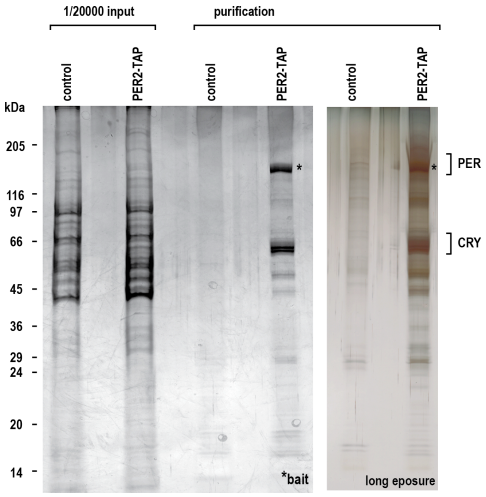
>20-fold higher luciferase activity in the immunoprecipitate as compared to the pre-immune serum control IP.
As a control cell line for non-specific luciferase precipitation in the IP, we performed the identical experiment using NIH3T3 cells stably expressing luciferase under the control of the CMV promoter. From extracts of these cells, anti-CAVIN-3 antibodies precipitated a similar amount of luciferase activity (less than 1.5-fold difference) than did the pre-immune serum. We thus concluded that the observed co-purification of luciferase activity in the *Per2::Luciferase* samples was not due to cross-reactivity of the antibodies with luciferase protein, but indeed reflected the endogenous CAVIN-3:PER2::Luc interaction.
Finally, we also controlled for the level of CAVIN-3 expression in the two cell lines because a considerably higher CAVIN-3 expression in the *Per2::Luciferase* primary cells would have compromised the interpretability of the observed enrichment. As shown in **Supplementary Fig. 3**, CAVIN-3 is in fact expressed at lower levels in the primary cells as compared to NIH3T3 cells, ruling out an unspecific enrichment that is merely due to higher CAVIN-3 expression in the *Per2::Luciferase* cells.
We hope that these new data, together with the data on the tagged proteins that we already presented in the first version of the manuscript, provide strong evidence that CAVIN-3 is indeed a novel *bona fide* interaction partner of PER2.

Other concerns:

2. In fig 1, the level of coimmunoprecipitated CRY seems to be higher than the overexpressed bait protein, PER2-TAP. The CRY must be endogenous and would be less abundant than the overexpressed PER-TAP. Further, the interaction cannot be preserved 100% throughout the purification process. This result needs to be explained.

Concerning band intensity in the silver gel, it turns out that the representation in Fig. 1C may indeed give the impression that CRYs and PER2 co-purified at stoichiometric amounts in spite of PER2 being overexpressed and the CRYs endogenous. However, we would like to draw the reviewer's attention to two points, which we had not well explained in the first version of the manuscript. First, CRYs migrate at a similar molecular weight as the abundant HSP70 proteins, which are found as major "contaminants" in most protein complex purifications. The mass-spec analysis of our purification indeed revealed significant HSP70 levels co-migrating with the CRYs. The CRYs thus probably only constitute a certain fraction to the whole signal strength around 70 kDa.
Second, it should also be considered that silver staining is generally not a quantitative technique. Different proteins may stain to quite different extents. In addition, highly phosphorylated proteins such as PER2 spread to a larger surface in the gel, which can change the visual impression as well. Finally, depending on the development time of the silver gel, relative band intensities can vary quite substantially. In the accompanying figure, we thus show a longer exposure of the same gel as in Fig. 1C that shows more clearly that the PER bands are after all probably more intense than the bands around 70 kDa.

Silver gel from Fig. 1C. Longer exposure (right panel)



To clarify these points within the manuscript we have now added the following sentences to the figure legend: "*Selected proteins detected in the bands are listed to the right of the panel. Part of the strong signal detected around 70 kDa probably originates from HSP70 proteins which were found as major constituents next to the CRYs within this region of the gel.*"

3. The authors used the constitutive CMV promoter to drive the expression of PER2-TAP. Why is there no protein at 0 and 1 hr after serum shock in fig 1B?

Below we repeat the response provide to reviewers 1 and 2.

This result was a surprise to us as well and we were initially excited by the possibility that there might be a serum-responsive element localized within the *Per2* cDNA. However, a number of follow-up studies then showed that stably transfected transgenes using our TAP-tag vector were frequently serum-responsive even in the absence of any *Per2* sequence.
Interestingly, similar observations have been made previously. Brightwell et al. for example found that (1) the expression of CMV-driven transgenes was strongly affected by the cell cycle (essentially silent in G0/G1, i.e. in a confluent dish of cells) and (2) the expression was strongly induced up to 10-fold within 2 hours of serum exposure (Brightwell et al, 1997). It would thus appear that in NIH3T3 cells, stably transfected transgenes driven from the viral CMV promoter are subject to silencing and re-activation upon serum treatment; the CMV promoter may thus not be as constitutive as expected after all.
In our TAP-tag purifications, cells were indeed also grown to confluence in order to obtain the maximum amount of cell extract for the experiment, and then subjected to the 50% horse serum shock to synchronize cellular oscillators. Also in the other experiments, such as in Fig. 1B, we let the cells grow to confluence before applying the serum shock. Whether the protein levels before serum shock appear almost undetectable (as in Fig. 1B), or clearly visible (as we have observed in other experiments), is likely to depend on the length of time the cells were in the confluent state without a medium change and thus may show some variability between individual experiments.

In order to comment on the serum effect, we have now added the following sentences on pages 5/6 of the manuscript: *"Surprisingly, PER2-TAP induction, driven by the CMV promoter, followed similar kinetics as endogenous PER2 protein (Fig. 1B). This effect was most likely due to the CMV-promoter's silencing and immediate early-like reactivation that has been previously reported to occur in fibroblasts (Brightwell et al, 1997)."*

4. In fig 1D and 1E, a more relevant negative control would be IP of extracts from CAVIN-3-transfected cells with anti-Flag antibody, since the anti-Flag antibody could pull down CAVIN-3 in the absence of PER2.

We agree that in Fig. 1D this would indeed have been a better control. In Fig. 1E, however, we did show such a control, i.e. the IP anti-HA from cells that only express V5-PER2, but not HA-CAVIN-3, followed by probing for precipitated V5-PER2.
We have not repeated the experiment Fig. 1D with the anti-FLAG, as we believe that the IP of the endogenous proteins is actually the better and more meaningful experiment and clearly shows specific interaction between CAVIN-3 and PER2 (see comments above and **Fig. 1F**).

5. The authors claim that CAVIN-3 does not oscillate, but it seems to oscillate as much as PER2 in NIH 3T3 cells. Even non-specific bands seem to be oscillating relative to the constitutive control U2AF65. PER2 oscillations in Fig2D are not consistent with those in fig 1B, which showed a dramatic difference between 0 and 4 hours after serum shock.

We have now repeated the experiment examining CAVIN-3 expression in serum-synchronized cells as shown in **Fig 2D**. We took samples before the serum shock (0), after the immediate early induction (8 hours after the shock), the first minimum (20) and fist circadian peak (28) of PER2. Again, we see that CAVIN-3 expression is rather constant. In the manuscript we describe these finds as follows: *"Although Cavin-3 mRNA levels showed some variability, there was no consistent trend towards circadian expression when comparing two independent time course series (Fig. 2A)."* and *"Similar to Cavin-3 mRNA, also CAVIN-3 protein accumulation was rather constant in serum-shocked NIH3T3 cells (Fig. 2D)."* on pages 7/8. We hope that the reviewer agrees with these conclusions.
Concerning the much stronger induction after serum-shock in Fig. 1B as compared to Fig. 2D, we have observed that this is mainly an effect of the confluency of the cells before the shock. Very confluent cells that have been without a medium change for several days thus have very low PER2 levels before the shock and the induction looks by far more dramatic (see Fig. 1B) than in other experiments such as in Fig. 2D. The situation is thus quite similar to what we already described in the response to point 3.

6. I am wondering why ICC has been done on exogenous CAVIN-3 when antibody to endogenous CAVIN-3 is available. The authors should show the interaction with endogenous proteins since the antibodies against the endogenous proteins are available. Time-course

studies in vivo, whether in cells or mouse tissues, should be performed as well, as these would be very informative towards a potential clock role of CAVIN-3.

As mentioned in the response to point 1, we have now added the interaction data on the endogenous proteins in **Fig. 1F**. We agree that time-course studies on the PER2:CAVIN-3 interaction in cells and in tissues would be highly interesting for a future, dedicated project. We are also aware that there is a multitude of exciting follow-up experiments possible regarding our first characterization of the CAVIN-3:PER2 interaction and the functional data that we present in our study. However, we deemed such detailed, comprehensive investigations beyond the scope of this manuscript; we hope that the reviewer can agree with this assessment.

3. I could not understand the rationale and significance of the mammalian two-hybrid data presented in fig 3, unless this is validated in vivo. CAVIN-3 may up- or down-regulate levels/activity of PER:CRY in the cytoplasm, as the authors showed, but how does this translate to a role in the in vivo circadian clock? How does this result in the altered periods shown in fig 4? There is a big gap between fig 3 and fig 4. I do not understand how data in fig 3 could lead to those in fig 4.

We have worked considerably on this part of the study and manuscript, since also Referee 1 was dissatisfied with the two-hybrid data (see also comments there, reviewer 1, point 2.).
With regard to reviewer 3's comments, we also noticed a logical gap between the former Figs. 3 (two-hybrid) and 4 (period length phenotype). We have changed the order of the data and first show the data on period length changes (now Fig. 3) before we turn to the two-hybrid data. We think that readability and the logical thread is now much better.

Within the two-hybrid data, we have made the following main additions:
We addressed and now discuss more carefully alternative interpretations of the two-hybrid data. Does a higher signal in the two-hybrid assay really reflect a stronger interaction? Can other mechanisms account for the observations as well? We have observed, for example, that CAVIN-3 overexpression (and higher BD-PER2:AD-CRY2 two-hybrid signals) are accompanied with a lower abundance of the BD-PER2 and AD-CRY2 proteins (**Fig. 4F**) and we have now confirmed this effect also for endogenous PER2 (**Supplementary Fig. 12**). We thus tested if the lower levels of BD-PER2/AD-CRY2 were alone sufficient for the dramatic effects in the two-hybrid assay. This, however, was apparently not the case, as the titration of AD-PER2 and BD-CRY2 to lower levels had barely any effect on the assay (**Supplementary Fig. 13**).
How could CAVIN-3 then influence the PER2:CRY2 proteins in molecular terms? CAVIN-3 was previously found to interact with PKCδ and in the first version of the manuscript we had presented evidence suggesting that this particular kinase was unlikely to be involved in the phenotypes we were seeing. However, we considered the possibility that other kinases acted through CAVIN-3 as well. We have thus mutated the two serine and a threonine residue in CAVIN-3's previously described kinase binding site (protein CAVIN-3-mut); as shown in **Fig. 5E, F**, CAVIN-3-mut no longer lengthens the circadian period in the reporter assays nor increases the PER2:CRY2 two-hybrid signal. These data show that there is a clear correlation between what we observe in the two-hybrid assay and CAVIN-3's effect in the clock *in vivo*. Moreover, they suggest that a (still unidentified) kinase distinct from PKCδ may be involved in the underlying mechanism.

In spite of these new data, we are aware that the two-hybrid data are to be interpreted with some caution and we have thus completely re-written the corresponding parts of the manuscript. To avoid any overstatement, we have also changed the title of the manuscript to *"CAVIN-3 regulates circadian period length and PER:CRY protein abundance and interactions"*.

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1st Editorial Decision 25 September 2012

Many thanks for transferring your revised manuscript to EMBO reports and I apologize again for the delay in getting back to you.

I have now had the time to take a final look at the study and the additional data that you kindly provided in response to the comments of the referees from The EMBO Journal.

As the results with the overexpression of Cavin3 in primary cells and the effects of it on the endogenous reporter support your conclusions on the role of Cavin3 in regulating the clock, I am now happy to accept your study for publication in EMBO reports.

Below you will find the official acceptance letter with relevant information on the next steps.

Please do not hesitate to contact me if you have any further questions.

Yours sincerely
Editor
EMBO reports

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