Manuscript EMBOR-2010-34506

The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in Drosophila

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Review timeline:

Submission date:	21 October 2010
Editorial Decision:	16 November 2010
Correspondence:	18 November 2010
Correspondence:	19 November 2010
Correspondence:	20 November 2010
Correspondence:	29 November 2010
Correspondence:	03 December 2010
Correspondence:	04 December 2010
Revision received:	08 March 2011
Editorial Decision:	16 March 2011
Revision received:	18 March 2011
Editorial Decision:	22 March 2011
Accepted:	22 March 2011

Transaction Report:

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

1st Editorial Decision 16 November 2010

Thank you for the submission of your research manuscript to our editorial offices. We have now received the enclosed reports from the three referees that were asked to assess it. As you will see - although all the referees find the topic of interest and referee 1 is more positive- referees 2 and 3 find the proof that CTRIP directly affects CLK and PER stability insufficient and raise a substantial number serious technical concerns that preclude a conclusive interpretation of some of the data. However, given that all find the study novel and interesting, we would like to invite revision of your manuscript, with the understanding that the referee concerns must be addressed and that acceptance of the manuscript would entail a second round of review.

As the reports are pasted below, I will not summarize them in detail here. However, I would like to point out that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the outcome of the next, final round of peer-review. In this case, it would be important to demonstrate that CTRIP directly modulates CLK and PER stability through ubiquitination (or that its E3 ligase activity is required) and address the

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numerous technical concerns that preclude a conclusive interpretation of the data provided. Please note that revised manuscripts must be submitted within three months of a request for revision, as they will otherwise be treated as new submissions.

In this case, I must also ask you to reformat your paper to conform to EMBO reports style and length requirements during the revision process. As a transfer from The EMBO Journal, your manuscript is presently longer than we can consider to publication and I must ask you to shorten it to a maximum of 27,500 characters (including spaces). The presentation of a merged Results and Discussion section, which we encourage, may help to eliminate some redundancy inherent to their presentation separately and aid in the shortening. In addition we can only accommodate a maximum of 5 figures in the main text of the manuscript.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

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REFEREE REPORTS

Referee #1 (Remarks to the Author):

This manuscript from the Rouyer lab describes a role for a novel E3 ubiquitin ligase (Ctrip) in the Drosophila circadian clock. Post-transcriptional regulation of circadian clocks is increasingly viewed as key to generating 24hr rhythms alongside the documented rhythmic expression of certain core clock genes. The authors provide compelling evidence that Ctrip is important in circadian rhythms in flies. Specifically, they show that:
1. Two different enhancer traps in the large Ctrip locus are expressed in the master pacemaker neurons.
2. Ctrip mutants show delays in clock protein oscillations.
3. Complementing the mutant analysis, 2 different RNAi transgenes that target different parts of Ctrip mRNA lengthen the period of adult rhythms.
4. Ctrip RNAi also affects molecular clock oscillations.

Furthermore, the authors go on to pinpoint a specific defect in the clock by showing that Clk protein levels are increased while Clk RNA expression is unchanged. This leads to increased Clk/Cyc activity since expression of a number of direct Clk/Cyc target genes is increased quite dramatically.

Overall, the data are very thorough and well-presented. For me, having 2 different RNAi transgenes that give the same phenotype as a mutant overrides the lack of validation of Ctrip RNA expression. Although there are no direct assays of Ctrip as a ubiquitin ligase for Per or Clk, the authors infer that this Ctrip is a ubiquitin ligase by homology and by careful analysis of the circadian phenotypes. Certainly it is clear from previous studies from the Edery group (and probably others that I am

overlooking) that Per and Clk are degraded by the proteosome and likely ubiquitinated.

10 years ago a manuscript like this would have been a Cell or Nature paper. I recommend acceptance immediately.

Having said that, I have a few minor comments worth addressing that would improve the manuscript:

1. Why is there no phenotype from Ctrip over-expression?
2. Why do flies have longer periods with RNAi expressed from Pdf-Gal4 than tim-Gal4?
3. If this protein is part of the core clock, why is it not expressed in other clock neurons (according to the enhancer trap)? It must be expressed in photoreceptor cells since there are effects on clock proteins here analyzed by Western blot / RNA.
4. What is a "WWE interaction domain"?
5. The nomenclature of 14R3, 17R3 is awkward and does not help the reader understand. What about Ctrip-RNAi e2 e6 for exon 2 or 6? Or Ctrip-i 2 / 6? Or something like that?
6. FlyBase calls Pdp1 with an upper case P not lower case since it was named by homology with mammalian proteins.
7. It is probably worth stating in the results that per, tim, vri and Pdp1 are direct Clk/Cyc targets and this is why their levels were measured.

Referee #2 (Remarks to the Author):

CLOCK-CYCLE complex activates circadian transcription of target genes, including the negative regulators period/timeless. In turn, PERIOD-TIMELESS complex represses CLK-CYC transcriptional activity by releasing it from the target gene promoter, but also by bridging Casein Kinase I (DBT) (and possibly other kinases) to CLK protein, thus promotes CLK phosphorylation. Phosphorylated CLK is presumably a better substrate for proteasome degradation. However, the E3 ligase that regulates CLK degradation is unknown. In this study, Lamaze et al. found that a HECT domain E3 ligase named CTRIP is involved in this process. Loss of function of CTRIP results in higher levels of CLK protein and also elevated PER/TIM in larval brain neurons as well as in adult fly heads, suggesting that the normal function of CTRIP is to promote degradation of these circadian clock proteins. This finding is important in the field of circadian biology. However, critical data are either missing or insufficient to support the conclusion that CTRIP controls CLOCK levels and PERIOD oscillations. Specific concerns are detailed below.

Major concerns:
1. The CTRIP transcript is based on flybase annotation, but not experimental evidence. A full-length transcript from fly heads needs to be shown (Figure 1 and Supplemental Figure 1 lack this information), especially when this locus contains multiple annotated transcripts.
2. The E3 ligase ubiquitination function of CTRIP is not demonstrated. It is critical to show that CTRIP binds to CLK/PER in vivo. Without this data, it is not clear if CTRIP acts directly on CLK/PER, or secondarily through other proteins.
3. Evidence is lacking that CLK/PER is ubiquitinated more or less by increasing or knocking down CTRIP. Since CLK/PER seems to have similar degradation mechanism in cell culture as that in flies, these experiments can be easily performed in S2 cells by transfection/RNAi.
4. While it is nicely shown that effect of ctrip knock-down on CLK is independent of PER, same experiment should be performed to support the claim that CTRIP act on PER/TIM at the post-transcriptional levels (per/tim mRNA levels are actually higher). This experiment can be carried out in S2 cells, due to the fact that PER/TIM levels are low in Clk mutant flies.
5. The important conclusion was drawn from mainly from DD data, rather than LD data. Since mutant flies have long period, attributing a particular phase difference to defects in degradation seems to be an over-interpretation. In fact, Figure 2B clearly shows that PER/TIM takes longer time to reach peak levels (and it comes back to trough even faster than control during the light phase). On the other hand, CLK levels are elevated throughout the day (Figure 3A), instead of an increase specifically during the degradation phase (when it is hyper-phosphorylated during late night and early morning) as it is predicted from a defect in degradation. This issue can be solved by assaying CLK/PER/TIM protein degradation in S2 cells (after inhibition of new protein synthesis).
6. Mapping of the phenotype to ctrip has not been adequately demonstrated. No transgenic rescue

data/deficiency complementation is presented in this study.

Other concerns:
1. The specificity of CLK antibodies needs to be established in this study (immunofluorescence and Western blot). GP47 has non-specific immunoreactivity (Houl et al. 2006 in J Biol Rhythms, and Houl et al. 2008 in BMC Neuroscience 9:119); while the commercial antibody sc-27070 revealed CLK cycling pattern (Figure 3A) that is quite different from published results.
2. The expression of CTRIP in lateral neurons is based on an intronic enhancer trap reporter, rather than a reporter that is in-frame with the endogenous transcript. It is well known that enhancer element sometime locates far away from the target gene and that intronic enhancer trap does not always report endogenous gene expression. A promoter reporter, or in situ data is required to conclude that CTRIP is "strongly" expressed in the LNv.
3. As stated in the manuscript, increased CLK levels does not always produce behavioral phenotype (Kim et al. 2002), normal behavioral phenotype of Y4 does not exclude the possibility that it may have similar CLK levels as 32-3. Since 32-3 harbors deletion of both hus1-like and ctrip, the real control for ctrip mutation should be Y4, which deletes hus1-like only.
4. It is difficult to judge the quantification of fluorescence signal without the supplement of real images. Sample size (n) is missing in Figure 2 and Figure 6A.
5. Without loading control band (house-keeping gene), one can not judge the Western blot results (Figure 3). Using equal amount of measurements (50ug) does not control the variability introduced in later procedures. Also, the experiment should have repeats.
6. How was qPCR data normalized? It is not clear in the Methods and Figure legends. s.e.m based on n=2 is not adequate.
7. The function of CTRIP in the proteasome degradation/UFD pathway needs to be clarified. Without experimental evidence that CTRIP directs N-terminal ubiquitination, one can not distinguish it from its function in polyubiquitination.
8. Nomenclature is not consistent. For example, genes should be italicized, but not specific alleles (Y4, 32.3, gal1118 etc.); it should be Clk/Pdf/Pdp1, instead of clk/pdf/pdp1. When a specific protein function is stated, it should be PER-independent, but not per-independent.
9. It is confusing when the authors attribute more phosphorylated PER and hypophosphorylated CLK both to deficits in proteasome degradation.

Referee #3 (Remarks to the Author):

A major aspect of circadian clock mechanisms involves the timely degradation of central clock proteins. Evidence from several systems indicates that E3 ubiquitin ligases play major roles in the degradation of clock proteins. In Drosophila, E3 ligases have been identified for regulating cycles in PER levels and the light-mediated degradation of TIM and CRY. The importance of regulating the stability of the CLK protein is not clear. While clk mRNA levels clearly oscillate, the protein appears rather constant with some low level oscillation. However, there is evidence that phosphorylated CLK is more unstable and CLK also appears to be limiting in abundance. The interesting aspect of this work is that it identifies a novel E3 ligase, CTRIP, which appears to target CLK. The overall quality of the work is impressive. Alterations in ctrip levels clearly result in strong effects on the period of activity rhythms with similar effects on the molecular cycles of key clock components. For example, mutations and RNAi both reveal that lowering ctrip levels results in period lengthening. In addition, CTRIP in the brain has a very limited spatial expression pattern, mainly in the PDF-expressing clock neurons. Thus, from a physiological perspective it is clear that a novel E3 ligase has been identified in the Drosophila clockworks (i.e., in the right place and alterations in its levels affects the pace of behavioral and molecular rhythms). The biochemical data indicating CLK as a major target for CTRIP-mediated degradation are good but indirect. Thus, the manuscript is novel and interesting because it identifies a new component of the Drosophila clock but the proposed mechanism, although supported, is more circumstantial. Below are more specific comments.

Major comments:
1. What is the evidence that CTRIP directly targets CLK for degradation? The case is built from solid but indirect evidence. In the 32-2 and RNAi lines, clk mRNA levels are not affected but CLK protein levels are higher. The simplest interpretation is that CTRIP levels affect CLK abundance at the posttranscriptional level, consistent with its predicted function. There seems to be a discrepancy

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in the amplitude of the rhythm between in situ and western data. It is much higher and even observed for the control at DD for western's but not for the corresponding in situ. Prior work is also a bit controversial as to whether there is cycling at the total protein level and it seems to be due to the extraction buffer used. The data shown for the western blots seems to suggest that the DNA bound version of CLK was not effectively extracted, or else the total levels should be rather constant (see Yu et al., 2006). While this does not argue against the clear increase in CLK levels in the RNAi lines, why the discrepancy between in situ and blotting and prior results? In any case, it would be reassuring to show at least some evidence for CTRIP directly affecting CLK levels. While this is difficult to obtain in vivo, can any evidence be obtained in S2 cells as was done recently for TIM and CRY degradation? e.g., do CTRIP and CLK interact, is the stability of CLK affected by overexpressing or inhibiting CTRIP. Is the ubiqutinylated status of CLK altered when comparing wildtype and RNAi extracts? Related to this, do the authors know how well their RNAi worked in flies? If CLK undergoes daily levels in abundance, as indicated by the results shown, one has to conclude that CTRIP is not important for controlling temporal changes in the stability of CLK but rather some base-line stability. This should be mentioned. It is not known that effects of CTRIP on CLK can be measured in S2 cells or that the ubiquitin status of CLK can be measured in vivo, but the authors should at least attempt to obtain some more direct evidence and at the very least should be able to say whether CLK and CTRIP interact when co-expressed in S2 cells.
2. Similar experiments as above should also be done for PER and TIM, as the evidence for CTRIP DIRECTLY controlling PER levels is weaker. For example, do CTRIP and SLMB have additive effects on PER stability?
3. The in situ results (Fig. 1) and the whole head extract data do not seem to reconcile. If ctrip is mainly only expressed in PDF cells, how does RNAi have effects on total PER, TIM and CLK, which are expressed in many other non-PDF clock cells (including the eyes)? What happens to CLK, PER, TIM levels with drivers that express RNAi of ctrip in non-PDF clock cells? Further, does RNAi of ctrip have preferential effects on CLK in PDF-expressing cells by in situ? e.g., compare DNs to LNvs.

Minor comments:
1. page 6, middle; I think they mean Figure 1C and not 1B.
2. Fig 1. Did the authors look at other body tissues?
3. Any idea why overexpressing ctrip has no effect on period length; this is a bit hard to understand as it is supposed to degrade CLK.
4. Any difference between nuclear and cytoplasmic levels of CLK in wildtype versus RNAi lines as it is proposed that ctrip might affect CLK stability in the nucleoplasm.

authors' correspondence 18 Nov 2010

Thank you for considering our manuscript. However, I have to say that I do not really understand the policy of the brother journals embo journal and embo reports. We have submitted our manuscript to the embo journal a month ago and we were told (see below) by the embo journal editor that, although the finding of new ubiquitin ligase specifically expressed in the clock neurons that controls CLK (first time) and PER was very interesting, the lack of evidence for a direct effect on CLK or for a ubiquitination defect in the mutant would not allow him to consider it. Based on these considerations, he suggested us to submit our manuscript to the embo reports. We are thus surprised to see that the additional data that you are asking for are the same (direct effect and ubiquitination on CLK) and even more (direct effect on PER). We just don't understand why we are asked to submit to the embo reports (short notes, impact factor 7) a paper including the complete molecular characterization of this new clcok gene. In addition to adding such new data, we will have to shorten the paper to have it fitting with the short format. We certainly agree that the data you are speaking about are important and would definitely improve the study, but we would have submitted it to Cell or Genes and Dev if we had such data, not as a short paper to the embo reports.

I thus would like to ask you whether you would consider the manuscript if we add some experiments showing that CLK stability is increased in S2 cells (we have some preliminary data in collaboration with another lab and we could reinforce them during the next few weeks), but without experiments with ubiquitination data and no definitive evidence for a direct effect (something that the title does

not claim, as opposed to the comment of the reviewer 2 among other major errors). Of course, we would also reply to the reviewers' concerns about the present experiments, including new data validating them. If you will not consider it without all the present requests, we believe we should just send it to another journal.

editor's correspondence 19 Nov 2010

Thank you for your email regarding the revision of your manuscript. I would like to first clarify our relationship with our sister journal, The EMBO Journal. As a journal dedicated to short reports, we do not require the full mechanistic elucidation of a give pathway and thus were happy to send your manuscript for peer-review. However, we do require that the conclusions are strongly supported by the data and, after our initial editorial decision, we do have to take the referee assessments into account. In this case, two of them feel that the implication of a new E3 ligase in the stability of the CLK and PER proteins would require the proof that ubiquitination is involved in addition to several other concerns that preclude a solid interpretation of the data.

Nonetheless, after reevaluating the file, I agree with you that requesting proof of a direct effect would be out of the scope of this manuscript. In my previous decision letter, I meant to open the door to an alternative approach that would be appropriate for us, but clearly did not explain it in sufficient detail. I do think that it is of importance to demonstrate that the E3 ligase activity of CTRIP is involved -directly or indirectly- in controling CLK levels. This could be done by performing the CLK half-life experiments in S2 cells that you are offering to include, in the presence of wild-type CTRIP and of a catalytically inactive mutant of CTRIP in parallel. As far as I am aware, this would be a reasonable experiment in the S2 cell setting. Is this something you would be able to provide? I think that such a demonstration that CTRIP controls CLK protein levels through its E3 ligase activity would address our concerns.

Of course, addressing the additional technical issues would also be required, but this is in your plans already. I find particularly important to clarify the detection of CLK protein levels and reconcile them with previous reports as much as possible, as well as the discrepancy between the in situ results (expression in PDF) and whole head extract data, in addition to providing western blot loading controls, replicates, statistical information, etc. In this regard, we do encourage the submission of fluorescence microscopy images as part of the supplementary information and, as they were specifically requested in this case, representative images should be provided.

I hope you find that our requests are reasonable and proceed with the revision of your study for EMBO reports, as we are in principle positive about its suitability for our journal. Please do not hesitate to contact me if you have any additional concerns/questions.

Yours sincerely

Editor
EMBO reports

authors' correspondence 20 Nov 2010

Thank you for your quick reply. We do have ongoing experiments in S2 cells that address CLK half-life control by ctrip. These experiments use ctrip RNAi in conditions where protein synthesis is blocked. The preliminary results indicate that CLK stability is increased in the presence of ctrip RNAi. We are very keen to complete the preliminary experiments and provide evidence in the paper hat decreasing ctrip affects CLK stability in such conditions. This would clearly be a step forward showing ctrip action on CLK stability, compared to our fly experiments.
You suggest to compare the effect of wild type ctrip with mutated ctrip to show that ctrip E3 ligase activity is indeed involved.
In the current version of the manuscript, we have indicated that ctrip overexpression has no behavioral effect, indicating that ctrip is not a limiting component. In agreement with the behavioral result, we only observed a very small decrease of CLK in ctrip overexpressing flies. In addition, we

have measured ctrip expression in S2 cells and the mRNA levels are already high, making rather unlikely that ctrip overexpression in S2 cells would give significant effects on CLK.
Finally, as indicated in the paper, the ctrip gene is a huge 12 exons gene and It would take a considerable amount of work to build wild-type and mutant constructs of this size (about 10 kb for the coding sequence, no cDNA including the whole ORF available), for an experiment that is so unlikely to give significant results.
We thus propose to add S2 cells experiments with ctrip RNAi, in addition to our answers to the reviewers concerns, which include some new experiments.
Please tell me whether you think that this would fit your request.

editor's correspondence 29 Nov 2010

I apologize for the time it has taken me to contact you regarding your revision. I have now contacted two of the referees for their input regarding your plans for revision and have had time to assess their responses and what we editorially believe would be sufficient. One referee still asks for evidence of direct ubiquitination, but we have decided that this would not be required in this case, in agreement with the other referee.

We would be satisfied with a careful experiment in S2 cells, although we would ask you to incorporate an additional control, as suggested by referee #3, which would be to also assess PER and TIM stability in S2 cells. TIM would serve as a negative control as it should not be affected and it would be relevant to know the PER results in this assay.

In addition, I would like to discuss with you another approach that was suggested, as it does seem to be relatively straightforward. In the S2 cell setting, you could immunoprecipitate CLK and probe with an anti-ubiquitin antibody to determine the ubiquitination status of CLK. I'm not sure if there is enough CLK in wild type S2 cells to do this, but, if possible, it would be a valuable piece of information that would strengthen the study, which is in both of our interests. Do let me know what you think about this last possibility.

I trust that you will find this an agreeable way forward and look forward to receiving your revised manuscript in due time. Do let me know if you have any additional questions or concerns.

Yours sincerely

Editor
EMBO reports

authors' correspondence 03 Dec 2010

We are happy to understand that you would be satisfied with an improved version of the paper that would include RNAi experiments in S2 cells. As I mentioned to you, we have preliminary results indicating that CLK is stabilized when ctrp RNAi is added. This will require some more experiments and we will add a negative control as requested. These experiments, in addition to some more limited requests of the reviewers, will take a few weeks of work and should allow us to submit an improved version in January. However, we have been discussing in depth with our collaborators about the PER experiments in S2 cells and the conclusion is that we're not willing to start a new set of experiments with PER transfected cells. First, as opposed to CLK, our RNAi fly results do not indicate that PER levels are higher but rather show that phosphorylated PER is present for a longer time in the morning, as already stated by the title of the paper. This indicates that ctrp affects PER stability in very particular conditions that require a certain level of phosphorylation by DBT and are very likely to depend on PER/TIM and/or PER/CLK interactions. This is certainly an interesting question but it is not in the scope of the paper and would be a research project by itself, demanding several months of work. You also mention testing CLK ubiquitination. The reviewers may not know but even PER ubiquitination is not clearly established although SLMB has been identified 8 years

ago (a single experiment has been published as supplemental material in Ko et al. 2002 - fair to say that this is not the most convincing experiment of this important paper). CLK ubiquitination is being investigated by different clock labs for several years and not a single experiment has been published. I certainly agree that showing CLK ubiquitination would be very interesting, but this is another study.
We show in the present paper that a new ubiquitin ligase is expressed specifically in the clock neurons. We show that downregulating this enzyme increases the behavioral period, increases CLK levels independently of PER, in addition to stabilizing phosphorylated PER at the end of the cycle. These results are also supported by our study with the ctrp mutants in the larval brain. We believe that the present results, confirmed by the S2 cells RNAi data, already provide a very significant advance in the circadian field, and are certainly adapted to a short report as EMBO reports publish. We thus ask you to tell us whether you think that such a version of the manuscript will be acceptable as a revised manuscript. If not, we would rather submit our work to another journal.

editor's correspondence 04 Dec 2010

Thank you for your detailed explanation of the situation. Given the circumstances, we would indeed be satisfied with a revised version that included a careful experiment in S2 cells as additional insight into the effect of CTRIP on CLK. Please note that, in addition, it must address all of the technical concerns of the referees -all of whom are active researchers in this field- in order to increase the conclusiveness of some of the data, which was also an issue in the previous version.

I look forward to receiving your revised version when it is ready.

Yours sincerely

Editor
EMBO reports

1st Revision - authors' response 08 March 2011

Please find enclosed the revised version of our manuscript entitled "The HECT domain E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in Drosophila" that we have submitted for publication in EMBO Reports.
As you have requested in your last email, we have added CLK degradation experiments in S2 cells. These new experiments show that CLK protein degradation is decreased in the presence of ctrip dsRNA, strongly supporting our results in flies. Our results thus unravel the first ubiquitin ligase regulating CLK levels in Drosophila. We have also added new experiments and controls that address specific points of the reviewers and modified our manuscript to answer the different issues raised by the reviewers. In addition, an important point has been added, which is the finding by another laboratory that this gene belongs to a set of genes that are enriched in the PDF clock cells, validating our enhancer trap results. Finally, the manuscript length and the number of figures (5) have been reduced to fit the short format of the journal.
We thus hope that this study will now be suited for publication in EMBO Reports.

Responses to referees:

Referee #1 (Remarks to the Author):

This manuscript from the Rouyer lab describes a role for a novel E3 ubiquitin ligase (Ctrip) in the Drosophila circadian clock. Post-transcriptional regulation of circadian clocks is increasingly viewed as key to generating 24hr rhythms alongside the documented rhythmic expression of certain core clock genes. The authors provide compelling evidence that Ctrip is important in circadian rhythms in flies. Specifically, they show that:
1. Two different enhancer traps in the large Ctrip locus are expressed in the master pacemaker neurons.
2. Ctrip mutants show delays in clock protein oscillations.
3. Complementing the mutant analysis, 2 different RNAi transgenes that target different parts of Ctrip mRNA lengthen the period of adult rhythms.
4. Ctrip RNAi also affects molecular clock oscillations.

Furthermore, the authors go on to pinpoint a specific defect in the clock by showing that Clk protein levels are increased while Clk RNA expression is unchanged. This leads to increased Clk/Cyc activity since expression of a number of direct Clk/Cyc target genes is increased quite dramatically.

Overall, the data are very thorough and well-presented. For me, having 2 different RNAi transgenes that give the same phenotype as a mutant overrides the lack of validation of Ctrip RNA expression. Although there are no direct assays of Ctrip as a ubiquitin ligase for Per or Clk, the authors infer that this Ctrip is a ubiquitin ligase by homology and by careful analysis of the circadian phenotypes. Certainly it is clear from previous studies from the Edery group (and probably others that I am overlooking) that Per and Clk are degraded by the proteosome and likely ubiquitinated.

10 years ago a manuscript like this would have been a Cell or Nature paper. I recommend acceptance immediately.

Having said that, I have a few minor comments worth addressing that would improve the manuscript:

1. Why is there no phenotype from Ctrip over-expression?
We do not have a definitive answer to that question. We now indicate in the text (p. 6) what we think is the more likely explanation: a limiting factor is likely to be required by CTRIP to act on clock proteins.

2. Why do flies have longer periods with RNAi expressed from Pdf-Gal4 than tim-Gal4?
They do not. One needs to compare genotypes with the same number of transgene copies in Table 1. For example, flies with one copy of pdf-gal 4 and one copy of each UAS-RNAi insertion have a 26.2 h period, flies with one copy of tim-gal 4 and one copy of each UAS-RNAi insertion have a 26.4 h period. Same thing for flies carrying a single RNAi insertion.

3. If this protein is part of the core clock, why is it not expressed in other clock neurons (according to the enhancer trap)? It must be expressed in photoreceptor cells since there are effects on clock proteins here analyzed by Western blot / RNA.
We certainly agree that ctrip must be expressed in other cells and this is now more clearly stated in the text (p. 4, see also response to reviewer 3). The two enhancer trap lines show much stronger expression in the PDF cells (and the recent Kula-Eversole 2010 paper reports an enrichment of "CG17735" mRNA in the PDF cells) but we do see (and report) weak expression in at least some other clock neurons when two copies of the gal4 are used to drive gfp expression. We have not seen expression of the gal1118 and gal1501 in the eye, but these are enhancer traps and may not reflect the complete expression pattern of ctrip, as frequently observed for enhancer traps. We did see molecular effects in head extracts by expression of ctrip RNAi under GMR-gal4 control, in agreement with expression of the gene in eye photoreceptors. We believe that only the availability of good antibodies will allow to give a clear answer to that question.

4. What is a "WWE interaction domain"?
The WWE domains has been identified in a short report by Aravind (2001): "The WWE domain: a common interaction module in protein ubiquitination and ADP ribosylation". For obvious space reasons we do not describe this finding in our paper but we cite this study when mentioning the WWE domain (p. 5).

5. The nomenclature of 14R3, 17R3 is awkward and does not help the reader understand. What about Ctrip-RNAi e2 e6 for exon 2 or 6? Or Ctrip-i 2 / 6? Or something like that?
We understand that complex nomenclature is a problem, and we actually use a simpler nomenclature, namely UAS-ctrip RNAi for the combination of two UAS RNAi constructs that we use throughout the paper. 14R3 and 17R3 thus only appear in the table and we believe that creating new names only for that would rather create more confusion.

6. FlyBase calls Pdp1 with an upper case P not lower case since it was named by homology with mammalian proteins.
This has been modified accordingly.

7. It is probably worth stating in the results that per, tim, vri and Pdp1 are direct Clk/Cyc targets and this is why their levels were measured.
Done.

Referee #2 (Remarks to the Author):

CLOCK-CYCLE complex activates circadian transcription of target genes, including the negative regulators period/timeless. In turn, PERIOD-TIMELESS complex represses CLK-CYC transcriptional activity by releasing it from the target gene promoter, but also by bridging Casein Kinase I (DBT) (and possibly other kinases) to CLK protein, thus promotes CLK phosphorylation. Phosphorylated CLK is presumably a better substrate for proteasome degradation. However, the E3 ligase that regulates CLK degradation is unknown. In this study, Lamaze et al. found that a HECT domain E3 ligase named CTRIP is involved in this process. Loss of function of CTRIP results in higher levels of CLK protein and also elevated PER/TIM in larval brain neurons as well as in adult fly heads, suggesting that the normal function of CTRIP is to promote degradation of these circadian clock proteins. This finding is important in the field of circadian biology. However, critical data are either missing or insufficient to support the conclusion that CTRIP controls CLOCK levels and PERIOD oscillations. Specific concerns are detailed below.

Major concerns:
1. The CTRIP transcript is based on flybase annotation, but not experimental evidence. A full-length transcript from fly heads needs to be shown (Figure 1 and Supplemental Figure 1 lack this information), especially when this locus contains multiple annotated transcripts.
We apologize for the confusion here. The structure of the head transcripts that we describe in the figure was determined by extensive RT-PCR analysis and 5' RACE extension experiments and sequencing of the PCR products. The obtained sequences were compared to Flybase (we actually obtained this information before the reporting of the merging of CG14656 and CG17735 in a new CG42574) in Flybase. This is now indicated in the text (p. 4), and we provide the sequence data as supplementary information (Fig S1). This includes, in addition to the splicing events reported in Fig 1, the sequence of the longest transcript, with all exon boundaries indicated. These were identical to the ones shown in flybase except for a 5' extension of exon 1. Our sequence also shows single nucleotide polymorphic changes, three of them inducing a change in the protein sequence. All this information is now reported in Fig S1, which includes the protein sequence predicted by our sequence analysis. In addition, Fig S2 indicates that the two types of transcripts that keep exon 4 (either 4b or 4ab), are expressed in the head about 10 times less than the transcript lacking this exon.

2. The E3 ligase ubiquitination function of CTRIP is not demonstrated. It is critical to show that CTRIP binds to CLK/PER in vivo. Without this data, it is not clear if CTRIP acts directly on CLK/PER, or secondarily through other proteins.
3. Evidence is lacking that CLK/PER is ubiquitinated more or less by increasing or knocking down CTRIP. Since CLK/PER seems to have similar degradation mechanism in cell culture as that in flies, these experiments can be easily performed in S2 cells by transfection/RNAi.
4. While it is nicely shown that effect of ctrip knock-down on CLK is independent of PER, same experiment should be performed to support the claim that CTRIP act on PER/TIM at the post-transcriptional levels (per/tim mRNA levels are actually higher). This experiment can be carried out in S2 cells, due to the fact that PER/TIM levels are low in Clk mutant flies.
These are important questions about CTRIP molecular function, but they are out of the scope of this

paper, as agreed by the editor.

5. The important conclusion was drawn from mainly from DD data, rather than LD data. Since mutant flies have long period, attributing a particular phase difference to defects in degradation seems to be an over-interpretation. In fact, Figure 2B clearly shows that PER/TIM takes longer time to reach peak levels (and it comes back to trough even faster than control during the light phase). On the other hand, CLK levels are elevated throughout the day (Figure 3A), instead of an increase specifically during the degradation phase (when it is hyper-phosphorylated during late night and early morning) as it is predicted from a defect in degradation. This issue can be solved by assaying CLK/PER/TIM protein degradation in S2 cells (after inhibition of new protein synthesis).
We do not say that CTRIP specifically affects the putative degradation phase of CLK, but show that CLK is higher during the all cycle (especially during the evening). This rather suggests that CTRIP controls overall CLK levels. As suggested by the reviewer, we now provide a CLK degradation assay in S2 cells in the presence of cycloheximide and show that CLK degradation kinetics is decreased by ctrip downregulation (p. 7 and Fig 3C).

6. Mapping of the phenotype to ctrip has not been adequately demonstrated. No transgenic rescue data/deficiency complementation is presented in this study.
We already provided evidence from a mutant (32.3), two different RNAi constructs and showed that ctrip UAS-induced RNA partially rescues the RNAi phenotype. Finally, a different type of ctrip RNAi has been shown to lengthen the behavioral period (Sathyanarayanan 2008).
We have now added CLK/PER quantifications in the PDF cells of larval brains heterozygous for 32.3 and two different deficiencies that validate our results. The same analysis was done in hus1-like null mutants and shows no effect of hus1-like loss. These experiments are reported in Fig S4 (text p. 5).

Other concerns:
1. The specificity of CLK antibodies needs to be established in this study (immunofluorescence and Western blot). GP47 has non-specific immunoreactivity (Houl et al. 2006 in J Biol Rhythms, and Houl et al. 2008 in BMC Neuroscience 9:119); while the commercial antibody sc-27070 revealed CLK cycling pattern (Figure 3A) that is quite different from published results.
- Immunofluorescence: to our knowledge, the non specific signals of the GP47 antibodies reported in the Houl papers are exclusively in non clock cells. In agreement with this, we have observed no GP47 labeling in the clock neurons of clk jrk mutants, as we now indicate in the legend of the new Fig S3, which provides images of CLK, PER and TIM staining in the larval and adult clock neurons.
- Western blots: we have added a clk jrk control in fig 3A. The cycling of CLK on western blots (with the same antibody) has already been reported in Richier et al. 2008. In addition, a nice CLK cycling (using CLK-V5 flies) on head extracts western blots is shown in Menet et al. 2010 (Figure 3). This looks very similar to what we show in the 2008 paper and in this study (see also response to reviewer 3).

2. The expression of CTRIP in lateral neurons is based on an intronic enhancer trap reporter, rather than a reporter that is in-frame with the endogenous transcript. It is well known that enhancer element sometime locates far away from the target gene and that intronic enhancer trap does not always report endogenous gene expression. A promoter reporter, or in situ data is required to conclude that CTRIP is "strongly" expressed in the LNv.
We do not have such a tool. However, our study is not based on a single enhancer trap but on two different gal4 insertions, which are located 12 460 bp apart in the ctrip gene. This is why we believe that their virtually identical expression pattern reflects ctrip expression. In addition, we are not sure that having 5 or 10 kb of upstream sequences for driving reporter gene expression would allow to better exclude the possibility that the observed pattern is the one of a neighboring gene. Only antibodies will give a definitive answer to that question, but up to now we could not produce good anti-CTRIP antibodies. Importantly, our expression data are confirmed by the finding that ctrip (specifically CG17735) mRNA is enriched in the PDF cells, as reported by Kula-Eversole et al. 2010.

3. As stated in the manuscript, increased CLK levels does not always produce behavioral phenotype (Kim et al. 2002), normal behavioral phenotype of Y4 does not exclude the possibility that it may have similar CLK levels as 32-3. Since 32-3 harbors deletion of both hus1-like and ctrip, the real control for ctrip mutation should be Y4, which deletes hus1-like only.

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Done with the published hus1-like null mutant (Fig S4, see point 6 above).

4. It is difficult to judge the quantification of fluorescence signal without the supplement of real images. Sample size (n) is missing in Figure 2 and Figure 6A.
- Images of fluorescence labelings for both larval and adult brains are now provided in Fig S3. - Sample size have been added in the legends.

5. Without loading control band (house-keeping gene), one can not judge the Western blot results (Figure 3). Using equal amount of measurements (50ug) does not control the variability introduced in later procedures. Also, the experiment should have repeats.
- A loading control has been added to the western blots of Figure 3.
- As now indicated in the methods, all western blot experiments have been repeated two or three times with very similar results.

6. How was qPCR data normalized? It is not clear in the Methods and Figure legends. s.e.m based on n=2 is not adequate.
- We now indicate how data were normalized in the methods section.
- We agree that s.e.m requires at least n=3 and apologize for the mistake. Most of the data have been averaged from three independent experiments, and s.e.m as well as sample size are now provided. In some cases, only two independent experiments were used, and for those we only report the two values on the graph as indicated in the corresponding legends.

7. The function of CTRIP in the proteasome degradation/UFD pathway needs to be clarified. Without experimental evidence that CTRIP directs N-terminal ubiquitination, one can not distinguish it from its function in polyubiquitination.
We certainly do not claim that ctrip is involved in N-terminal ubiquitination, but only suggest that it might be the case, since TRIP12 has been shown to be involved in N-terminal ubiquitination. We have anyway tone down references to N-terminal ubiquitination in the text.

8. Nomenclature is not consistent. For example, genes should be italicized, but not specific alleles (Y4, 32.3, gal1118 etc.); it should be Clk/Pdf/Pdp1, instead of clk/pdf/pdp1. When a specific protein function is stated, it should be PER-independent, but not per-independent.
Changes were made accordingly.

9. It is confusing when the authors attribute more phosphorylated PER and hypophosphorylated CLK both to deficits in proteasome degradation.
We are not sure to understand exactly what the reviewer means. We only say that deficient ubiquitin-proteasome function could be responsible for more protein being present in the mutants. We do not think that proteasome degradation should only target phosphorylated PER and/or CLK, we just do not know. Isn't it the case that, in principle, any form of any protein could be ubiquitinated by a specific ubiquitin ligase and targeted for proteasome degradation ?

Referee #3 (Remarks to the Author):

A major aspect of circadian clock mechanisms involves the timely degradation of central clock proteins. Evidence from several systems indicates that E3 ubiquitin ligases play major roles in the degradation of clock proteins. In Drosophila, E3 ligases have been identified for regulating cycles in PER levels and the light-mediated degradation of TIM and CRY. The importance of regulating the stability of the CLK protein is not clear. While clk mRNA levels clearly oscillate, the protein appears rather constant with some low level oscillation. However, there is evidence that phosphorylated CLK is more unstable and CLK also appears to be limiting in abundance. The interesting aspect of this work is that it identifies a novel E3 ligase, CTRIP, which appears to target CLK. The overall quality of the work is impressive. Alterations in ctrip levels clearly result in strong effects on the period of activity rhythms with similar effects on the molecular cycles of key clock components.
For example, mutations and RNAi both reveal that lowering ctrip levels results in period lengthening. In addition, CTRIP in the brain has a very limited spatial expression pattern, mainly in the PDF-expressing clock neurons. Thus, from a physiological perspective it is clear that a novel E3 ligase has been identified in the Drosophila clockworks (i.e., in the right place and alterations in its

levels affects the pace of behavioral and molecular rhythms). The biochemical data indicating CLK as a major target for CTRIP-mediated degradation are good but indirect. Thus, the manuscript is novel and interesting because it identifies a new component of the Drosophila clock but the proposed mechanism, although supported, is more circumstantial. Below are more specific comments.

Major comments:
1. What is the evidence that CTRIP directly targets CLK for degradation? The case is built from solid but indirect evidence. In the 32-2 and RNAi lines, clk mRNA levels are not affected but CLK protein levels are higher. The simplest interpretation is that CTRIP levels affect CLK abundance at the posttranscriptional level, consistent with its predicted function.

There seems to be a discrepancy in the amplitude of the rhythm between in situ and western data. It is much higher and even observed for the control at DD for western's but not for the corresponding in situ.
It is difficult to compare cycling amplitudes in western blots and brain immunolabelings since we do not know whether the antibodies recognize the same forms of the proteins, which are clearly in very different conditions between fixed tissues on one hand and denaturing buffer on the other hand. This even more difficult when two different antibodies must be used in the two types of analyzes, like here for CLK. Finally, the RNAi expression levels are very likely to be different in eyes and neurons. We thus rather think that the western and in situ results do correlate quite well.

Prior work is also a bit controversial as to whether there is cycling at the total protein level and it seems to be due to the extraction buffer used. The data shown for the western blots seems to suggest that the DNA bound version of CLK was not effectively extracted, or else the total levels should be rather constant (see Yu et al., 2006). While this does not argue against the clear increase in CLK levels in the RNAi lines, why the discrepancy between in situ and blotting and prior results?
As indicated to referee 2 (other concern 1), robust CLK cycling in head extracts has been recently shown in Menet et al 2010 (fig 3), in flies expressing a V5-tagged CLK. We have previously shown CLK levels cycling with the same antibody in Richier et al. 2008. We certainly cannot exclude that this cycling is related to the extraction procedure that we use or to the one that is used in the Menet paper, but it at least reflect changes in the CLK protein accessibility. It is also supported by the cycling of anti-CLK immunolabeling that we detect (more clearly in the ctrip mutants or ctrip RNAi flies) in the larval and adult brain. As indicated in the text, CLK immunolabeling cycling has also been reported in the larval brain by Hung et al. 2009. Again, this might reflect changes in the protein accessibility and not real levels, as it coud be said for any assay based on immunolabeling.

In any case, it would be reassuring to show at least some evidence for CTRIP directly affecting CLK levels. While this is difficult to obtain in vivo, can any evidence be obtained in S2 cells as was done recently for TIM and CRY degradation? e.g., do CTRIP and CLK interact, is the stability of CLK affected by overexpressing or inhibiting CTRIP. Is the ubiqutinylated status of CLK altered when comparing wildtype and RNAi extracts?
We have now added a CLK degradation assay in S2 cells (see response to referee 2, point 5), which confirms that CTRIP indeed affects CLK stability.

Related to this, do the authors know how well their RNAi worked in flies?
The best evidence that we have is that the molecular phenotype of ctrip mutants and ctrip RNAi flies is very similar. In addition, we have shown that overexpressing ctrip RNA partially rescues the RNAi behavioral phenotype. Finally, a different type of ctrip RNAi has been shown to lengthen the behavioral period (Sathyanarayanan 2008).
We have tried to estimate the effect of ctrip RNAi on ctrip mRNA by QRT-PCR, and did see a very small decrease. However, the same experiment in flies overexpressing ctrip indicates that when ctrip mRNA is induced in tim-expressing cells (four fold increase in tim-gal4 UAS-ctrip flies compared to wild-type), a two fold decrease is observed in flies expressing both UAS-driven ctrip mRNA and ctrip RNAi. This is now mentionned in the legend of table S1, but we'll be happy to provide the data if requested.
We thus believe that the high ctrip expression levels in head extracts (about 10 times more than per, indicated in the text p. 4) reflects contributions of both clock and non-clock cells (see discussion, p. 9) with tim-gal4 driven RNAi expression only altering the contribution of tim-expressing cells (which is much higher in tim-gal-UAS ctrip flies).

If CLK undergoes daily levels in abundance, as indicated by the results shown, one has to conclude that CTRIP is not important for controlling temporal changes in the stability of CLK but rather some base-line stability. This should be mentioned.
Yes and No. CLK levels are increased at all times, in agreement with a base-line stability control. However, the increase is stronger at the end of the day than at the end of the night, suggesting a possible regulation of ctrip levels and/or function. This is now discussed (p. 10).

It is not known that effects of CTRIP on CLK can be measured in S2 cells or that the ubiquitin status of CLK can be measured in vivo, but the authors should at least attempt to obtain some more direct evidence and at the very least should be able to say whether CLK and CTRIP interact when co-expressed in S2 cells.
These are certainly very interesting questions but they are out of the scope of this paper, as agreed by the editor.

2. Similar experiments as above should also be done for PER and TIM, as the evidence for CTRIP DIRECTLY controlling PER levels is weaker. For example, do CTRIP and SLMB have additive effects on PER stability?
PER and TIM show no oscillations in slmb mutants and the slmb phenotype is stronger than the ctrip phenotype. Unfortunately, this leaves no hope for seeing a ctrip molecular defect in a slmb mutant background.

3. The in situ results (Fig. 1) and the whole head extract data do not seem to reconcile. If ctrip is mainly only expressed in PDF cells, how does RNAi have effects on total PER, TIM and CLK, which are expressed in many other non-PDF clock cells (including the eyes)?
See response to referee 1, point 3.

What happens to CLK, PER, TIM levels with drivers that express RNAi of ctrip in non-PDF clock cells? Further, does RNAi of ctrip have preferential effects on CLK in PDF-expressing cells by in situ? e.g., compare DNs to LNvs.
We analyzed CLK and PER levels at CT0 in the PDF-negative DN1 neurons of 32.3 larvae. This eliminated the possibility that differences between clock neurons might be a consequence of different RNAi expression levels in different subsets of cells in tim-gal4 UAS-ctrip RNAi flies. As in the PDF-expressing sLNvs (Fig 2A), CLK and PER levels were increased in the DN1s of 32.3 mutants (Fig S4). CTRIP thus appears to act similarly in at least some PDF-negative neurons.

Minor comments:
1. page 6, middle; I think they mean Figure 1C and not 1B.
Corrected.
2. Fig 1. Did the authors look at other body tissues?
No enhancer trap expression could be observed outside the brain but QRT-PCR indicates that ctrip RNA levels are about 10 times higher than per RNA levels in head RNAs, suggesting broad expression (see above). The effect of ctrip RNAi in the head extracts show that ctrip is expressed in more cells than the ones detected by the enhancer-traps. Our results also indicate that ctrip is expressed in embryonic S2 cells.

3. Any idea why overexpressing ctrip has no effect on period length; this is a bit hard to understand as it is supposed to degrade CLK.
see response to referee 1 (point 1)

4. Any difference between nuclear and cytoplasmic levels of CLK in wildtype versus RNAi lines as it is proposed that ctrip might affect CLK stability in the nucleoplasm.
As indicated in the text, we could not see any obvious effect of ctrip loss or downregulation on subcellular localization of the clock proteins.

2nd Editorial Decision 16 March 2011

Thank you for the submission of your revised manuscript to our offices. We have now received feedback from referees 2 and 3, who in principle support publication. Nonetheless, referee 2 has a couple of minor concerns that would need to be addressed before we can proceed with the official acceptance of your manuscript, except the one related to the title, which I think is a matter of opinion.

As is our standard practice, I have edited the title and abstract of your manuscript to make them more accessible to our wide readership. Please find the edited versions at the end of this letter and incorporate them into your final versions if you agree with the changes (or specify which modifications you would make).

In addition, I have realized that all of the methods section has been moved to Supplementary Information. We require that the essential information needed to understand the experiments performed be included in the main text; longer, more detailed information necessary to repeat the experiments should then be included in supplementary information. I would encourage the presentation of a merged the Results and Discussion section, which would help to eliminate some redundancy inherent to their presentation separately and provide space to include the Methods.

I also need to ask you to provide the editorial office with scan for the full blots corresponding to figure 3A, in which both the control band and the CLK band can be seen. Lastly, some of the lanes of gels presented in Fig 3A and Fig 4B appear to have been spliced together. Please indicate that samples were run on different gels using a vertical bar (as you do for the control in Fig 3A and in Fig S6).

I look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS

Referee #2 (Remarks to the Author):

Judging from the western blot image, the effect on CLK is rather minor (compared to loading control band). Thus a title "The HECT domain E3 ubiquitin ligase CTRIP modulates CLOCK levels and PERIOD oscillations in Drosophila" is more appropriate.

remaining concern:

1)please clarify if the added control experiment for CLK antibody specificity was performed with same condition to the original western blot assay.
2)please provide quantification (normalized over loading control) for the western blot experiments

Referee #3 (Revision Comments):

The authors have addressed a main concern with the S2 cell CLK degradation assay. Given all the other data in flies, the evidence is very strong that CTRIP directly regulates CLK levels, a finding that is clearly of importance to the circadian field and of general interest.

Edited title and abstract:

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The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in Drosophila

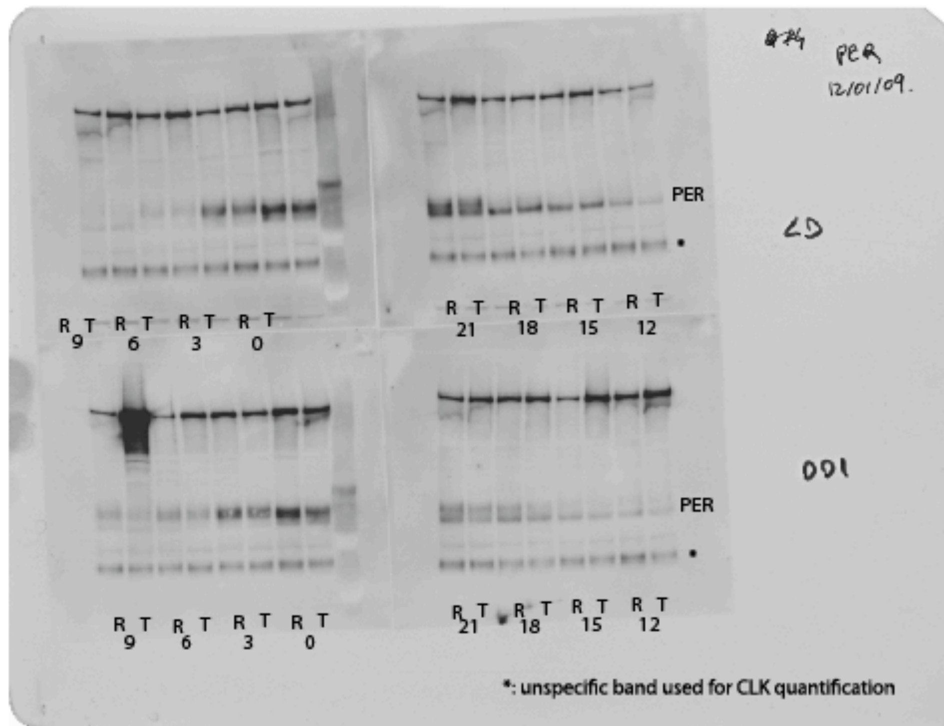
In the Drosophila circadian clock, the CLOCK/CYCLE complex activates the period and timeless genes, which negatively feedback on CLOCK/CYCLE activity. The 24 hour pace of this cycle largely depends on CLOCK stability. RING-domain E3 ubiquitin ligases have been shown to destabilize PERIOD and TIMELESS. Here, we identify a clock function for the circadian trip (ctrip) gene, which encodes a HECT-domain E3 ubiquitin ligase. Ctrip expression in the brain is largely restricted to clock neurons and its downregulation leads to long period activity rhythms in constant darkness. This altered behavior is associated with high CLOCK levels and persistence of phosphorylated PERIOD during subjective day. Importantly, the control of CLOCK protein levels does not require PERIOD. Thus, CTRIP seems to regulate the pace of the oscillator by controlling the stability of both the activator and the repressor of the feedback loop.

2nd Revision - authors' response 18 March 2011

Please find enclosed the revised version of our manuscript entitled "The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in Drosophila" that we have submitted for publication in EMBO Reports.
As you have requested in your last email, we have replaced title and abstract with your version, except for the second sentence which refers to "clock proteins" not to "the CLOCK protein". We have also merged the results and discussion sections, and put the essential information of the methods back in the main text.
As requested have joined scans of the CLK and PER western blots used in Fig3A as supplementary material for the editorial office, and have modified Fig3A and 4B according to your request.

As requested by reviewer 2, we have specifically indicated in the legend of Fig3A that controls were processed under the same conditions and have added a normalized quantification of CLK levels in head extracts (illustrated in Fig3A) as a new Sup. Fig6.

We thus hope that our paper will now be suited for publication in EMBO Reports.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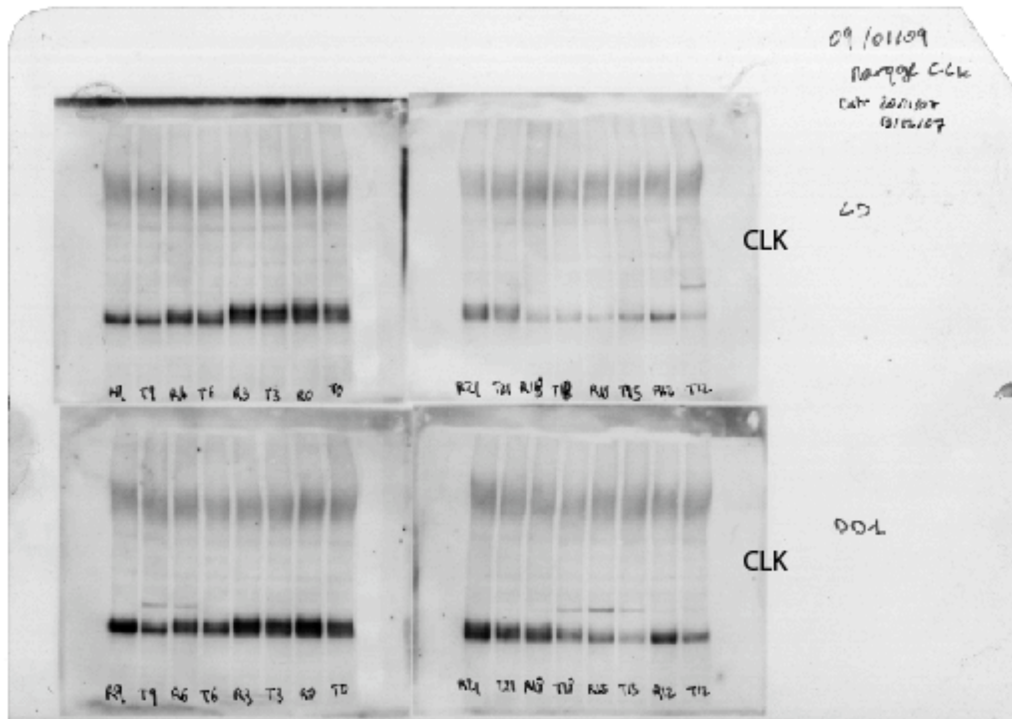
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3rd Editorial Decision 22 March 2011

Thank you for submitting your revised manuscript to EMBO reports. I am very pleased to accept it for publication in the next available issue of the journal.

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Yours sincerely,

Editor
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