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Nipped-A Regulates the *Drosophila* Circadian Clock via Histone Deubiquitination

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

1st Editorial Decision 9th Jan 2019

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received a full set of referee reports on your manuscript, which are included below for your information.

As you can see from the comments, all referees express interest in the described role of Nipped-A in regulation of the Drosophila circadian clock via histone ubiquitination. However, the reviewers raise a number of substantive concerns regarding the conclusiveness of the data and the breadth of analysis that need to be addressed before they can support publication here. Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees. In particular, please address these comments:
- Provide further support for the specificity of Nipped-A mediated H2B ubiquitination (referee #1, point 2; referee #2, point 5; referee #3, point 6). Genome-wide analysis of Nipped-A binding (referee #1, point 1) will not be required for re-consideration.
- Provide information on screening strategy leading to Nipped-A identification (referee #1, point 3; referee #3, point 1)
- Improve the statistical analysis and include requested controls (referee #1, minor point 1; referee #2, point 3; referee #3, points 3 and 4)
- Provide further support to the involvement of Not and the SAGA complex in H2B ubiquitination via Nipped-A (referee #1, minor points 5-7 and 10; referee #2, points 6 and 7)
- Please clarify the effect of Nipped-A on Clk expression and activity (referee #2, points 2 and 4; referee #3, point 5)

REFEREE REPORTS:

Referee #1:

In this paper, Bu and colleagues show the impact of Nipped-A, the fly homolog of TRRAP, on the

circadian period in the Drosophila Clock. They found that knock down of Nipped-A lengthens the period of fly locomotor rhythms due to the reduction of tim and Pdp1e mRNAs resulting in PER protein degradation. Interestingly, they claim that the phenotype is not associated with NIPPED-A-mediated acetylation of histones but with NIPPED-A-mediated regulation of histone H2B ubiquitination at the tim and Pdp1e loci by recruiting the deubiquitinase NOT. Although this paper proposes a new role of Nipped-A for determination of the circadian period length in fly, the underlying mechanism is not solved and the story appears premature to justify publication in this journal. The following points need to be considered by the authors.

Major concerns:
1. The authors claim that NIPPED-A binds on the tim and Pdp1e loci in Figure 4C, but we do not find any essential controls for showing the specificity. The global binding profile of NIPPED-A should be examined by other methods , for example, ChIP-seq analysis or ChIP-PCR which would demonstrate how the binding is specific to some loci or genome-wide events. If a specific binding is supported, the authors should explain how NIPPED-A targets specifically the tim and Pdp1e loci. If a genome-wide binding is supported, they need to explain how NIPPED-A regulates selectively the transcription of tim and Pdp1e genes.

2. Figure 5B: if the authors claim that Nipped-A RNAi affects the pattern of ub-H2B levels, the significances should be confirmed with Student's t-test. In addition, the ub-H2B levels of other regions (e.g. per locus) are to be examined in order to show the specific regulation of the transcription of tim and Pdp1e genes.

3. The authors do not provide any statements on strategy and method for screening of mutant flies; how did they find Nipped-A mutant? Which kind of mutation(s) did they identified in the gene?

Minor concerns:
1. In this study, the authors demonstrate only a part of the statistical differences, they appear to be randomly picked up from the results of Tukey's test. All significant differences should be indicated.

2. Figure 1E, F; Why cryG4-16 alleles are introduced? They wished to induce RNAi expression both during developmental stage and in the adult. No explanation for this is found.

3. Which RNAi lines are used in Figure 1A, 2, 5A, 6C?

4. What does Ptim stand for in Figure 3A?

5. The period of behavioral rhythm is lengthened by knocking down Nipped-A. This phenotype is not recapitulated by knocking down not in Figure 6A. More detailed explanations about this difference need to be stated in the text.

6. The authors describe that knocking down not reduces tim and Pdp1e mRNA in Figure 6C. Do the ub-H2B levels increased in not RNAi lines?

7. Figure 6D, E: not overexpression shortened the period length in both intact and Nipped-A-knocked down lines. Therefore, it remains unclear whether not overexpression rescues the long period phenotype by Nipped-A reduction or not overexpression and knocking down Nipped-A independently regulate the period of locomotor rhythm. Furthermore, the authors describe knocking down not alone does not alter the period, however, this statement is not appropriate because knocking down not does alter the period length significantly as shown in Figure 6A.

8. The authors illustrate NIPPED-A binding on E-box in the model shown in Figure 7, which does not reflect the data shown in Figure 4C.

9. The authors speculate that NIPPED-A regulates the circadian period independent of Cry. Although they demonstrate that cry mRNA level is not affected by knocking down Nipped-A, it is uncertain whether Cry protein levels are affected in Nipped-A-knocked down lines.

10. Does Nipped-A overexpression shorten the period length? Is the phenotype in Nipped-A-overexpressed line rescued by knocking down not?

Referee #2:

The paper by Bu et al. identifies the NippedA component of the SAGA transcription complex as a regulator of the expression of the timeless and pdp1e clock genes in Drosophila. Downregulating NippedA induces a lengthening of the behavioral period and specifically decreases the transcription of tim and pdp1e. Although NippedA does not appear to be circadianly controlled, it binds to chromatin at the tim and pdp1e loci where it promotes H2B2 deubiquitylation, in agreement with its putative function in transcriptional activation. Accordingly, the Not deubiquitylating enzyme appears to have similar effects at the behavioral and molecular levels.
This is an interesting study that builds upon a nice behavioral phenotype resulting from NippedA downregulation. The authors do a lot of efforts to identify the role of these SAGA components in circadian timing and I believe that they make the case for such a role. With such broadly acting proteins, the main difficulty is to show that the observed effects are the consequences of a specific function in the circadian oscillator, and I think that additional experiments (and controls) would be required to support the idea that SAGA plays a specific role in tim and pdp1e transcriptional control.

Main points

1. Introduction :
"CLK/CYC also activate the transcription of two additional transcription factors, vrille (vri) and PAR-domain protein 1ε/δ (Pdp1ε/δ), with the former activating while the latter repressing clk transcription."
vri actually represses clk transcription while pdp1 activates it (see Gunawardhana and Hardin, 2017)

2. Since pdp1 is known to activate clk transcription, why do clk mRNA levels remain unaffected in the nipped-A RNAi flies (fig 2A)?

3. Figure 3 and table S2. Period is similarly affected by nippedA RNAi in tim0/+ and +/+ whereas rhythmicity (power, % rhythmic flies) is much more affected in tim0/+ than in +/+ flies. What could explain this?
In the nippedA RNAi rescue experiments with either tim, per or pdp1e, the authors use Ptim for tim and UASpdp1 for pdp1e. It is not indicated how much tim overexpression is induced by Ptim (why not using UAStim?). Also, introducing a UASpdp1 might compete with the UAS-nippedA RNAi for Gal4 activity and a control with a neutral UAS (such as GFP) should be provided to exclude that the recue observed with UASpdp1 and UASper is not due a to a decrease of RNAi expression.

4. In Fig S4, the authors indicate that downregulation of nippedA increases CLK-binding to to tim and pdp1 E-boxes. To me, the clearest effect seems to be an advanced binding (high binding at CT6 in nippedA RNAi flies). How could this be associated with a long behavioral period?

5. Fig 5. The ub-H2B2 CHIP experiments show a slight increase of ubiquitylation at the tim and pdp1 loci. To be interpreted as the specific molecular basis of the behavior of nippedA-downregulated flies, the same experiment should be done with the clk promoter (as the authors did for the nippedA CHIP in fig 4).

Fig6A, the behavioral consequences of not downregulation are not easy to interpret. The authors indicate that the power of the rhythms is decreased by not RNAi, but not RNAi can either increase or decrease the period, depending on the transgene (1 or 2) and the controls (Gal4 or UAS) that are used for the comparison. I believe that it raises questions about interpreting small period differences, in particular when rhythms are weak. This is particularly important for the following not-overexpression experiments (D, E) where about 1h period changes are interpreted as rescue of nippedA downregulation. In addition, as mentioned above for figure 3, multiple UAS can compete for gal4 activity, and additional controls are required for interpreting such experiments. Since the authors emphasize the change in the rhythm power when downregulating not, they need to keep the same parameter when interpreting rescue experiments.

6. Fig6C is convincing at showing that tim (and pdp1e) mRNA levels are low in not RNAi flies, as in nippedA RNAi flies but the specificity of this effect should be shown as for nippedA, by doing the same assay with per and clk for example (as in fig 2 for nippedA RNAi).

Minor points

- English should be improved
- Methods: To my knowledge, the Clocklab software is not a product of the Trikinetics company

Referee #3:

This study by Bu et al. characterized the role of NIPPED-A in regulating Drosophila circadian rhythms. Nipped-A is the Drosophila ortholog of TRRAP, a gene associated with schizophrenia in humans. As the authors are interested in understanding the molecular mechanisms underlying circadian disruptions observed in human patients with psychiatric diseases, they performed a Drosophila behavioral screen in which they knocked down genes previously reported to be associated with psychiatric diseases using RNAi (in clock neurons) and assayed for circadian rhythm defects. They observe that RNAi knock down of Nipped-A results in lengthening of circadian output rhythms. Furthermore, they report that NIPPED-A protein regulates the transcription of timeless and Pdp1e (two key clock genes) by facilitating deubiquitination of H2B at the promoters of these clock genes. They postulate that NIPPED-A function in clock gene regulation is mediated by its interaction with the deubiquitinase NON-STOP. Finally, they noted that the role of NIPPED-A in clock regulation provides a possible explanation for circadian disruptions often observed in schizophrenia patients.

Overall, this is a significant body of work that adds to the literature on epigenetic and transcriptional regulation of circadian clock genes and circadian physiology. Some of the results are rather unexpected, e.g. tim mRNA is affected in Nipped-A RNAi transgenic fly lines, but not per mRNA; CLK ChIP signals at tim and Pdp1e promoters are elevated in flies expressing Nipped-A RNAi although these same flies show decrease in tim and Pdp1e mRNA levels. Although the authors took time to discuss some of these unexpected results in the discussion, addition of some validation/control experiments will improve the rigor of this manuscript. Finally, since NIPPED-A is part of the SAGA complex, which is involved in broad transcriptional control of the genome, highlighting it as a link between psychiatric diseases and circadian disruptions may be a bit of a stretch or at least premature. See below for specific comments.

Major comments:
1. The authors should provide more details with regard to their RNAi behavioral screen in flies. How were the genes chosen? Did they perform a literature search on genes associated with human psychiatric diseases? How many did they screen? What is the percentage that show behavioral phenotypes?

2. The authors should elaborate on the discrepancy on the changes in period length when knocking down Nipped-A in adult stage only (1h lengthening) vs throughout development into adult stage (3h lengthening).

3. Please show results of validation experiments confirming that TRRAP antibody specifically recognizes Drosophila NIPPED-A and can be used for Western blots and immunoprecipitations. This is important not only for Western blot analysis, but it will also enhance confidence in the ChIP assay results.

4. Please provide significance indications (e.g. use asterisks) on bar graphs in figures, rather than just in figure legends, e.g. Figure 4, 5, S5, S6.

5. The authors noted that "knocking down Nipped-A significantly increases CLK binding at Pdp1e promoter, while a trend of increase is also observed at tim promoter". This result is unexpected as

Nipped-A RNAi treatment leads to reduction in tim and Pdp1e mRNA levels. The authors indicated that perhaps this is due to an unknown compensatory mechanism. Before making this conclusion, I would like to see if these two events show any causality. For example, the authors did not observe a change in per mRNA levels in Nipped-A RNAi flies. This would indicate that CLK ChIP signals at per promoter should not be altered in Nipped-A RNAi flies.

6. The authors can strengthen the link between H2B ubiquitination and clock gene expression level by showing that genes that did not show changes in expression in Nipped-A RNAi flies, e.g. per, also have no differences in H2B ubiquitination when Nipped-A is knocked down.

Minor comments:
1. Does knock down of Nipped-A in flies also result in phenotype analogous to human psychiatric diseases? If not, the case for linking clock disruption and schizophrenia based on results presented here may be weak. Also, the role of tim in the circadian clock is different in flies and in mammals.

2. The authors should include information outlining the association of TRRAP with schizophrenia in the Introduction IF they choose to stay with the focus of "understanding circadian disruptions in patients with psychiatric disorders". Saving it for the Discussion is too late. Alternatively, if they shift the focus of the manuscript to epigenetic regulation of circadian rhythms by NIPPED-A/TRRAP, then it is ok to highlight that connection in the Discussion.

3. In the Introduction, the authors cited a 2008 review on circadian clock. Perhaps a more updated review paper or review papers should be cited instead.

4. Introduction, line 5, no parenthesis is necessary for "and in many other animals including humans".

5. Page 5, line 4: I think there should be 6 RNAi lines, not 5?

6. Page 5, lines 1-2: What is the difference between "clock cells" and "circadian neurons" for tim-gal4 and cry-gal4?

7. SAGA is a protein complex that regulates transcription broadly. Do the authors have any hypothesis that can explain its specificity on certain clock genes? Perhaps they can elaborate briefly in the discussion.

1st Revision - authors' response 8th Jul 2019

We thank the editor and the reviewers for their thoughtful comments and suggestions which have made the manuscript much stronger. We have addressed these comments and suggestions as described below. The original reviews are listed point-by-point. Our responses are in italic font. Edits made in the text of the manuscript are marked in red.

Referee #1:

In this paper, Bu and colleagues show the impact of Nipped-A, the fly homolog of TRRAP, on the circadian period in the Drosophila Clock. They found that knock down of Nipped-A lengthens the period of fly locomotor rhythms due to the reduction of tim and Pdp1e mRNAs resulting in PER protein degradation. Interestingly, they claim that the phenotype is not associated with NIPPED-A-mediated acetylation of histones but with NIPPED-A-mediated regulation of histone H2B ubiquitination at the tim and Pdp1e loci by recruiting the deubiquitinase NOT. Although this paper proposes a new role of Nipped-A for determination of the circadian period length in fly, the underlying mechanism is not solved and the story appears premature to justify publication in this journal. The following points need to be considered by the authors.

Major concerns:
1. The authors claim that NIPPED-A binds on the tim and Pdp1e loci in Figure 4C, but we do not find any essential controls for showing the specificity. The global binding profile of NIPPED-A should be examined by other methods , for example, ChIP-seq analysis or ChIP-PCR which would demonstrate how the binding is specific to some loci or genome-wide events. If a specific binding is supported, the authors should explain how NIPPED-A targets specifically the tim and Pdp1e loci. If a genome-wide binding is supported, they need to explain how NIPPED-A regulates selectively the transcription of tim and Pdp1e genes.
We have examined the binding pattern of NIPPED-A by ChIP-PCR and detected binding at the per locus (Fig 4C), although knocking down Nipped-A does not affect per mRNA level. We believe that as part of the SAGA complex, NIPPED-A may have a relatively broad genome-wide binding pattern, but tim and Pdp1e are particularly sensitive to Nipped-A deficiency as knocking down Nipped-A specifically affects H2B ubiquitination at tim/Pdp1e loci (Fig 5B). It has been shown in yeast that not all SAGA-dependent genes require Tra1, the yeast homolog of NIPPED-A (Helmlinger et al, 2011). The SAGA complex can be assembled and recruited to some genes but not others in the absence of Tra1. This may also be the case for tim and Pdp1e. We have discussed about this in the first paragraph of Page 15 (marked in red).

2. Figure 5B: if the authors claim that Nipped-A RNAi affects the pattern of ub-H2B levels, the significances should be confirmed with Student's t-test. In addition, the ub-H2B levels of other regions (e.g. per locus) are to be examined in order to show the specific regulation of the transcription of tim and Pdp1e genes.
We have now used Student's t-test in addition to ANOVA to analyze the difference in ub-H2B levels between Nipped-A RNAi and control flies. We observed statistical significance at tim and Pdp1e loci. We have also examined the effects of knocking down Nipped-A on ub-H2B levels at per and clock loci and found no significant difference. These results are included in Fig 5B.

3. The authors do not provide any statements on strategy and method for screening of mutant flies;
how did they find Nipped-A mutant? Which kind of mutation(s) did they identified in the gene?
We have now added a short paragraph in the beginning of the results section regarding how we conducted our RNAi screen which is marked in red: "We initiated a RNAi screen of Drosophila homologs of genes reported to be associated with psychiatric conditions in human to identify genes that are involved in circadian regulation. We knocked down the expression of these candidate genes in all clock cells (including neurons and glial cells) using a timGAL4 driver or mainly in circadian neurons using a cryptochrome (cry)GAL4-16 driver (Emery, So et al., 1998, Emery, Stanewsky et al., 2000), and assessed the effects of these manipulations on fly locomotor rhythm. So far we have tested 24 genes and have identified Nipped-A, the Drosophila homologue of human TRRAP, to be involved in determining the period length of fly locomotor rhythm under constant darkness (DD)."

Minor concerns:
1. In this study, the authors demonstrate only a part of the statistical differences, they appear to be randomly picked up from the results of Tukey's test. All significant differences should be indicated.
We apologize for not having done this thoroughly. We have now added additional statistical analysis to Fig 3 and 6, Appendix Fig S3, as well as Appendix Table S1, S4-S7, and S9.

2. Figure 1E, F; Why cryG4-16 alleles are introduced? They wished to induce RNAi expression both during developmental stage and in the adult. No explanation for this is found.
We apologize for not explaining this clearly in the original manuscript. We have now added an explanation (marked in red) regarding this in the last paragraph of Page 6: "To test whether NIPPED-A functions in the adult circadian system, we used a temperature sensitive tubulin (tub)GAL80^{ts} in combination with cryGAL4-16 to knock down Nipped-A specifically during the adult or developmental stage (McGuire, Mao et al., 2004). tubGAL80^{ts} represses the

transcriptional activities of GAL4 at permissive temperature (18ºC), thus GAL4-driven transcription can only occur under restrictive temperature (29ºC).”

3. Which RNAi lines are used in Figure 1A, 2, 5A, 6C?
This has been indicated in the figures now.

4. What does Ptim stand for in Figure 3A?
Ptim is a tim cDNA construct driven by tim promoter. We have added an explanation for this in the legend of Fig 3 (marked in red).

5. The period of behavioral rhythm is lengthened by knocking down Nipped-A. This phenotype is not recapitulated by knocking down not in Figure 6A. More detailed explanations about this difference need to be stated in the text.
We have conducted additional experiments and found that knocking down not reduces the mRNA levels of not only tim and Pdp1e, but also the 4 other core clock genes (Fig 6C). This could contribute to the difference in phenotype compared to knocking down Nipped-A.

6. The authors describe that knocking down not reduces tim and Pdp1e mRNA in Figure 6C. Do the ub-H2B levels increased in not RNAi lines?
Yes, we have verified that knocking down not indeed increases ub-H2B levels at tim and Pdp1e loci. These results are in Fig.6D

7. Figure 6D, E: not overexpression shortened the period length in both intact and Nipped-A-knocked down lines. Therefore, it remains unclear whether not overexpression rescues the long period phenotype by Nipped-A reduction or not overexpression and knocking down Nipped-A independently regulate the period of locomotor rhythm. Furthermore, the authors describe knocking down not alone does not alter the period, however, this statement is not appropriate because knocking down not does alter the period length significantly as shown in Figure 6A.
We agree with the reviewer and now describe the result as “over-expressing not shortens the long period phenotype caused by Nipped-A deficiency”. Knocking down not with pdfGAL4 driver lengthens the period by ~0.3h compared to pdfGAL4-Udcr2/+. Although this effect is modest, we agree that it is significant and thus inappropriate to say that knocking down not does not alter the period. We have changed this to “knocking down not alone does not substantially alter the period”. Despite this small period lengthening, we believe the synergistic effect on period when knocking down both Nipped-A and not is robust, which supports the idea that NIPPED-A and NOT cooperate to time the clock.
To further validate that SAGA DUB module functions together with NIPPED-A to determine period length, we tested for genetic interaction between Nipped-A and Sgf11, another component of the SAGA DUB module that binds with NOT. Knocking down or mutating Sgf11 does not appear to exhibit a prominent effect on the period, whereas knocking down or mutating Sgf11 synergistically enhances the period lengthening caused by Nipped-A deficiency. This indicates that Nipped-A acts in synergy with Sgf11 to set the pace of the clock. These results are now included in Fig.EV4 and Appendix Table S10-11.

8. The authors illustrate NIPPED-A binding on E-box in the model shown in Figure 7, which does not reflect the data shown in Figure 4C.
Thank you for pointing this out. We have now modified our model figure.

9. The authors speculate that NIPPED-A regulates the circadian period independent of Cry. Although they demonstrate that cry mRNA level is not affected by knocking down Nipped-A, it is uncertain whether Cry protein levels are affected in Nipped-A-knocked down lines.
We would like to test this but unfortunately we do not have a CRY antibody, and there is no commercial antibody that works. Since cry mutants do not exhibit circadian phenotypes in DD, we believe the effects we show here for NIPPED-A in determining period length is independent of CRY. We have also added a couple sentences in the second paragraph of Page 13 regarding this (marked in red).

10. Does Nipped-A overexpression shorten the period length? Is the phenotype in Nipped-A-overexpressed line rescued by knocking down not?
Over-expressing Nipped-A does not significantly shorten the period length, but it does rescue the long period phenotype caused by knocking down Nipped-A. These results are in Fig EV1 and Appendix Table S2. We reason that there may be a ceiling for NIPPED-A level and thus the over-expressed NIPPED-A may not be functional, as they may not be able to be recruited to the chromosome.

Referee #2:

The paper by Bu et al. identifies the NippedA component of the SAGA transcription complex as a regulator of the expression of the timeless and pdp1e clock genes in Drosophila. Downregulating NippedA induces a lengthening of the behavioral period and specifically decreases the transcription of tim and pdp1e. Although NippedA does not appear to be circadianly controlled, it binds to chromatin at the tim and pdp1e loci where it promotes H2B2 deubiquitylation, in agreement with its putative function in transcriptional activation. Accordingly, the Not deubiquitylating enzyme appears to have similar effects at the behavioral and molecular levels.
This is an interesting study that builds upon a nice behavioral phenotype resulting from NippedA downregulation. The authors do a lot of efforts to identify the role of these SAGA components in circadian timing and I believe that they make the case for such a role. With such broadly acting proteins, the main difficulty is to show that the observed effects are the consequences of a specific function in the circadian oscillator, and I think that additional experiments (and controls) would be required to support the idea that SAGA plays a specific role in tim and pdp1e transcriptional control.

Main points

1. Introduction :
"CLK/CYC also activate the transcription of two additional transcription factors, vrille (vri) and PAR-domain protein 1ε/δ (Pdp1ε/δ), with the former activating while the latter repressing clk transcription."
vri actually represses clk transcription while pdp1 activates it (see Gunawardhana and Hardin, 2017)
Thank you for pointing this out. We have fixed this.

2. Since pdp1 is known to activate clk transcription, why do clk mRNA levels remain unaffected in the nipped-A RNAi flies (fig 2A)?
We believe this is because the remaining PDP1ε is sufficient for maintaining normal clk expression. On the other hand, we are able to specifically rescue the period phenotype of Nipped-A deficiency by over-expressing Pdp1ε, and we have verified that over-expressing Pdp1ε enhances clk mRNA level. This in turn leads to increased per and tim expression, thus reverting the long period phenotype. These results are now included in Appendix Fig S3B. We have added some discussion regarding this in the first paragraph of Page 14 (marked in red).

3. Figure 3 and table S2. Period is similarly affected by nippedA RNAi in tim0/+ and +/+ whereas rhythmicity (power, % rhythmic flies) is much more affected in tim0/+ than in +/+ flies. What could explain this?
Our hypothesis is that the lengthened period observed in flies with Nipped-A knocked down is at least in part due to decrease in TIM and consequently PER. This could lead to a delay in PER accumulation which ultimately slows down the pace of the clock. The effect of tim^{01}/+ on PER may not be strong enough to influence period length, but may act together with Nipped-A to affect the power of the rhythm via other mechanisms that are yet unclear. We have added some discussion regarding this in the first paragraph of Page 14 (marked in red).

In the nippedA RNAi rescue experiments with either tim, per or pdp1e, the authors use Ptim for tim and UASpdp1 for pdp1e. It is not indicated how much tim overexpression is induced by Ptim (why not using UAStim?). Also, introducing a UASpdp1 might compete with the UAS-nippedA RNAi for Gal4 activity and a control with a neutral UAS (such as GFP) should be provided to exclude that the recue observed with UASpdp1 and UASper is not due a to a decrease of RNAi expression.
We have now included tim mRNA level in Ptim flies in Appendix Fig S4A. We did not use UAStim because based on our previous experience and published data, over-expression with UAStim leads to arrhythmicity (DiAngelo et al, 2011). Therefore here for our rescue experiments we used Ptim which over-expresses tim at a modest level. We have used a UASGFP as a control for the rescue experiments and expressing GFP does not shorten the period of Nipped-A RNAi flies (Appendix Table S3), indicating that the rescue effects of UASpdp1 and UASper are not due to a decrease of RNAi expression.

4. In Fig S4, the authors indicate that downregulation of nippedA increases CLK-binding to to tim and pdp1 E-boxes. To me, the clearest effect seems to be an advanced binding (high binding at CT6 in nippedA RNAi flies). How could this be associated with a long behavioral period?
We agree with the reviewer that CLK binding may be advanced, and we observe this trend for tim, Pdp1 and per but not vri. These results are in Appendix Fig S6. This may reflect some compensatory mechanism caused by reduction of TIM, PDP1e and PER in these flies. Although there is increased (and perhaps advanced) binding of CLK at these gene loci, the corresponding pre-mRNA levels are reduced and do not appear to be phase advanced, indicating that the increased/advanced binding of CLK is not able to compensate for the transcription defects caused by knocking down Nipped-A.

5. Fig 5. The ub-H2B2 CHIP experiments show a slight increase of ubiquitylation at the tim and pdp1 loci. To be interpreted as the specific molecular basis of the behavior of nippedA-downregulated flies, the same experiment should be done with the clk promoter (as the authors did for the nippedA CHIP in fig 4).
We have conducted this experiment. Knocking down Nipped-A does not significantly alter ub-H2B at per and clk loci. These results are now included in Fig 5B.

Fig6A, the behavioral consequences of not downregulation are not easy to interpret. The authors indicate that the power of the rhythms is decreased by not RNAi, but not RNAi can either increase or decrease the period, depending on the transgene (1 or 2) and the controls (Gal4 or UAS) that are used for the comparison. I believe that it raises questions about interpreting small period differences, in particular when rhythms are weak. This is particularly important for the following not-overexpression experiments (D, E) where about 1h period changes are interpreted as rescue of nippedA downregulation. In addition, as mentioned above for figure 3, multiple UAS can compete for gal4 activity, and additional controls are required for interpreting such experiments. Since the authors emphasize the change in the rhythm power when downregulating not, they need to keep the same parameter when interpreting rescue experiments.
*Only one not RNAi line shows significantly altered period compared to both UAS and GAL4 controls, and this period change is modest (<1hr). Therefore we are not able to draw a firm conclusion regarding whether knocking down not affects period length, but it clearly affects the power of the rhythm. We have included UASGFP as controls and found that over-expressing GFP does not shorten the period of Nipped-A RNAi flies. These results are in Appendix Table S3. Over-expressing not does not significantly alter the power of the rhythm on WT background, although it does significantly increase the power of the rhythm in pdf*G4-U*dcr2/+;*U*Nipped-ARNAi-4/+. However, the changes are modest and may not be of biological significance.*
To further validate that SAGA DUB module functions together with NIPPED-A to determine period length, we tested for genetic interaction between Nipped-A and Sgf11, another component of the SAGA DUB module that binds with NOT. Knocking down or mutating Sgf11 does not appear to exhibit a prominent effect on the period, whereas knocking down or

mutating Sgf11 synergistically enhances the period lengthening caused by Nipped-A deficiency. This indicates that Nipped-A acts in synergy with Sgf11 to set the pace of the clock. These results are now included in Fig.EV4 and Appendix Table S10-11.

6. Fig6C is convincing at showing that tim (and pdp1e) mRNA levels are low in not RNAi flies, as in nippedA RNAi flies but the specificity of this effect should be shown as for nippedA, by doing the same assay with per and clk for example (as in fig 2 for nippedA RNAi).
We have now conducted this experiment and found that knocking down not reduces the mRNA level of all 6 core clock genes. These results are included in Fig 6C. This is probably because the transcription of all of these genes requires the SAGA complex (at least the DUB module of SAGA), but only tim and Pdp1e are susceptible to the lack of NIPPED-A. It has been shown in yeast that not all SAGA-dependent genes require Tra1, the yeast homolog of NIPPED-A (Helmlinger et al, 2011). The SAGA complex can be assembled and recruited to some genes but not others in the absence of Tra1. This may also be the case for tim and Pdp1e.

Minor points

- English should be improved
We have asked a native English speaker to read through the manuscript and made some edits.

- Methods: To my knowledge, the Clocklab software is not a product of the Trikinetics company
Thanks for pointing this out. We have fixed it.

Referee #3:

This study by Bu et al. characterized the role of NIPPED-A in regulating Drosophila circadian rhythms. Nipped-A is the Drosophila ortholog of TRRAP, a gene associated with schizophrenia in humans. As the authors are interested in understanding the molecular mechanisms underlying circadian disruptions observed in human patients with psychiatric diseases, they performed a Drosophila behavioral screen in which they knocked down genes previously reported to be associated with psychiatric diseases using RNAi (in clock neurons) and assayed for circadian rhythm defects. They observe that RNAi knock down of Nipped-A results in lengthening of circadian output rhythms. Furthermore, they report that NIPPED-A protein regulates the transcription of timeless and Pdp1e (two key clock genes) by facilitating deubiquitination of H2B at the promoters of these clock genes. They postulate that NIPPED-A function in clock gene regulation is mediated by its interaction with the deubiquitinase NON-STOP. Finally, they noted that the role of NIPPED-A in clock regulation provides a possible explanation for circadian disruptions often observed in schizophrenia patients.

Overall, this is a significant body of work that adds to the literature on epigenetic and transcriptional regulation of circadian clock genes and circadian physiology. Some of the results are rather unexpected, e.g. tim mRNA is affected in Nipped-A RNAi transgenic fly lines, but not per mRNA; CLK ChIP signals at tim and Pdp1e promoters are elevated in flies expressing Nipped-A RNAi although these same flies show decrease in tim and Pdp1e mRNA levels. Although the authors took time to discuss some of these unexpected results in the discussion, addition of some validation/control experiments will improve the rigor of this manuscript. Finally, since NIPPED-A is part of the SAGA complex, which is involved in broad transcriptional control of the genome, highlighting it as a link between psychiatric diseases and circadian disruptions may be a bit of a stretch or at least premature. See below for specific comments.

Major comments:
1. The authors should provide more details with regard to their RNAi behavioral screen in flies.

How were the genes chosen? Did they perform a literature search on genes associated with human psychiatric diseases? How many did they screen? What is the percentage that show behavioral phenotypes?
This is a continuous screen of genes that have been published to be associated with psychiatric conditions. So far we have screened 24 and Nipped-A is the only gene with period phenotype. There is another gene with reduced power but the phenotype is not very strong and needs further validation. We have now added a short paragraph in the beginning of the results section regarding how we conducted our RNAi screen which is marked in red: "We initiated a RNAi screen of Drosophila homologs of genes reported to be associated with psychiatric conditions in human to identify genes that are involved in circadian regulation. We knocked down the expression of these candidate genes in all clock cells (including neurons and glial cells) using a timGAL4 driver or mainly in circadian neurons using a cryptochrome (cry)GAL4-16 driver (Emery, So et al., 1998, Emery, Stanewsky et al., 2000), and assessed the effects of these manipulations on fly locomotor rhythm. So far we have tested 24 genes and have identified Nipped-A, the Drosophila homologue of human TRRAP, to be involved in determining the period length of fly locomotor rhythm under constant darkness (DD)."

2. The authors should elaborate on the discrepancy on the changes in period length when knocking down Nipped-A in adult stage only (1h lengthening) vs throughout development into adult stage (3h lengthening).
We have now added a paragraph in the discussion regarding this (second paragraph of discussion session, marked in red) :" We clearly demonstrate a role for NIPPED-A in circadian period length determination in adults. However, UASdcr2/tubGAL80^{ts};cryGAL4-16/UASNipped-ARNAi-4 raised at permissive temperature and tested at restrictive temperature show ~1h lengthening of the period (compared to UASdcr2/tubGAL80^{ts};cryGAL4-16/+), whereas UASdcr2/+;cryGAL4-16/UASNipped-ARNAi-4 flies in the same experiment show nearly 2h longer period (compared to UASdcr2/+;cryGAL4-16/+). This implicates that knocking down Nipped-A during development exerts influence on adult period. It is possible that NIPPED-A protein has a low turnover rate, and thus knocking down Nipped-A during development could affect NIPPED-A level in adults, leading to a more severe phenotype."

3. Please show results of validation experiments confirming that TRRAP antibody specifically recognizes Drosophila NIPPED-A and can be used for Western blots and immunoprecipitations. This is important not only for Western blot analysis, but it will also enhance confidence in the ChIP assay results.
Specificity of NIPPED-A antibody on Western blots is demonstrated in Fig 4B and the input lanes of Appendix Fig S5. In Nippd-A RNAi flies, the signal is weaker that in control flies. This is also the case for immunoprecipitation demonstrated in Appendix Fig S5.

4. Please provide significance indications (e.g. use asterisks) on bar graphs in figures, rather than just in figure legends, e.g. Figure 4, 5, S5, S6.
We have now conducted Student's t-test in addition to ANOVA, and have indicated significant differences on the bar graphs in these figures.

5. The authors noted that "knocking down Nipped-A significantly increases CLK binding at Pdp1e promoter, while a trend of increase is also observed at tim promoter". This result is unexpected as Nipped-A RNAi treatment leads to reduction in tim and Pdp1e mRNA levels. The authors indicated that perhaps this is due to an unknown compensatory mechanism. Before making this conclusion, I would like to see if these two events show any causality. For example, the authors did not observe a change in per mRNA levels in Nipped-A RNAi flies. This would indicate that CLK ChIP signals at per promoter should not be altered in Nipped-A RNAi flies.
We have now shown that knocking down Nipped-A increases CLK binding at tim, Pdp1e and per E-box, but not at vri E-box. These results are now included in Appendix Fig S6. We believe this is consistent with reduced tim and Pdp1e transcription in these flies. Although per mRNA is not reduced, its protein level is significantly reduced and this PER deficiency may trigger some kind of compensatory response to increase CLK binding, similar to tim and Pdp1e.

6. The authors can strengthen the link between H2B ubiquitination and clock gene expression level by showing that genes that did not show changes in expression in Nipped-A RNAi flies, e.g. per, also have no differences in H2B ubiquitination when Nipped-A is knocked down.
We have done this and found that knocking down Nipped-A does not affect H2B ubiquitination at per and clk loci. These results are now included in Fig 5B.

Minor comments:
1. Does knock down of Nipped-A in flies also result in phenotype analogous to human psychiatric diseases? If not, the case for linking clock disruption and schizophrenia based on results presented here may be weak. Also, the role of tim in the circadian clock is different in flies and in mammals.
It has not been reported that Nipped-A deficiency in flies results in phenotypes analogous to human psychiatric diseases. We are currently testing this. We agree with the reviewer that based on our results here, the link between clock disruption and schizophrenia is still weak. Therefore, we have tuned down relevant discussion both in the abstract and in the discussion section. Although in mammals, whether tim plays a role in the clock is still an issue of debate, H2B-ub has been shown to influence Per1/2 expression in mouse fibroblasts. We have added more detailed discussion regarding this in the second paragraph of Page 16 (marked in red): "A previous study in mouse liver demonstrates rhythmic H2B monoubiquitination of circadian E-box genes including Per1 and Per2, which may be regulated by Ddb1-Cullin-4 ubiquitin ligase (Tamayo et al., 2015). Reducing H2B ubiquitination in fibroblast culture results in shortened period and enhanced Per1/2 mRNA levels. The authors propose a role for DDB1-CULLIN-4-mediated H2B monoubiquitination during the transcriptional repression phase of the circadian cycle. Here we showed that knocking down Nipped-A leads to increased H2B ubiquitination accompanied by lengthened period and reduced tim/Pdp1ε mRNA levels, which is mediated by the DUB module of the SAGA complex. These results support a role for the SAGA complex and H2B deubiquitination in the transcriptional activation phase of the circadian cycle."

2. The authors should include information outlining the association of TRRAP with schizophrenia in the Introduction IF they choose to stay with the focus of "understanding circadian disruptions in patients with psychiatric disorders". Saving it for the Discussion is too late. Alternatively, if they shift the focus of the manuscript to epigenetic regulation of circadian rhythms by NIPPED-A/TRRAP, then it is ok to highlight that connection in the Discussion.
We thank the reviewer for this suggestion and have shifted the focus to epigenetic regulation. Therefore we have removed some description regarding psychiatric disease and circadian rhythm from the introduction section. Moreover, we added a paragraph on the role of chromatin modification in the clock (the second paragraph of introduction section, marked in red).

3. In the Introduction, the authors cited a 2008 review on circadian clock. Perhaps a more updated review paper or review papers should be cited instead.
We have replaced this citation with a 2019 review paper.

4. Introduction, line 5, no parenthesis is necessary for "and in many other animals including humans".
We have fixed this.

5. Page 5, line 4: I think there should be 6 RNAi lines, not 5?
Thank you for pointing this out. We have edited this.

6. Page 5, lines 1-2: What is the difference between "clock cells" and "circadian neurons" for tim-gal4 and cry-gal4?
timGAL4 has a broad expression pattern in all clock cells, which are cells that express the core clock genes and have a functional clock. These include neurons and non-neuron cells such as glial cells. cryGAL4, on the other hand, is expressed mainly in the CRY+ neurons in the brain

which show persistent molecular oscillations in DD and are believed to be particularly important in driving circadian rhythms in behavior and physiology.

7. SAGA is a protein complex that regulates transcription broadly. Do the authors have any hypothesis that can explain its specificity on certain clock genes? Perhaps they can elaborate briefly in the discussion.
It has been shown in yeast that not all SAGA-dependent genes require Tra1, the yeast homolog of NIPPED-A (Helmlinger et al, 2011). The SAGA complex can be assembled and recruited to some genes but not others in the absence of Tra1. This may also be the case for tim and Pdp1e, rendering them particularly sensitive to NIPPED-A deficiency. We have added a brief discussion regarding this in the first paragraph of Page 15 (marked in red).

2nd Editorial Decision 6th Aug 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by all original referees, who find that their main concerns have been addressed and are now broadly in favour of publication of the manuscript pending a minor revision.

Specifically, reviewer #1 suggests further experiments to strengthen the data on specific Nipped-A association with its target loci. I have consulted with the other two reviewers on this issue, and they both found that, while addition of the data requested by reviewer #1 would potentially clarify the circadian regulation of Nipped-A activity, this aspect would not be required for acceptance here. Specifically, reviewer #3 indicated that the presented results are within range obtained by other researchers, but additionally pointed out that normalisation of Ub-H2B ChIP signal over total H2B ChIP would be informative, but not absolutely required. Based on these considerations, the experiments requested by reviewer #1 will not be required for acceptance of the manuscript, but you are welcome to add the data requested by reviewer #1 and the normalisation of the ub-H2B ChIP if you have the data available. Alternatively, reviewer #2 has suggested to add a comment in the manuscript text to indicate that more sensitive methods would have to be used to reveal existence of a potential a temporal control of Nipped-A binding to its target genes.

I would further ask you to address the following textual and editorial issues in the final version of the manuscript.

REFEREE REPORTS:

Referee #1:

In the original manuscript, the authors claimed that NIPPED-A specifically regulates tim and pdp1e transcription among the clock genes, probably by specific binding of NIPPED-A to the E-box regions in these gene loci. The model was originally based on NIPPED-A-ChIP-PCR analysis of tim and pdp1e gene loci with no negative control data. In response to my comment on this issue, they show that NIPPED-A binds to not only tim and pdp1e loci but also per locus in the revision, and they now propose new model as they state: "NIPPED-A may have a relatively broad genome-wide binding pattern, but tim and Pdp1e are particularly sensitive to Nipped-A deficiency". Furthermore, their conclusion that the DUB module of the SAGA complex regulates the expression of clock genes is supported by newly added data on Sgf11.

I think that the manuscript has been improved in this revision. However, all the ChIP-PCR data with the anti-NIPPED-A antibody show very flat signals in any genomic positions across the day (new Fig. 4c). Although these signals are indeed higher than those with control IgG, I am concerned about the possibility that the antibody may have very adhesive property causing high background signals. This reviewer suggests the authors to approach the mechanism underlying selective effects of NIPPED-A deficiency on tim and pdp1e transcript levels. For example, ChIP-seq analysis using the anti-NIPPED-A antibody may identify significant binding peaks when compared with the broad and

flat binding signals in the per and tim loci. Alternatively, the broad genome-wide binding of NIPPED-A would be verified by ChIP analysis using a transgenic line in which NIPPED-A is tagged with Myc or Flag. I believe that their conclusion should be strengthened if the authors employ such an option.

Typo,
Sgf11 (1L) at page11 line14 should read Sgf11 (eleven).

Referee #2:

The authors have significantly improved their manuscript by adding new experiments and controls. The behavioral effect clearly shows that NippedA is involved in the fly circadian oscillator. Although the molecular mechanisms underlying NippedA clock function remain very partially characterized, the new experiments reinforce the main conclusion that tim is likely the first target of NippedA. I believe that the work adds a significant step in understanding how chromatin modifiers control circadian transcription and I would only recommend small changes to this new version.

- p8: On the other hand, knocking down Nipped-A in a tim01 heterozygous mutant background dramatically reduces the power of the rhythm, while knocking down Nipped-A in a wild-type (WT) background or tim01/+ does not substantially decrease the power (Sehgal, Price et al., 1994) (Fig 3B; Appendix Table S4).
tim0 heterozyous show no effect of NippedA downregulation but tim0/+ do? Please correct.

p13: Unfortunately, due to lack of CRY antibody, we are not able to examine whether there is any alteration of CRY protein.
Surprising. Several anti-CRY antibodies have been described in the literature.

- The Ptim and FlySAM-Nipped A constructs should be precisely described (exact sequence information) except if they have been already published.

Referee #3:

I am happy to see that the authors have performed the suggested control and validation experiments to further improve the rigor of their results and conclusions. This manuscript is a significant body of work and should be of interest to the circadian biology community and the gene regulation community at large.

Minor comment:
As part of their revision, the authors addressed the phenotypic differences between Nipped-A RNAi KD in adult vs developmental stage (second paragraph of discussion) by suggesting that the potential slow turnover rate of NIPPED-A could have led to the stronger effects with RNAi starting during development. I don't necessarily agree with this assumption. It is possible that NIPPED-A have developmental effects, given the pleiotropic function of NIPPED-A in transcriptional regulation. I think the authors do not really have to discuss protein turnover. It is acceptable to indicate possible developmental effects. That will not take away from the adult stage effect they observed.

2nd Revision - authors' response 25th Aug 2019

We thank the reviewers for their thoughtful comments and suggestions. We have addressed these comments and suggestions as described below. The original reviews are listed point-by-point. Our responses are in italic font. Edits made in the text of the manuscript are marked in red.

Referee #1:
In the original manuscript, the authors claimed that NIPPED-A specifically regulates tim and pdp1e transcription among the clock genes, probably by specific binding of NIPPED-A to the E-box regions in these gene loci. The model was originally based on NIPPED-A-ChIP-PCR analysis of tim and pdp1e gene loci with no negative control data. In response to my comment on this issue, they show that NIPPED-A binds to not only tim and pdp1e loci but also per locus in the revision, and they now propose new model as they state: "NIPPED-A may have a relatively broad genome-wide binding pattern, but tim and Pdp1e are particularly sensitive to Nipped-A deficiency". Furthermore, their conclusion that the DUB module of the SAGA complex regulates the expression of clock genes is supported by newly added data on Sgf11.
I think that the manuscript has been improved in this revision. However, all the ChIP-PCR data with the anti-NIPPED-A antibody show very flat signals in any genomic positions across the day (new Fig. 4c). Although these signals are indeed higher than those with control IgG, I am concerned about the possibility that the antibody may have very adhesive property causing high background signals. This reviewer suggests the authors to approach the mechanism underlying selective effects of NIPPED-A deficiency on tim and pdp1e transcript levels. For example, ChIP-seq analysis using the anti-NIPPED-A antibody may identify significant binding peaks when compared with the broad and flat binding signals in the per and tim loci. Alternatively, the broad genome-wide binding of NIPPED-A would be verified by ChIP analysis using a transgenic line in which NIPPED-A is tagged with Myc or Flag. I believe that their conclusion should be strengthened if the authors employ such an option.
We thank the reviewer for these wonderful suggestions which will be addressed in our future study, as the editor has told us that these experiments will not be required for the publication of the current manuscript. We have added a sentence in the discussion section regarding this: "More sensitive methods will be needed to reveal a potential temporal binding of NIPPED-A to its targets."
Typo,
Sgf1l (1L) at page11 line14 should read Sgf11 (eleven).
We have fixed this.

Referee #2:
The authors have significantly improved their manuscript by adding new experiments and controls. The behavioral effect clearly shows that NippedA is involved in the fly circadian oscillator. Although the molecular mechanisms underlying NippedA clock function remain very partially characterized, the new experiments reinforce the main conclusion that tim is likely the first target of NippedA. I believe that the work adds a significant step in understanding how chromatin modifiers control circadian transcription and I would only recommend small changes to this new version.
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tim0 heterozyous show no effect of NippedA downregulation but tim0/+ do? Please correct.
We apologize for this confusion. We have now deleted "or tim^{01}/+" in this sentence.
p13: Unfortunately, due to lack of CRY antibody, we are not able to examine whether there is any alteration of CRY protein.
Surprising. Several anti-CRY antibodies have been described in the literature.
We apologize for not making this clear. There is no working CRY antibody that is commercially available, and we were not able to obtain the working CRY antibodies generated by individual labs. We have changed this sentence to: "Unfortunately, we were not able to obtain a working CRY antibody to examine whether there is any alteration of CRY protein."
- The Ptim and FlySAM-Nipped A constructs should be precisely described (exact sequence information) except if they have been already published.
Ptim has been published and we have referenced this in the text (McDonald, Rosbash et al., 2001). We have added more detailed information in the methods section regarding how FlySAM-Nipped-A was generated: "UASflySAM2.0-Nipped-A is generated by Tsinghua Fly Center following previously published methods (Jia et al., 2018). sgRNA (GCAGTAAACATGCAAATAAG) targeting upstream sequence of Nipped-A was cloned into flySAM2.0 vector. The construct was then injected into y sc v nanos-integrase;attP40 embryos."

Referee #3:
I am happy to see that the authors have performed the suggested control and validation experiments to further improve the rigor of their results and conclusions. This manuscript is a significant body of work and should be of interest to the circadian biology community and the gene regulation community at large.
Minor comment:
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We thank this reviewer for the suggestion. We have modified the relevant discussion and removed the part regarding protein turnover: "…This implicates that knocking down Nipped-A during development exerts influence on adult period, suggesting a role for NIPPED-A in modulating the development of the clock."

3rd Editorial Decision 28th Aug 2019

Thank you for incorporating the requested changes in the final manuscript. I am now pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Luoying Zhang
Journal Submitted to: THE EMBO Journal
Manuscript Number: EMBOJ-2018-101259

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen based on previous experience, experiment type, and on anticipated variation from studies using related methods.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was chosen based on previous experience, experiment type, and on anticipated variation from studies using related methods.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample or animal was excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization was used.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was done.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The range of variability and the standard error were comparable among groups.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Citation and/or catalog number have been provided for all antibodies used in this study in Appendix Table S12.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No, it does not.