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## Structure of the Frequency-Interacting RNA Helicase: a protein interaction hub for the circadian clock

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Editor: David del Alamo

### 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

24 March 2016

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Thank you for the submission of your research manuscript "Structure of the Frequency-Interacting RNA Helicase: a protein interaction hub for the circadian clock". I have evaluated your study according to the scope of The EMBO Journal and I regret to say that the outcome is not a positive one.

I certainly appreciate that your study provides the crystal structure of FRH both in the apo form and bound to ADP and RNA and particularly that you identify novel key residues involved in its role as a circadian clock regulator in *N. crassa*. However, considering that the roles of FRH in the circadian clock are largely known and that similar structures of homologous helicases are already available, I believe that your findings, while highly appealing to the immediate field, lack the wide interest to a greater readership that we pursue in a general molecular biology title such as The EMBO Journal and I believe that your manuscript would be better suited to a more structural biology oriented venue. We will therefore not be able to further proceed with the publication process.

Please note that this decision is by no means a reflection of the quality of your study, but rather a question of scope. I regret to have to disappoint you this time and I truly hope for the rapid publication of your manuscript somewhere else.

We appreciate your careful consideration our manuscript. While I understand the points you make, I firmly believe that there are important aspects of the study's significance that were overlooked or perhaps not fully conveyed. Although, as you note, the structures of related helicases are known, our study is emphatically not about helicase activity. Exactly the same point could have been made about structural work on Cryptochromes whose role in circadian clocks was described in much greater detail than for FRH, and yet the structural work on CRY has been cited 47 times in short time since its publication. The novelty we report comes from the understanding of how a house-keeping helicase has been co-opted into an essential component of the circadian clock.

The interest lies in the comparison of FRH to other helicases, specifically Mtr4; and it has not escaped our attention that an RNA helicase co-purifies with PER in mammalian cells (Padmanabhan K et al Science 2012). The structural elements of FRH that impart clock function are unique and have not been structurally defined previously. Also, although FRH has certainly been recognized as an important component of the circadian oscillator, its role has been controversial. We help resolve this debate, but also contribute new information about FRH function. We support the notion that ATPase activity is not essential for the clock, provide a structural basis for the chaperone activity of FRH toward FRQ; but most importantly, we demonstrate that the FRH KOW domain directly mediates interactions between the white-collar transcriptional activator and FRQ. That is, FRH is not just a passive stabilizer of FRQ, it is a critical component of the repression mechanism. This function derives from unique elements of the KOW domain and is not present in the other helicases. Through structure-guided mutagenesis, we go on to discover a short-period mutant of the clock. Mutations that cause period changes are not common and those that generate short periods are rare. Again, these findings suggest that the interactions made by FRH are critical for establishing circadian timing.

Taken together we believe that these are significant advances in cementing FRH within the central mechanism of the clock, and will be appreciated by a large audience that includes those interested in structure, molecular evolution, circadian biology, chaperone function, gene regulation and helicase enzymology. We emphasize that the study of the circadian clock is not a small field, and there are exceedingly few structures of essential clock components, indeed only two of quasi-full length proteins (CRY and the FRH structure reported here).

Finally, I note that it is not uncommon for EMBO J to publish structures closely related to others that are known, provided that the new structures contribute to novel biological understanding. A case in point would be the article in the current issue on complement C3b complexes (Forneris et al. (2016) EMBO J. current online issue). Here, structures of complement complexes are presented, but they are all quite closely related to a previously determined structure (C3b-FH). Nevertheless, the new data provide important insight into complement regulation and it is the differences among the various structures that are perhaps the most valuable in this respect. Our paper on FRH falls within similar bounds, although unlike the C3b study, we also provide biological data to support the structure and expand its implications.

Perhaps, you have considered these points, but if not, I submit that our study truly has a scope of importance that is worthy of EMBO J. I believe that if you allow the paper to be reviewed, the referees will concur.

Thank you for the submission of your manuscript entitled "Structure of the Frequency-Interacting RNA Helicase: a protein interaction hub for the circadian clock" and for your patience during the review process. We have now received and analyzed the reports from the referees, which I copy below.

As you can see from their comments, both referee #1 and #3 are very supportive of your work, while referee #2 is not convinced that the manuscript should be published in The EMBO Journal, mostly due to novelty concerns. In any case, all three referees point out to a number of concerns that will

require your attention before your manuscript can be published in The EMBO Journal. I will not repeat here the referee concerns, which I believe are rather straightforward and mainly refer to clarifications and issues related to data presentation. I believe the concerns of the referees are reasonable and addressable, but please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points.

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## REFEREE REPORTS

### Referee #1:

Frequency-interacting RNA helicase (FRH) organises a complex consisting of Frequency (FRQ) and Casein kinase 1a that is involved in the circadian system of *Neurospora*. The understanding of the role of this helicase is difficult because there are other biological functions of this helicase as well. Here, the authors gained new insights in the organisation and function of this complex. The method of choice was structure determination of FRH and direct comparison with the related yeast helicases Mtr4 and Ski2. The relevant interaction sites were located to the N-terminus of FRH and the so-called KOW domain by structure comparison and complementation experiments in *Neurospora*. Indeed, mutations devoid of the ability to interact with FRQ and WCC1 and 2 became arrhythmic. Hence, there is an excellent agreement between the structure predictions and the biological function of FRH.

The data appear of good quality (although I can't judge the quality and resolution of the structure) and the interpretation of the results fair and in agreement with published literature. My only minor criticism concerns the abstract, which is difficult to understand for non-specialists. In particular, there is no good differentiation between already known and newly obtained data.

### Referee #2:

#### Summary

The manuscript by Conrad et al describes crystal structures of the Frequency-Interacting RNA Helicase (FRH), which plays an essential role in the *Neurospora* circadian clock. Previous publications showed that ATPase and helicase activity is not required for FRH clock function. Instead, FRH provides an interaction platform for the clock protein Frequency (FRQ), the clock transcription factors WC1 and WC2 forming the White-Collar complex (WCC), the photoreceptor Vivid and CK1a. FRH is a DEAD-box RNA helicase that belongs to the SF2-family of RNA-helicases with significant homology to Mtr4 (57%) and Ski2 (36%). Crystal structures of Mtr4 and Ski2 are known as well as structures of the TRAMP (Trf4-Air2-Mtr4) and Ski (Ski2-Ski3-Ski8) complex, which regulate exosome activity in the cytosol (Ski) and the nucleus (TRAMP). While distant homologs of Trf4 and Air2 are present in *Neurospora*, a role of FRH in RNA processing has not been reported yet. However, FRH promotes degradation of the *frq* RNA and interacts with exosome components. A FRH mutation (R806H) in the KOW domain, that severely affects FRH clock function (leads to arrhythmicity) but does not affect cell viability, was previously identified in a genetic screen and used to define FRH clock functions. The R806H mutation affected WCC-binding, phosphorylation and activity, but did not affect FRQ-binding (Shi et al, *Genetics* 2010). Instead, N-terminal residues 100 to 150 of FRH bind to residues 695-778 of FRQ (Hurley et al, *Mol Cell* 2013).

In this manuscript, two crystal structures of the N-terminally truncated FRH constructs FRH $\Delta$ 100 and FRH $\Delta$ 114 are reported, one obtained from crystals with a larger unit cell at 3.1 Å resolution and one from crystals with a smaller unit cell (same space group P212121) at 3.2 Å resolution. In addition, a 3.8 Å resolution structure of a complex of FRH $\Delta$ 100 with ADP and RNA is reported. The structures are validated by the analysis of mutants for their effect on circadian conidiation (race tube assay to determine circadian rhythmicity and period length in *Neurospora*) and FRH interactions with FRQ, WCC1 and WCC2 (co-IP). The FRH structures reveal conformational heterogeneity of the unstructured FRQ-binding N-terminal region (residues 100 to 150), suggesting that FRQ-FRH complex formation leads to a mutual ordering of the interacting FRH and FRQ

regions. The position of the KOW domain varies between the small and large cell FRH-structures due to movements of the arch/elbow domain. SAXS analyses confirm the conformational heterogeneity and the monomeric state of FRH in solution. In the ADP and RNA bound structure, ADP is in a similar position as in the Mtr4 helicase, but interacting residues are out of place. Four modelled rAdenin RNA nucleotides align with the 3'-strand of unwound DNA in the DNA-bound structure of the archael HEL308 helicase.

The structure-based mutational analyses of residues in the vicinity of R806 and in the region 100 - 150 confirms the importance of the KOW and N-terminal domain in FRH clock function and dissects the contributions of these residues/molecule regions to interactions with FRQ, WC1 and WC2 as well as to circadian rhythmicity. In particular, mutation of Val142 (N-terminal region), which contacts the ADP-binding region, leads to a shortened period (18.7 h) and affects binding of FRQ and WCC. In the KOW domain, the previously reported R806H mutation and structure-based mutations of the adjacent R712 (R712A or R713A ?) and K811 (K811A) lead to arrhythmicity and differentially affect WCC and FRQ interactions. Interestingly, the K811A mutation affects binding to WC2 more strongly than binding to WC1 and FRQ. The adjacent K766A mutation only moderately affects circadian clock function (20 h period) and binding to WC2. The FRH structure shows, that K766, R806, R712 and K811 are part of a positively charged surface patch in the KOW domain. In Mtr4, residues corresponding to R806 and R712 mediate interactions with Air2.

The presented FRH structures and their validation by mutant analyses provide some interesting new insights into the role of FRH in the circadian clock. However, compared to what could already be derived from the known structures of the Mtr4 and Ski2 helicases and previous mutational analyses, the new data and insights presented in this manuscript seem more appropriate for publication in a more specialized journal. For potential publication in EMBO journal, I would want to see a more reliable substrate (ADP/RNA) bound FRH structure and/or biochemical data on RNA helicase and RNA binding activity of FRH, which justify more conclusions on the FRH enzymatic mechanism and its potential role in RNA processing and clock function. Also, the presentation of the structures (figure labelling, description, accuracy, correctness) should be improved. As it is, the description of the FRH structure is difficult to follow and in some points inconsistent and faulty (see point by point discussion below). Labelling of the domains and some secondary structure elements in the figures would be very helpful for the reader to follow the detailed description of the FRH structures. For the domain descriptions, a more consistent nomenclature would also be helpful. The language and length of the manuscript are mostly ok.

Issues point-by-point:

Introduction:

The introduction is generally ok.

However, the description of the FRH-domains on p. 3, paragraph starting with "FRH belongs to the DEAD-like ..." is difficult to understand. It would help to use a domain nomenclature that is more consistent with figure 1B and refer to this domain schematic.

Results:

Inconsistent domain nomenclature and poor figure labelling and figure referencing is a reoccurring issue in this manuscript that severely affects the clarity of the results presentation, in particular the description of the FRH structures. E.g. "arch domain", "helix-arch domain", "winged helical domain", "elbow", "helicase core", "helicase domain", "pro arch", "DExH core" should be used in accordance with the referenced figures.

p. 4, paragraph "the DExH core domain": the authors do not refer to any figure here. Might move the description of the RecA domain motives into the introduction, if there is nothing special to add here for FRH.

p.5, 2nd paragraph, RecA domains: some of the secondary structure elements that are referred to as "connected" are quite unconnected according to Figure S1. Also  $\beta$ 20 is behind  $\alpha$ 20.

p. 5 KOW motif/domain description: here also a figure with more labeled secondary structure elements would help understanding, e.g. in Fig. 4.

p.6 , end of first paragraph: may refer to Figure 6 for positive patch.

p.6, substrate paragraph: inconstant RNA-oligo, see below. A more precise description of what can

be seen from the ligands (e.g. modelled RNA with 4 Adenin nucleotides) would be good. Also, a reasoning, why co-crystallization was done with a single stranded RNA (polyA or mixed sequence depending on which is correct) would be good (e.g. prior knowledge from Mtr4 and Ski2 helicase literature). The fact that the ATP-binding loop (170-187) and F179 are displaced from ADP (the authors mention crystal contacts as possible reason) and resemble more the Mtr4 apo structure, could also be due to incomplete binding of ADP. The difference density for ADP and RNA is not very good, the resolution not very high (3.8 Å) and the B-factor for the ligands is very high (192) (see "experimental" below).

The conclusion about the RNA-substrate position being close to the KOW domain in FRH is drawn from an overlay of 4 RNA nucleotides built into interrupted density with some nucleotides of the DNA substrate bound to the archaeal HEL308 SF2 Helicase. Based on the presented data, this conclusion does not seem justified. It requires better data/better quality electron density for the FRH-bound RNA.

p.8, upper paragraph: I would not necessarily call the V142G exchange "conservative". Also, on p.11 (Discussion) the authors say that the mutation is V142A instead of V142G. Which is correct? p. 9 and Figures 6 and 7: there also is a numbering discrepancy for residue Arg172/Arg173. The authors should consistently call this arginine either Arg172 or Arg173.

#### Experimental:

##### Co-crystallization with RNA-oligo:

First of all there is a discrepancy in the manuscript regarding the cocrystallized RNA oligo. In the results section (p. 4 line 3 and p.6 paragraph "substrate recognition" the authors claim that they used a 13-nt polyA (A13) RNA. In the Mat & Meth section (p. 19) the authors say, that they used a mixed 13mer with the sequence 5'-rGAUCCGCUCGAUC-3'. The authors should clarify, which RNA oligo was used in the crystals leading to the presented ADP-RNA-cocrystal structure and also explain this choice and its functional implications.

The 3.8 Å resolution and quality of the difference electron density (2sigma mFo-DFc) shown in Fig. 5a is not sufficient to derive which base sequence was used. Also the authors should describe better in either the main text or at least in the Figure legend of Fig. 5a, how much of the co-crystallized RNA oligo they modelled in (it is now modelled as 4 rAdenins based on quite interrupted difference density). Showing some 2mFo-DFc density in the surrounding of the RNA and ADP binding sites in addition to the mFo-DFc density will also help to appreciate the significance of the ligand density (this can also be included as a supplementary figure). Also, the ligand B-factor is very high (192, table S1).

##### Large and small cell structures, discrepancy:

there is a discrepancy regarding the correlation between "large and small cell" and "FRHdelta100 and FRHdelta114". In the Mat & Meth section (p. 19) the authors say "both the large and small cell structure reported in absence of cofactors were obtained from FRHdelta114". This is also implied by STable1. In contrast, in the results section (p.4 1st paragraph) the authors refer to the FRHdelta100 (3.2 Å resolution) structure as the small cell structure and the FRH114 structure (3.1 Å resolution) is associated with the larger cell. The authors should clarify, which is correct.

#### Mat & Methods:

Expression and purification: Some critical parameters are missing. Which plasmid was used for expression? Was it an N- or C-terminal His6-Tag? At which pH were Ni-NTA affinity, size exclusion and anion exchange chromatography performed?

Crystallization: A statement about the 2 cell sizes including the correct correlation between cell size and crystallized construct should be included. Also, the space group and the number of molecules per asymmetric unit should be mentioned here, because the authors describe symmetry contacts in the text and mention a monomer-dimer issue for FRH.

RNA-sequence: differs from main text (see above).

p. 20, lane 12: Figures 8 and S9 are referred to for SAXS: Figure 8 does not exist, S9 is something different. The authors probably mean Fig. 2b and S3.

Table S1, data statistics: In all three structures, the data quality in the highest resolution shells is marginal ( $R_{\text{merge}} > 100\%$ ,  $I/\sigma I < 2$ ). Based on these numbers, a more conservative resolution cutoff seems more appropriate. Please comment.

References:

Reference 17 and 19 is the same.

Figures:

Generally, better labelling of domains and secondary structure elements in the structure figures would help. Also, in some composite figures the assignment to sub-panels is not very clear.

Fig. 1A: Which structure is shown: large or small cell structure/FRHdelta100 or FRHdelta114? The current figure legend of 1A should be moved into the main text, e.g. to the introduction or discussion. Instead, the legend should say, which structure is presented and the domains may be mentioned.

Figure 1 would also benefit from labelling of domains in accordance with the text. E.g. in the introduction p. 3 the authors describe the "DEXH/Dc or HELICc helicase superfamily domain" and the "rRNA pro-arch domain", which are not labelled or mentioned in Fig. 1. Correlating this nomenclature e.g. with RecA-1, RecA-2 or Winged helix/Stalk/KOW areas or labelling the "pro-arch domain" and "DEXH/Dc domain" in Fig. 1 or Fig. 2 would help understanding.

It would also help to label some secondary structure elements (e.g. helices 21,22,25,26 of the stalk) in Fig. 1A in addition to consistent colour coding with fig. 1b and fig S1.

Also, labelling the position for ADP and RNA binding sites in an overview figure would be helpful.

Fig. 2: as Figure 1B, better domain labelling of Fig. 2A would help understanding. The authors claim that the RMSD between the two FRH crystal forms is larger (3.9 Å) than between FRH and Mtr4 (2.9 Å). They do not say which FRH crystal form is compared to Mtr4 (small or large cell)?

Fig. 2B: Which FRH construct is shown for SAXS? The figure legend of Fig. S3 implies that Fig. 2B shows the FRHdelta114 construct, but nothing is said in the Fig. 2B or its legend. Also, please explain the plot. E.g. say as in Figure legend of Fig. S3, that the reader is looking at a "FoxS analysis fit (red line) of FRHdelta114 data (empty circles)".

Fig. 3: Which crystal form of FRH is shown? On page 4 the authors say that the N-terminus makes crystal contacts that differ between the two FRH crystal forms and refer to Fig. 3. Fig. 3 shows only one FRH crystal form.

Fig. 4: labelling of more secondary structure elements of the RecA domains and KOW domain would be extremely helpful to reconcile the discussion on p. 5.

Fig. 6: Which structure is shown, small or large cell, FRHdelta100 or FRHdelta114? The discussed KOW and Arch domain movement observed between structures obtained from crystals with small and large unit cells (Fig. 2) (correlation with FRHΔ100 and FRHΔ114?) may affect the positioning of the mutated KOW positive residues. Also, the mutated positive residues could be highlighted in the electrostatic surface potential presentation. The authors could also discuss the different location of R806 and R712 (top) compared to K811 and K766 (more to one side), which may explain the stronger binding defect of K811 and K766 towards WC2 than towards WC-1. A close-up view of the surrounding of V142 may also help to better understand the effect of this mutation.

Fig. S3, SAXS: Showing the 2 constructs in one blot is a bit hard to grasp, but ok. It would help, if the authors would at least say in the figure legend, that "FRH1,2,3" corresponds to FRHdelta114 and "FRH7a,b,c" corresponds to FRHdelta110.

Figure S7: It would be good to adjust the residue numbering in the plots to the real sequence numbering of FRH and FRQ.

Referee #3:

Neurospora FRH, a homolog of yeast Mtr4, is required for circadian rhythmicity, and has ATPase and helicase functions that are essential for cell viability. A controversy in the literature exists as to whether or not the ATPase and helicase activities of FRH are needed for clock function. To distinguish these possibilities, and to get a better understanding of how FRH operates in the clock, the authors combine structural and mutational analysis of FRH to show conclusively that the helicase function of FRH is not necessary for its role in the molecular oscillator. Instead, they find that amino acids in the KOW and N-terminal domains of FRH mediate interactions between FRH and the clock components FRQ, WC-1 and WC-2, and show through mutant studies, that these dynamic interactions are necessary for proper running of the clock. As such, this study provides foundational structural studies of FRH, which in turn led to significant new information on the role of FRH in interactions between the clock components. Overall, the experiments are well done and described. I have a few questions and comments that require clarification.

Major questions/comments:

1. In the abstract, and in other places in the manuscript (bottom of page 6 and suppl Figure 2), it is stated that the FRH N-terminus is highly variable in structure in the absence of FRQ. However, unless I am missing some key information, this is only speculation based on the variable N-terminal structure in the large and small cell structures of the large and small cell structures, and previous data from the authors showing that FRQ binds to this region (aa 100-150) of FRH. What experimental data supports that binding of FRQ stabilizes the structure to a particular confirmation?
2. What is the rationale for using the 100 and 114 N-terminal amino acid deletion constructs for the structural analysis, particularly with data indicating that FRQ binds to this region? Could the lack of the N-terminal amino acids be responsible for the flexibility of the N-terminus in the deletions? Also, can you rule out that the N-terminus is not necessary for dimerization of FRH in solution?
3. The authors should include statistical analysis of the data in figure 7B

Minor questions/comments:

3. The period of the clock runs faster in the V142G mutant. It was speculated in the discussion that this may be due to changes in phosphorylation/stability of FRQ (decreased stability for a faster clock?), which would be due to a change in the interaction between FRH and FRQ. Would changes in FRQ phosphorylation/stability also be predicted to lead to weaker interactions between FRH and the WCC (Fig 7). An alternative explanation may be that the weaker interactions of FRH with the WCC and FRQ may render WCC more active in the mutant, thus increasing the rate of the oscillator. Note also in the abstract, it states that the V142A mutant alters FRQ binding to FRH, but it would be more consistent to indicate that the mutation alters binding to WCC as well.
4. In the last sentence of the first paragraph of the results on page 4, "with respect to the DExH core" - this is not represented in Figure 2. Should this instead say DExH core of the DSHCT? Also, sometimes DExH is DEXH in the manuscript.
5. In Figure 3, it would be helpful to use A, B, C, in the figure and legend.
6. On page 9 top, it is stated that R712 contributes to the positively charged surface of the KOW, but R713 was mutated and assayed for changes in the rhythmicity and clock protein interactions. What was the rationale for using 713 and not 712?
7. It is interesting that the interactions of K766A FRH to at least WC-1 is increased, but this appears to have no affect on period. Is this increased interaction statistically significant, and if so, is their any explanation that could be provided to help understand the data? See point 3 above.
8. Materials and Methods page 20. "Race tube assays were performed as described with sight modification" - the modi

*We thank the reviewers for their thoughtful critiques. Below, we describe the modifications we have made to the paper to address their concerns.*

Referee #1:

Frequency-interacting RNA helicase (FRH) organises a complex consisting of Frequency (FRQ) and Casein kinase 1a that is involved in the circadian system of *Neurospora*. The understanding of the role of this helicase is difficult because there are other biological functions of this helicase as well. Here, the authors gained new insights in the organisation and function of this complex. The method of choice was structure determination of FRH and direct comparison with the related yeast helicases Mtr4 and Ski2. The relevant interaction sites were located to the N-terminus of FRH and the so-called KOW domain by structure comparison and complementation experiments in *Neurospora*. Indeed, mutations devoid of the ability to interact with FRQ and WCC1 and 2 became arrhythmic. Hence, there is an excellent agreement between the structure predictions and the biological function of FRH.

The data appear of good quality (although I can't judge the quality and resolution of the structure) and the interpretation of the results fair and in agreement with published literature. My only minor criticism concerns the abstract, which is difficult to understand for non-specialists. In particular, there is no good differentiation between already known and newly obtained data.

*As recommended we have reworded the abstract to better distinguish and highlight the new information provided by this study.*

Referee #2:

Summary

The manuscript by Conrad et al describes crystal structures of the Frequency-Interacting RNA Helicase (FRH), which plays an essential role in the *Neurospora* circadian clock. Previous publications showed that ATPase and helicase activity is not required for FRH clock function. Instead, FRH provides an interaction platform for the clock protein Frequency (FRQ), the clock transcription factors WC1 and WC2 forming the White-Collar complex (WCC), the photoreceptor Vivid and CK1a. FRH is a DEAD-box RNA helicase that belongs to the SF2-family of RNA-helicases with significant homology to Mtr4 (57%) and Ski2 (36%). Crystal structures of Mtr4 and Ski2 are known as well as structures of the TRAMP (Trf4-Air2-Mtr4) and Ski (Ski2-Ski3-Ski8) complex, which regulate exosome activity in the cytosol (Ski) and the nucleus (TRAMP). While distant homologs of Trf4 and Air2 are present in *Neurospora*, a role of FRH in RNA processing has not been reported yet. However, FRH promotes degradation of the *frq* RNA and interacts with exosome components. A FRH mutation (R806H) in the KOW domain, that severely affects FRH clock function (leads to arrhythmicity) but does not affect cell viability, was previously identified in a genetic screen and used to define FRH clock functions. The R806H mutation affected WCC-binding, phosphorylation and activity, but did not affect FRQ-binding (Shi et al, *Genetics* 2010). Instead, N-terminal residues 100 to 150 of FRH bind to residues 695-778 of FRQ (Hurley et al, *Mol Cell* 2013).

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SAXS analyses confirm the conformational heterogeneity and the monomeric state of FRH in solution. In the ADP and RNA bound structure, ADP is in a similar position as in the Mtr4 helicase, but interacting residues are out of place. Four modelled rAdenin RNA nucleotides align with the 3'-strand of unwound DNA in the DNA-bound structure of the archael HEL308 helicase.

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The presented FRH structures and their validation by mutant analyses provide some interesting new insights into the role of FRH in the circadian clock. However, compared to what could already be derived from the known structures of the Mtr4 and Ski2 helicases and previous mutational analyses, the new data and insights presented in this manuscript seem more appropriate for publication in a more specialized journal. For potential publication in EMBO journal, I would want to see a more reliable substrate (ADP/RNA) bound FRH structure and/or biochemical data on RNA helicase and RNA binding activity of FRH, which justify more conclusions on the FRH enzymatic mechanism and its potential role in RNA processing and clock function. Also, the presentation of the structures (figure labelling, description, accuracy, correctness) should be improved. As it is, the description of the FRH structure is difficult to follow and in some points inconsistent and faulty (see point by point discussion below). Labelling of the domains and some secondary structure elements in the figures would be very helpful for the reader to follow the detailed description of the FRH structures. For the domain descriptions, a more consistent nomenclature would also be helpful. The language and length of the manuscript are mostly ok.

*We acknowledge the reviewer's opinion, but we respectively disagree with their assessment of the impact of our study. The novelty we report comes from the understanding of how a house-keeping helicase has been co-opted into an essential component of the circadian clock. The interest lies in the comparison of FRH to other helicases, specifically Mtr4, but the critical findings are unrelated to helicase activity. The structural elements of FRH that impart clock function are unique and have not been structurally defined previously. Also, although FRH has certainly been recognized as an important component of the circadian oscillator, its role has been controversial. We help resolve this debate, but also contribute new information about FRH function.*

*The FRH crystal structure was extremely challenging to obtain, with years of work resulting in only a few crystals diffracting to even moderate resolution. This was especially true for the RNA complex. Thus, the probability of obtaining a higher resolution RNA structure on a reasonably short time frame is not high. We believe that holding back the current results is not in the best interests of the field. Furthermore, the RNA binding and helicase activity of the protein are not the focus of this study. It has been clearly shown that FRH helicase activity is not essential for clock function, and as such, we focus on aspects of FRH function that are distinct for the clock.*

*We appreciate the concerns about domain nomenclature, and have improved this aspect of the paper. Part of the challenge in definition is that the field has not been entirely consistent in terms of how the various elements of these helicases are referenced, and there are several synonyms and subclasses for the various domains. Nonetheless, as described below, we have made modifications to make the designations more clear.*

*We have also updated several of the Figures following the specific recommendations given.*

Issues point-by-point:

Introduction:

The introduction is generally ok.

However, the description of the FRH-domains on p. 3, paragraph starting with "FRH belongs to the DEAD-like ..." is difficult to understand. It would help to use a domain nomenclature that is more consistent with figure 1B and refer to this domain schematic.

*We have rewritten the sentence to include the RecA nomenclature as below and have also added Pfam domain references:*

*"The protein comprises several domains (Fig 1B): 1) the characteristic DExH/Dc or HELICc helicase superfamily domains (Pfam:PF00270) of RecA-like modules 1 and 2 that harbor the nucleotide and ATP binding sites; 2) the DSHCT C-terminal domain found in Dob1/Ski2/hely-like DEAD box helicases (PF08148); 3) the arch domain required for 5.8S rRNA processing by Mtr4 (PF13234) that itself contains the helical stalk, elbow and the KOW (Kyrpides, Ouzounis and Woese) modules (Jackson et al, 2010; Weir et al, 2010); and 4) a winged-helix domain (PF00633) that connects the arch to the helicase + DSHCT core."*

Results:

Inconsistent domain nomenclature and poor figure labelling and figure referencing is a reoccurring issue in this manuscript that severely affects the clarity of the results presentation, in particular the description of the FRH structures. E.g. "arch domain", "helix-arch domain", "winged helical domain", "elbow", "helicase core", "helicase domain", "pro arch", "DExH core" should be used in accordance with the referenced figures.

*Many of these terms are synonymous designations that have been used by others; however, we appreciate that the large number of assignments can be confusing and we have simplified them or better specified alternative referrals throughout the paper. Helicase domain is a functional term of which the DExH designation is a subclass. RecA-1 and RecA-2 together compose the DExH helicase core. We refer now to the "arch domain" as containing the helical stalk, elbow and KOW modules and relate this to the alternative definition of "arm" and "fist".*

*e.g. on p. 4 "The arch domain extends up into an "arm and fist" structure, with the helical stalks forming the "arm", an "elbow" at the turn between helices and the KOW region as the "fist",..."*

p. 4, paragraph "the DExH core domain": the authors do not refer to any figure here. Might move the description of the RecA domain motives into the introduction, if there is nothing special to add here for FRH.

*We have made reference to Fig 4 and Fig S3 in this paragraph. The structure of the FRH helicase core is a result of the study, thus we prefer leave this description in the Results section.*

p.5, 2nd paragraph, RecA domains: some of the secondary structure elements that are referred to as "connected" are quite unconnected according to Figure S1. Also  $\beta$ 20 is behind  $\alpha$ 20.

*There is only one discontinuity in structure as we noted at the bottom of page 5.*

*"Overall, the FRH polypeptide is continuous with the exception of an undefined surface loop in the RecA-2 domain between  $\alpha$ 9 and  $\alpha$ 10. The ~30 residue missing loop contains ten lysine residues amongst a sequence dominated by flexible and polar side chains."*

p. 5 KOW motif/domain description: here also a figure with more labeled secondary structure elements would help understanding, e.g. in Fig. 4.

*We have increased the labeling of Fig. 4 and also added a close-up of the KOW module as Fig. 6E.*

p.6 , end of first paragraph: may refer to Figure 6 for positive patch.

*Added citation to Fig 6 as requested.*

p.6, substrate paragraph: inconstant RNA-oligo, see below. A more precise description of what can be seen from the ligands (e.g. modelled RNA with 4 Adenin nucleotides) would be good. Also, a reasoning, why co-crystallization was done with a single stranded RNA (polyA or mixed sequence depending on which is correct) would be good (e.g. prior knowledge from Mtr4 and Ski2 helicase literature). The fact that the ATP-binding loop (170-187) and F179 are displaced from ADP (the

authors mention crystal contacts as possible reason) and resemble more the Mtr4 apo structure, could also be due to incomplete binding of ADP. The difference density for ADP and RNA is not very good, the resolution not very high (3.8 Å) and the B-factor for the ligands is very high (192) (see "experimental" below).

*We agree that low occupancy of the ADP could contribute to the open binding configuration. We have modified the sentence to read. "The reason for these differences is unclear, but may stem from incomplete occupancy of ADP or crystal contacts made by the 170-187 loop to other FRH molecules." With respect to the B-factors, the values are indeed high, but they are also difficult to estimate at such low resolution. We added a panel (Fig 5B) that shows 2Fo-Fc electron density for both substrates and a surrounding region of the protein to give a better sense for the relative strength of the respective electron density. (The substrates do not contribute to the model in the 2FoFc calculation).*

The conclusion about the RNA-substrate position being close to the KOW domain in FRH is drawn from an overlay of 4 RNA nucleotides built into interrupted density with some nucleotides of the DNA substrate bound to the archaeal HEL308 SF2 Helicase. Based on the presented data, this conclusion does not seem justified. It requires better data/better quality electron density for the FRH-bound RNA.

*Our data shows that single stranded RNA binds in the analogous position as the single stranded DNA of the unwinding complex of HEL308. This binding mode is consistent with what is expected based on the homology and superposition of the respective RecA domains. Furthermore, the unwound duplex structure is also compatible with this superposition. While this is only a model, we believe that the reader will appreciate the comparison as it provides a reasonable framework to understand how FRH may interact with duplex RNA. The superposition shows that a dramatic conformational change is not necessary for substrate to bind FRH in the expected manner. We believe that this is a point worth making; however, to de-emphasize the issue we now only show one such figure in the main text (Fig 5D) and have placed the superposition in the Appendix (Fig S5C).*

p.8, upper paragraph: I would not necessarily call the V142G exchange "conservative". Also, on p.11 (Discussion) the authors say that the mutation is V142A instead of V142G. Which is correct? p. 9 and Figures 6 and 7: there also is a numbering discrepancy for residue Arg172/Arg173. The authors should consistently call this arginine either Arg172 or Arg173.

*We apologize for the numbering discrepancies they have been corrected (We believe the reviewer refers to Arg712, not 172). The V142G sentence was changed to read: "Thus, the relatively minor V142G substitution recapitulates previous deletion analysis that identified the 100-150 segment as an important determinant for FRQ binding." Certainly this substitution is minor compared to removing 50 residues.*

Experimental:

Co-crystallization with RNA-oligo:

First of all there is a discrepancy in the manuscript regarding the cocrystallized RNA oligo. In the results section (p. 4 line 3 and p.6 paragraph "substrate recognition" the authors claim that they used a 13-nt polyA (A13) RNA. In the Mat & Meth section (p. 19) the authors say, that they used a mixed 13mer with the sequence 5'-rGAUCCGCUCGAUC-3'. The authors should clarify, which RNA oligo was used in the crystals leading to the presented ADP-RNA-cocrystal structure and also explain this choice and its functional implications.

*We apologize for the confusion. In fact, crystallization of FRH was screened with many different RNA nucleotides, the RNA sequence given in the methods section was the one that gave crystals with the highest resolution diffraction. However, the sequence in the structure was modeled as poly-A because at this resolution we cannot distinguish the bases and did not want to imply a definitive sequence placement. We have corrected the results section on p.7, added more information there and provided details on forming the co-crystals and refining the structure in the Materials and Methods.*

The 3.8 Å resolution and quality of the difference electron density ( $2\sigma$  mFo-DFc) shown in Fig. 5a is not sufficient to derive which base sequence was used. Also the authors should describe better in either the main text or at least in the Figure legend of Fig. 5a, how much of the co-crystallized RNA oligo they modelled in (it is now modelled as 4 rAdenins based on quite interrupted difference density). Showing some  $2mFo-DFc$  density in the surrounding of the RNA and ADP binding sites in addition to the mFo-DFc density will also help to appreciate the significance of the ligand density (this can also be included as a supplementary figure). Also, the ligand B-factor is very high (192, table S1).

*We have added the requested electron density map as Fig. 5B. See the response to a similar point about the ADP ligand above.*

Large and small cell structures, discrepancy:

there is a discrepancy regarding the correlation between "large and small cell" and "FRHdelta100 and FRHdelta114". In the Mat & Meth section (p. 19) the authors say "both the large and small cell structure reported in absence of cofactors were obtained from FRHdelta114". This is also implied by STable1. In contrast, in the results section (p.4 1st paragraph) the authors refer to the FRHdelta100 (3.2 Å resolution) structure as the small cell structure and the FRH114 structure (3.1 Å resolution) is associated with the larger cell. The authors should clarify, which is correct.

*FRHD114 was crystallized in both cell sizes. FRHD100 was crystallized with RNA/ADP in the smaller cell size. (FRHD100 was also crystallized in the small cell size in the absence of the RNA/ADP, but those crystals gave weaker, 3.28 Å resolution diffraction data). We have been careful to designate in the text and figure legends which structures pertain to which statements.*

Mat & Methods:

Expression and purification: Some critical parameters are missing. Which plasmid was used for expression? Was it an N- or C-terminal His6-Tag? At which pH were Ni-NTA affinity, size exclusion and anion exchange chromatography performed?

*These details were added into the methods section: pET28a vector, N-terminal His6-Tag, pH 7.6.*

Crystallization: A statement about the 2 cell sizes including the correct correlation between cell size and crystallized construct should be included. Also, the space group and the number of molecules per asymmetric unit should be mentioned here, because the authors describe symmetry contacts in the text and mention a monomer-dimer issue for FRH.

*The FRHD114 was crystallized in both cell sizes. The FRHD100 crystallized with RNA/ADP in the smaller cell size. The space group was P212121 and there is one molecule per asymmetric unit in both crystal forms. This information and the difference in unit cell sizes are now reported in the methods section, as requested.*

RNA-sequence: differs from main text (see above).

*Corrected, see response above.*

p. 20, lane 12: Figures 8 and S9 are referred to for SAXS: Figure 8 does not exist, S9 is something different. The authors probably mean Fig. 2b and S3.

*Thank you for the correction. Indeed, Fig. 8 should have been Fig. 2B, but Fig. S9 was correct.*

Table S1, data statistics: In all three structures, the data quality in the highest resolution shells is marginal ( $R_{\text{merge}} > 100\%$ ,  $I/\sigma I < 2$ ). Based on these numbers, a more conservative resolution cutoff seems more appropriate. Please comment.

*Resolution limits were determined by application of the CCP4 scaling program AIMLESS. Furthermore, the  $CC1/2$  values are all above 0.500 for the highest resolution shells quoted. Thus, there is signal in these data sets to the stated resolution limits, despite the admittedly high  $R_{\text{merge}}$  and low  $I/\sigma I$  values. We have added the  $CC1/2$  values for each data set to Table S1.*

References:

Reference 17 and 19 is the same.

*Corrected.*

Figures:

Generally, better labelling of domains and secondary structure elements in the structure figures would help. Also, in some composite figures the assignment to sub-panels is not very clear.

*Additional labels were added to Figures, Fig. 1B, 5 and 6 were remade.*

Fig. 1A: Which structure is shown: large or small cell structure/FRHdelta100 or FRHdelta114? The current figure legend of 1A should be moved into the main text, e.g. to the introduction or discussion. Instead, the legend should say, which structure is presented and the domains may be mentioned.

Figure 1 would also benefit from labelling of domains in accordance with the text. E.g. in the introduction p. 3 the authors describe the "DEXH/Dc or HELICc helicase superfamily domain" and the "rRNA pro-arch domain", which are not labelled or mentioned in Fig. 1. Correlating this nomenclature e.g. with RecA-1, RecA-2 or Winged helix/Stalk/KOW areas or labelling the "pro-arch domain" and "DEXH/Dc domain" in Fig. 1 or Fig. 2 would help understanding.

It would also help to label some secondary structure elements (e.g. helices 21,22,25,26 of the stalk) in Fig. 1A in addition to consistent colour coding with fig. 1b and fig S1.

*Labels were added to Fig 1B as requested and we have made the domain nomenclature more consistent throughout the paper, as described above.*

Also, labelling the position for ADP and RNA binding sites in an overview figure would be helpful. Labeled overviews are shown in Fig. 5 and Fig. 6.

*Nomenclature is somewhat problematic as it lacks consistency in previous publications and the structural databases. Nonetheless, we adjusted the designations to reflect groupings more cohesively – the “arch domain” now refers to the elbow, helical stalks, and KOW modules and the DEXH helicase is defined to include two RecA-like domains.. These changes provide clarification and consistency with the previous Mtr4 publications.*

Fig. 2: as Figure 1B, better domain labelling of Fig. 2A would help understanding. The authors claim that the RMSD between the two FRH crystal forms is larger (3.9 Å) than between FRH and Mtr4 (2.9 Å). They do not say which FRH crystal form is compared to Mtr4 (small or large cell)? Fig. 2B: Which FRH construct is shown for SAXS? The figure legend of Fig. S3 implies that Fig. 2B shows the FRHdelta114 construct, but nothing is said in the Fig. 2B or its legend. Also, please explain the plot. E.g. say as in Figure legend of Fig. S3, that the reader is looking at a "FoxS analysis fit (red line) of FRHdelta114 data (empty circles)".

*The FRH structure used throughout for comparison is FRHD114 on account of its slightly higher resolution. This is now stated clearly in each legend.*

Fig. 3: Which crystal form of FRH is shown? On page 4 the authors say that the N-terminus makes crystal contacts that differ between the two FRH crystal forms and refer to Fig. 3. Fig. 3 shows only one FRH crystal form.

*Again, this is the higher resolution FRHD114 structure. This point was clarified by adding the designation to the figure legend. The difference between the conformation of the N-terminus in the two crystal forms is now shown more clearly in new Fig 6C.*

Fig. 4: labelling of more secondary structure elements of the RecA domains and KOW domain would be extremely helpful to reconcile the discussion on p. 5.

*Labels have been added as requested.*

Fig. 6: Which structure is shown, small or large cell, FRHdelta100 or FRHdelta114 ? The discussed KOW and Arch domain movement observed between structures obtained from crystals with small and large unit cells (Fig. 2) (correlation with FRHD100 and FRHD114 ?) may affect the positioning of the mutated KOW positive residues. Also, the mutated positive residues could be highlighted in the electrostatic surface potential presentation. The authors could also discuss the different location of R806 and R712 (top) compared to K811 and K766 (more to one side), which may explain the stronger binding defect of K811 and K766 towards WC2 than towards WC-1. A close-up view of the surrounding of V142 may also help to better understand the effect of this mutation.

*Figure 6 has been remade with two close-ups of the requested regions. Although the position of the KOW is different in the two crystal forms, the structure of the module itself is not. We do not know the KOW positioning in the complex with the WCC and thus either of these FRH conformations could be relevant to that assembly.*

Fig. S3, SAXS: Showing the 2 constructs in one blot is a bit hard to grasp, but ok. It would help, if the authors would at least say in the figure legend, that "FRH1,2,3" corresponds to FRHdelta114 and "FRH7a,b,c" corresponds to FRHdelta110.

*The legend of Fig S9 (SAXS data) was adjusted to label the data more clearly.*

Figure S7: It would be good to adjust the residue numbering in the plots to the real sequence numbering of FRH and FRQ.

*The numbering was adjusted, as requested.*

Referee #3:

Neurospora FRH, a homolog of yeast Mtr4, is required for circadian rhythmicity, and has ATPase and helicase functions that are essential for cell viability. A controversy in the literature exists as to whether or not the ATPase and helicase activities of FRH are needed for clock function. To distinguish these possibilities, and to get a better understanding of how FRH operates in the clock, the authors combine structural and mutational analysis of FRH to show conclusively that the helicase function of FRH is not necessary for its role in the molecular oscillator. Instead, they find that amino acids in the KOW and N-terminal domains of FRH mediate interactions between FRH and the clock components FRQ, WC-1 and WC-2, and show through mutant studies, that these dynamic interactions are necessary for proper running of the clock. As such, this study provides foundational structural studies of FRH, which in turn led to significant new information on the role of FRH in interactions between the clock components. Overall, the experiments are well done and described. I have a few questions and comments that require clarification.

Major questions/comments:

1. In the abstract, and in other places in the manuscript (bottom of page 6 and suppl Figure 2), it is stated that the FRH N-terminus is highly variable in structure in the absence of FRQ. However, unless I am missing some key information, this is only speculation based on the variable N-terminal structure in the large and small cell structures of the large and small cell structures, and previous data from the authors showing that FRQ binds to this region (aa 100-150) of FRH. What experimental data supports that binding of FRQ stabilizes the structure to a particular confirmation?

*The structures provide strong evidence that the N-terminus of FRH does not have defined structure in the absence of FRQ. The conformation of the key 100-150 region is completely different in the two crystal forms and has little defined secondary structure (i.e. is essentially unfolded - Fig 2 and Fig S3). Electron density for these residues is weak and their thermal factors are high, indicating that they are sampling conformational space even within the crystal lattice. This is in keeping with the PONDR sequence predictions of Fig. S8. Furthermore, as Fig. 3 shows, the extended conformation of the N-terminal region is stabilized by non-native interactions to other molecules in the crystal lattice. In absence of these contacts, this region is almost certainly disordered. That said, we do not know the degree to which this region structures upon binding FRQ; but by nature of forming an interaction, it must order to some extent. The mutagenesis data suggests that the*

*interaction is specific and FRQ is substantially stabilized by the interaction (Hurley et al; Mol. Cell 2013).*

*At the top of page 6 we have added "In the absence of these contacts the N-terminus is likely to be highly dynamic."*

2. What is the rationale for using the 100 and 114 N-terminal amino acid deletion constructs for the structural analysis, particularly with data indicating that FRQ binds to this region? Could the lack of the N-terminal amino acids be responsible for the flexibility of the N-terminus in the deletions? Also, can you rule out that the N-terminus is not necessary for dimerization of FRH in solution?

*Unfortunately, the recombinant full-length protein was not well behaved. However, the deletion analyses of Hurley et al (Mol Cell 2013) show that the first 100 residues are not important for clock function; thus it is unlikely that they contribute to forming a critical dimerize.*

*We have added the following sentence to the Methods section:  
"Full-length recombinant FRH was not well behaved."*

3. The authors should include statistical analysis of the data in figure 7B

*This information was added to Figure 7B as requested.*

Minor questions/comments:

3. The period of the clock runs faster in the V142G mutant. It was speculated in the discussion that this may be due to changes in phosphorylation/stability of FRQ (decreased stability for a faster clock?), which would be due to a change in the interaction between FRH and FRQ. Would changes in FRQ phosphorylation/stability also be predicted to lead to weaker interactions between FRH and the WCC (Fig 7). An alternative explanation may be that the weaker interactions of FRH with the WCC and FRQ may render WCC more active in the mutant, thus increasing the rate of the oscillator. Note also in the abstract, it states that the V142A mutant alters FRQ binding to FRH, but it would be more consistent to indicate that the mutation alters binding to WCC as well.

*We thank the reviewer for this insight. Furthermore, it is interesting to note that, like V142G, the KOW mutants also reduce affinity among the components, but do not affect period. We have added the following sentences to the discussion to address these points: "Conversely, the shortened period caused by V142G could derive from a weakened interaction with the WCC that effectively increases WCC activity. However, the KOW variants, which have qualitatively similar effects on FRQ and WCC binding, do not shorten period. Thus, affinity of the clock components for each other is not the only parameter important for proper function."*

*The requested change has also been made in the abstract.*

4. In the last sentence of the first paragraph of the results on page 4, "with respect to the DExH core" - this is not represented in Figure 2. Should this instead say DExH core of the DSHCT? Also, sometimes DExH is DEXH in the manuscript.

*The DExH helicase core comprises the two RecA domains, but not the DSHCT domain. Additional labels have been added to make this designation more clear. On p.6 we do define the helicase core: "The DExH helicase core of FRH (Fig 4 and Appendix Fig. S3) is composed of two RecA domains arranged similarly to those of other RNA helicases...." The sentence in question has been changed to read: "with respect to the DExH helicase core + DSHCT domain by over 20° (Fig 2)."*

5. In Figure 3, it would be helpful to use A, B, C, in the figure and legend.

*Added as requested.*

6. On page 9 top, it is stated that R712 contributes to the positively charged surface of the KOW, but R713 was mutated and assayed for changes in the rhythmicity and clock protein interactions. What was the rationale for using 713 and not 712?

*As described above, this was a mistake, the residue in question is in all cases, Arg712; we have made this correction.*

7. It is interesting that the interactions of K766A FRH to at least WC-1 is increased, but this appears to have no effect on period. Is this increased interaction statistically significant, and if so, is there any explanation that could be provided to help understand the data? See point 3 above.

*Yes, the increased interaction to WC-1 of the K766A substitution is significant, but there does not appear to be an effect on period length, perhaps indicating that a stable interaction between WC-1 and FRH is required up to a certain threshold of affinity. This data also supports the assertion that WC-1 and WC-2 interact with FRQ/FRH at least somewhat independently. We have noted the increased binding of the K766A mutant in the results and added the following sentence to the discussion:*

*“The need for a stable interaction between WC-1 and FRH:FRQ up to a certain threshold of affinity may be reflected in the behavior of the FRH K766A variant, which binds WC-1 more tightly than WT, but does not affect period.”*

8. Materials and Methods page 20. "Race tube assays were performed as described with sight modification"

*We have updated the Materials and Methods section.*

3rd Editorial Decision

23 May 2016

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for your contribution and congratulations on a successful publication.



YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Brian R. Crane

Journal Submitted to: EMBO J

Manuscript Number: EMBOJ-2016-94327

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

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  $\chi^2$  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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

**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 sizes were based on previous work that established the level of replicates required to generate reproducible results. Effects sizes were unknown prior to the experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
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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.	N/A
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Any comment?
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

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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).	Anti-V5 antibody supplied by invitrogen. Anti-FRQ, Anti-WC-1, and Anti-WC-2 as referenced.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Neurospora cell lines supplied by the Fungal Genetics Stock Center. New lines generated in this study were verified by PCR sequencing and western-blotting of gene products.

\* 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.	N/A
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.	N/A

#### 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 accession codes for deposited data. See author guidelines, under '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	Crystallographic coordinates and diffraction data has been deposited in the Protein Data Bank.
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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: <b>Primary Data</b> Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 <b>Referenced Data</b> Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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

23. 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.	N/A
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