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Defining the functional determinants for RNA surveillance by RIG-I

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Transfer Note:

Please note that this manuscript was originally submitted to The EMBO Journal, where it was peer-reviewed. It was then transferred to EMBO reports and the original editor's decision, as well as the referees' comments are shown below.

Original decision and referees' comments – EMBO Journal 30 April 2013

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see below, the referees are quite divergent in their opinions on the suitability of the paper for publication in The EMBO Journal. While referee #1 finds that the advance and insight provided over previous work is not sufficient for publication in the EMBO Journal, referee #2 is much more supportive. Given these two reports, I have also asked for further editorial advice on the paper and reports from a good expert in the field. While the advisor appreciates that the analysis contains interesting information, s/he also finds that the novel biological insight provided and the

implications of the data set for the biological function of RIG-I too unclear at the moment. Given the comments provided by the referees and advisor, I am afraid that we have come to the conclusion that the advance provided is not sufficient to consider publication in the EMBO Journal.

However given the interest in RIG-I and in its structural and biochemical characterization, I have taken the opportunity to discuss the manuscript and reports with our "sister" journal EMBO Reports. EMBO Reports is interested in considering a suitably revised manuscript for publication using the present referee reports. I would like to encourage you to discuss this option and what specific experiments are needed further with the EMBO Reports senior editor Nonia Pariente (pariente@embo.org).

I am sorry that I can't be more positive for the EMBO Journal, but I hope that you will consider the EMBO Reports option. If you have any further questions regarding this please don't hesitate to contact me.

REFEREE REPORTS:

Referee #1:

Comment on Kohlway et al., 2013

In their manuscript „Defining the functional determinants for RNA recognition by RIG-I in-vitro and in-vivo" Kohlway et al. claim to identify the first time a minimal RIG-I activating motif designed by rational design according to crystallography data. The authors compare crystals of RIG-I (ΔCARDs:1-229) with a 5´-OH GC-10 -mer with an empty ATP binding pocket, with ternary complexes from the structure previously reported (Luo et al, 2011) containing SO4-in the ATP binding pocket, with a new ternary complex of RIG-I (ΔCARDs:1-229) and 5´-OH GC-10, in which the ATP binding pocket is occupied with ADP_Mg2+ . The three different crystals are proposed to reflect a scanning movement of Hel2i during duplex recognition characterized by the movement of the amino acids Q511 and K508. They observe that during the scanning process Q511 from the helicase domain HEL2i interacts with different 2´-OH groups of the bottom strand (5 and 4) of the duplex ligand as indicated in Fig 1E of the manuscript while K508 interacts with a phosphate in the top strand (only in conformation 3). In the next step using sedimentation velocity as a read-out, RIG-I-RNA binding and RIG-I/RNA complex stoichiometry in dependence of dsRNA length of GC-rich sequences were analyzed. The authors find that one RIG-I protein binds one 5'ppp(dsRNA) terminus: 1:1 stoichiometry with hairpins and 1:2 if two triphosphates are present (pppRNA/pppRNA-duplex). pppRNA hairpins and blunt ended OH-dsRNA of different length were tested for RIG-I ATPase inducing capacity. The authors found an optimum of 12 bp length for OH-dsRNA and ppp-dsRNA activating the ATPase and concluded that 10 bp are the minimum length for ATPase stimulation (with and without ppp). Of note, ATPase activity was similar for OH- and pppRNA (pppRNA exhibited tighter binding than OH-RNA). Since the authors see no considerable influence of the length (as soon as >10 bp) on ATPase activity, they conclude that not length but number of termini is important for RIG-I activation. Experiments with fractionated poly IC were performed to support this interpretation. Finally, with RIG-I expressing and IFNbeta-reporter plasmid transfected HEK293 cells were stimulated with RIG-I ligands. On the first view confusingly, in disparity to ATPase assays, short OH-dsRNA or ppp-dsRNA (up to 12mer, below 22mer) did not induce RIG-I activity while 10mer hairpins induced a substantial RIG-I signal.

While the crystal data have a potential to exhibit interesting details of a putative RIG-I scanning mechanism, the second part of the manuscript aiming to identify a minimal RIG-I activation motif does not support the major finding mentioned in the abstract. On the contrary, in most parts the results confirm the literature but are interpreted in another direction. In contrast to what the authors imply in their abstract, there exist many studies aiming at determining the minimal activation motif and length of RIG-I activation (Marques, 2006, Hornung, 2006, Schlee 2009, Schmidt 2009, Binder, 2011)

Major comments

Movement of the Hel2i domain contributes to RNA duplex recognition by RIG-I
1. The authors claim that Q511 in the ADP bound state interacts with a 2´-OH group of the bottom strand of basepair 7 (ie pos 4 in Fig 1 E) and this shift - in comparison to the sulfate bound state (here, Q511 interacts with base pair 6) is an important aspect of Hel2i duplex recognition. In their previous structure however (Luo et-al, 2011) with a 8 bp ppp-hairpin no such change was observed. Q511 forms a H-bond with the 6th base pair of bottom strand nucleoside 2´-OH in the ADP bound state as well(4AY2.pdb vs 2YKG.pdb).

Is there an explanation for this discrepancy? Is this structural comparison valid only when the same 5´- OH GC 10 mer is used with and without ADP ?

2. General comparison of previously published structures already revealed (Kolakofsky et al., 2012, RNA review) that " in the nucleotide-free and ADP complexes (Luo et al. 2011, 2012), the helicase is in an open state with the disordered Hel-2 not contributing to RNA binding and Hel2i making different contacts due to its slightly rotated position" when compared to crystals containing nonhydrolyzable ATP analogs such as ADP -BeF3 (nature , 3TMI.pdb) and ADP-ALF3 (Cussack Cell 4a36 .pdb)
However the Q511 site chain within the YEQ511WIVT sequence of hRIG-I of the Hel-2i subunit involved in interaction with the duplex ligand is not properly resolved in 3TMI.pdb. This leads to modeling of an E510- 2´-OH interaction at base pair 7, whereas for Q511 only the main chain is indicated. Additionally, the human Hel-2i sequence YE510Q511/WIVT is changed to YEHWIVV in duck RIG-I (Cusack Cell 4a36 .pdb) leading to different local interactions in this area.
It would be an important contribution to see the change in the position of Q511 between various open and tightly bound forms as determined in 3zd6.pdb and 3zd7.pdb with the same GC10 ligand complexed with h-RIG-I as described in this manuscript, because well resolved structures may clear open issues in 3TMI and 4A36.

(minor comment to this issue: In order to simplify comparisons with different ligands with reported structures we propose that base pair numbering starts with the first base pair on the CTD site of the duplex such a scheme makes numbering of interacting position independent from duplex length)

Kinetic analysis of ATPase activity reveals the optimal RNA length for RIG-I stimulation

3. ATP hydrolysis cannot be correlated in a straightforward way with signalling activity (Kolakofsky et al., 2012 RNA review). For instance (Bamming& Horvath 2009) report a mutation in the motif III region of the helicase (LTA410 S to LAA410 A), which cannot hydrolyse ATP but has even a constitutive signaling activity .

It has been observed (Schmidt et al., 2009 ,PNAS) that 10mer dsRNA may stimulate ATP hydrolysis (Fig. 3 C/E) but does not activate RIG-I in cells. Obviously the transient release of CARDs effected by 10 mer duplex RIG-I interactions is not sufficient for type I IFN induction. The structural explanation for the minimal requirement of ppp-dsRNA 19-20 mers (Schlee et al., 2009) remains still unclear.

In conclusion the ATPase data are not appropriate for supporting a 10mer minimal dsRNA RIG-I activation motif.

Defining the optimal length of RNA ligands for interferon production in cells.

4. "To satisfy all of these objectives, we synthesized a family of structurally well-defined RNA hairpins in which the duplex length was varied, but one terminus was blocked by the presence of a structured, RNA tetraloop. In this way, we could vary duplex length, but keep the number of duplex termini constant (at 1)."..." The short 'GC' 5'triphosphorylated and 5'hydroxyl palindromic RNAs did not elicit a significant IFN-β response (Supplementary Fig. 2), potentially because they fall apart quickly and are then degraded by cellular nucleases. However, the 5'triphosphorylated hairpins containing 10, 20, and 30 bp duplexes elicited IFN responses comparable to that of poly I:C (Fig. 7B). The hairpins are an ideal stimulant for RIG-I because they are more thermodynamically stable and likely to re-anneal after being unwound by other cellular helicases"

Hairpins are in equilibrium with their self-complementary duplexes
(Nakano et al., 2007 NAR): A 10mer hairpin (4mer loop) will be transformed into an 24mer dsRNA, explaining identical RIG-I inducing activity as the pppGC22 sequence.
Therefore not a 10mer but - as published previously - a 24mer is most probably the activating RIG-I ligand in the in vitro transcription mix.

5. GC-rich sequences could easily fold into higher order structures which were shown to be very potent RIG-I ligands even at very small concentrations (Binder et al., 2011).

6. OH-dsRNA and ppp-dsRNA should be presented in the same figure. It is confusing and contradicts the whole literature that ppp-dsRNA should be as active as OH-dsRNA.

Referee #2:

The manuscript by Kohlway et. al., provides a comprehensive study of the minimal determinants for RIG-I RNA binding and ATPase activity in vitro correlated with the ability of the RNA ligand to activate interferon production in cells. The work both confirms and extends previous structure based results from the same authors and others. The novelty is to use stable RNA hairpins as RNA ligands enabling demonstration that a minimal 10bp RNA is able to stimulate interferon induction. The major results are: (a) two new RIG-I ligand structures which together with previous structures show how the hinged Hel2i domain can reach a variable distance (up to 9bp) along the duplex from the CTD capped end, dependent on the occupation of the ATP binding site, (b) RIG-I is an end-binder and forms a 1:1 complex with dsRNA (or 2:1 with a double ended dsRNA > 20 bp) that does not oligomerize (this has been shown before e.g. Kowalinski et al 2011, SuppFig3*) (c) the maximal ATPase activity is achieved with a minimal 10bp RNA and is not enhanced by protein-protein interactions on the same RNA under conditions where a longer RNA can bind more than one RIG-I, (d) the 5' triphosphate contributes to a 10-fold lower Km for RNA compared to 5'OH (i.e. initially binds tighter) but does not significantly change the Kcat or Km for ATP. Thus 5'OH RNA can still activate RIG-I but at a higher concentration, (e) Using fractions of poly(I:C) of different sizes, it is shown that RIG-I ATPase and interferon induction activity is higher for shorter poly(I:C) (shown before) according to the concentration of free ends that RIG-I can preferentially bind, (f) in cell base assays, hairpins from 10bp in length induce robust interferon activation (comparable with poly(I:C)) whereas short duplex RNAs do not (consistent with previous results), probably because of much lower stability. Finally the authors present a refined structure-based model of RNA/ATP induced RIG-I activation (SuppFig 4), incorporating the idea of 'scanning' of Hel2i with the CARDs being released only if Hel2i senses the presence of at least 10 bp. The authors are careful to say that whilst the minimal RNA activated RIG-I complex does not oligomerize in itself, it may do in subsequent steps e.g.via poly ubiquitin binding.
The paper is clearly written and presents a picture of RIG-I activation that is consistent with structure, biochemistry and biology (interferon induction) and clarifies several outstanding issues in the field. It thus merits publication after taking into the account the following points.
(1) The reason for the requirement of ATP binding for RIG-I activation (the K270A mutant does not induce interferon) could be discussed more, since several structures show an RNA bound form in the absence of nucleotide. The authors suggest that the ensemble of RIG-I structures now available show that the hinged Hel2i domain can reach a variable distance (up to 9-10bp) along the duplex from the CTD capped end and that only the ATP bound forms are extended enough along the duplex to sense the full 10bp and be able to release the CARDs. It would be very interesting to have a direct measure of CARD release to confirm this and the prediction that the K270A mutant can still bind RNA but does not release the CARDs. Furthermore, whilst the movies are useful, Supp Fig3 is too complicated to make out much. Indeed a more detailed, quantitative, structural analysis of the conformational differences between the ensemble of structures would be very useful, since it is not only the Hel2i that changes but also the contact of Hel2 with the RNA and the motifs (other than motif I and motif II) forming the ATP binding site.
(2) Much of the paper characterises the ATPase activity of RIG-I but skirts around discussion of the relevance of this activity for activation. Is a genuine and well characterised ATP hydrolysis mutant (that still binds ATP) active in interferon induction ? Also the notion of 'scanning' of Hel2i needs to be clarified. Is this envisaged as a stochastic process requiring ATP hydrolysis or does binding one ATP molecule induce a change to the extended bidning mode that is sufficient to release the

CARDS.
(3) It would be interesting to assess the relative stability of short RNA hairpins and duplexes in cells. Could this be done by transfecting RNAs with FRET labels at 5' and 3' end ? This is biologically relevant since RIG-I is thought to detect panhandles of negative strand RNA viruses, which are often quite short and probably only marginally stable as duplexes in the absence of the viral polymerase and nucleoprotein forming an RNP; in which case how does RIG-I get access to the panhandle (c.f. recent paper of Weber et al., Cell Host Microbe. 2013, 13(3):336-46.)?
Other point:
*Top of Page 11. The results shown in Figure 2 are not only consistent with the crystallographic results of Kowalinski et al but also solution binding experiments where a 2:1 complex was demonstrated with 61-mer dsRNA (Supp Figure 3).

The crystalloghraphic statistics looks OK although the completeness and signal to noise in the outer shell of the conformation 2 is below standard.

Advisor comments:

"...for the definition of the minimal RIG-I agonist, our reservations stem from the fact that the paper is written very much from a structural biology/in vitro biochemistry perspective yet tries to derive rules about biological function in cells that are not necessarily correct. For example, the authors largely equate RIG-I activation with ATPase activity. That may be a biochemical use of the term but most people in the field would equate RIG-I activation with induction of type I IFN, which does not always correlate with ATPase activity as reviewer 1 points out. Another example is that the authors imply that their data would suggest that "that a multimeric form of RIG-I is not required for activation on RNA". Again, that may be true biochemically when there are no accessory factors and the readout is simply ATPase activity but may not be relevant in cells where oligomerisation may occur following polyubiquitination by TRIM25, which is clearly key for RIG-I function and IFN induction. In that regard, the authors could try to add to their assays lysates of RIG-I-deficient cells as a source of ancillary factors. The last figure is an attempt to get at biological function but it is inadequate, with not even a positive control. Finally, the paper is difficult for biologists to understand (e.g., KmATP vs. KmRNA) yet it appears to aim at a biological readership by trying to establish rules about the triggering of IFN responses. In sum, we agree that the paper has lots of interesting information and details about the behaviour of RIG-I in vitro but the actual implications of the data for biological function are a little unclear...."

Submission to EMBO reports – author's response to original comments 13 June 2013

We thank the reviewers and the advisor for their analysis of the paper and for their helpful comments on the manuscript.

REVIEWER 1:

1) General comment A: "On the first view confusingly, in disparity to ATPase assays, short OH-dsRNA or ppp-dsRNA (up to 12mer, below 22mer) did not induce RIG-I activity while 10mer hairpins induced a substantial RIG-I signal."

In our manuscript we stated, "The short 'GC' 5'triphosphorylated and 5'hydroxyl palindromic RNAs did not elicit a significant IFN-β response (**Supplementary Fig. 2**), potentially because they fall apart quickly and are then degraded by cellular nucleases." However, given their stability on native gels, the stability of the short duplexes during the *in vitro* ATPase was not in question. Furthermore, some of the short RNA duplexes (GC12 in particular) did in fact elicit an IFN response above background, as we showed in Supplementary Fig. 2 of the original manuscript.

However, the response was not as strong as that for the poly I:C control and the hairpins, so we elected not to emphasize it in the main text. To reduce any confusion and shorten the revised manuscript, data on the IFN response by the short duplexes was removed.

2) General comment B: "In contrast to what the authors imply in their abstract, there exist many studies aiming at determining the minimal activation motif and length of RIG-I activation (Marques, 2006, Hornung, 2006, Schlee 2009, Schmidt 2009, Binder 2011)."

We never stated or implied that other studies had not attempted to determine the minimal activation motif and length of RIG-I activation. However we did state that "the minimal functional RNA PAMP for robust RIG-I activation has never been defined." None of the cited studies aimed to determine the minimal activation motif (in terms of length) of RIG-I, and only the Schmidt 2009 paper provided evidence that RNAs < 19 nucleotides might be functional (more about that paper below).

It is useful to consider a summary of the shortest RNA molecules used in each paper cited by the referee: Marques 2006 (21 nucleotides), Hornung 2006 (19 nucleotides), Schlee 2009 (19 nucleotides), Binder 2011 (>40 nucleotides). In these cases, all of the RNAs tested were larger than 19 nucleotides, which is twice the RIG-I site size. Furthermore, there are inconsistencies in the papers listed. For example: 1) Marques 2006 attempts to use RIG-I unwinding as a metric of functional binding, but it is now well established that RIG-I does not unwind RNA 2) Hornung 2006 claims that ssRNA of 19 nucleotides can elicit a response, but again the generally accepted consensus, based on data from many labs, is that RIG-I requires dsRNA (see also Schmidt 2009) 3) Binder 2011 is focused on perceived cooperative interactions among RIG-I molecules on very long RNA substrates. The only listed paper that provides insights into the possible minimal size of the RNA PAMP is Schmidt 2009. However that study, which implicated RNA duplexes as short as 10 bp, was aimed at proving that dsRNA is required for RIG-I ATPase and interferon activation, and it does not attempt to provide a systematic dissection of the RIG-I PAMP size. While Schmidt 2009's general findings corroborate our story, we feel it complements our paper rather than overlaps with it.

3) Specific comment #1. The authors claim that Q511 in the ADP bound state interacts with a 2´-OH group of the bottom strand of basepair 7 (ie pos 4 in Fig 1 E) and this shift - in comparison to the sulfate bound state (here, Q511 interacts with base pair 6) is an important aspect of Hel2i duplex recognition. In their previous structure however (Luo et-al, 2011) with a 8 bp ppp-hairpin no such change was observed. Q511 forms a H-bond with the 6th base pair of bottom strand nucleoside 2´-OH in the ADP bound state as well (4AY2.pdb vs 2YKG.pdb). Is there an explanation for this discrepancy? Is this structural comparison valid only when the same 5´- OH GC 10 mer is used with and without ADP?

As we documented in the Structure paper (2012), the duplex region was very short (8 bp) and the UUCG tetraloop introduced small deviations in the helical conformation of base pairs immediately adjacent to the loop. It is therefore only appropriate to compare the register of single-nucleotide protein contacts when examining RIG-I states that are bound to the same RNA molecule (in this case, GC10).

4) Specific Comment #2. It would be an important contribution to see the change in the position of Q511 between various open and tightly bound forms as determined in 3zd6.pdb and 3zd7.pdb with the same GC10 ligand complexed with h-RIG-I as described in this manuscript, because well resolved structures may clear open issues in 3TMI and 4A36.

We also agree that a more detailed analysis of the conformational differences between the ensemble of structures would be useful. However, given the length limitations of EMBO Reports, we limited ourselves to the initial structural analysis provided by our three structures complexed with GC10: Fig 1b shows the changes of Q511 in the three conformations relative to the RNA duplex backbone. Fig 1e is a cartoon illustration of the differences at the HEL2i and RNA interface among the three conformations.

5) Specific Comment #3. ATP hydrolysis cannot be correlated in a straightforward way with

signaling activity (Kolakofsky et al., 2012 RNA review). For instance (Bamming& Horvath 2009) report a mutation in the motif III region of the helicase (LTA410 S to LAA410 A), which cannot hydrolyse ATP but has even a constitutive signaling activity. It has been observed (Schmidt et al., 2009, PNAS) that 10mer dsRNA may stimulate ATP hydrolysis (Fig. 3 C/E) but does not activate RIG-I in cells. Obviously the transient release of CARDs effected by 10 mer duplex RIG-I interactions is not sufficient for type I IFN induction. The structural explanation for the minimal requirement of ppp-dsRNA 19-20 mers (Schlee et al., 2009) remains still unclear. In conclusion the ATPase data are not appropriate for supporting a 10mer minimal dsRNA RIG-I activation motif.

Reviewer 1 claims that "ATP hydrolysis cannot be correlated in a straightforward way with signaling activity". We never claimed that it could be. Indeed, the second reviewer applauds the conservatism by which we interpret our data. We acknowledge that the correlation between ATPase activity and cell culture interferon-β response is weak based on several papers in the literature, but at no point in our paper do we try and establish a strong connection. Our paper contains sedimentation velocity data that reports on the RNA binding properties directly, and our study does not rest on a tight link between ATPase activity and the IFN response (although in our hands, using rigorous enzymological techniques for monitoring ATPase activity, the correlation between ATPase activity and the IFN response is quite good). As we now explain more carefully in our paper, ATPase activity is simply being used as a sensitive probe of functional RNA binding to the protein. We have validated this assay, along with other direct RNA binding assays, in this and other work (see Vela, JBC 2012). In contrast to the reviewer's claims, we make no effort to establish a connection between RIG-I ATPase activity and the IFN response. Our focus in this paper is on RNA binding.

It is important to correct a point made by the referee that was meant to undermine a connection between ATP hydrolysis and the IFN response: "It has been observed (Schmidt et al., 2009, PNAS) that 10 mer dsRNA may stimulate ATP hydrolysis but does not activate RIG-I in cells. Obviously the transient release of CARDS effected by 10 mer duplex RIG-I interactions is not sufficient for type 1 IFN induction. In fact, exactly the opposite is demonstrated and described in Schmidt (2009), which was a study that was originally designed to refute the finding that single-stranded RNA was a RIG-I ligand. In that work, the authors show that short duplexes are made by a template switch mechanism during the T7 RNA transcription of "single stranded RNAs" used by others, resulting in short 10-base pair hairpins that demonstrably stimulate the IFN production in cells. These preliminary findings are in fact fully consistent with our work, and are clearly stated on page 12069, column 2, line 2 of Schmidt (2009) PNAS: "However, when 5-, 10-, or 15-nts complementary RNA were hybridized to the 5'-end of syn-ppp-ss2.2s RNA, 10 nts were sufficient to rescue IFN induction, suggesting that the extent of base-pairing as well as its relative position to the 5'-triphosphate-end are important determinants of immunostimulatory activity."

6) Comment #4. The short 'GC' 5'triphosphorylated and 5'hydroxyl palindromic RNAs did not elicit a significant IFN-? response (Supplementary Fig. 2), potentially because they fall apart quickly and are then degraded by cellular nucleases. However, the 5'triphosphorylated hairpins containing 10, 20, and 30 bp duplexes elicited IFN responses comparable to that of poly I:C (Fig. 7B). The hairpins are an ideal stimulant for RIG-I because they are more thermodynamically stable and likely to re-anneal after being unwound by other cellular helicases". Hairpins are in equilibrium with their self-complementary duplexes (Nakano et al., 2007 NAR): A 10mer hairpin (4mer loop) will be transformed into an 24mer dsRNA, explaining identical RIG-I inducing activity as the pppGC22 sequence. Therefore not a 10mer but - as published previously - a 24mer is most probably the activating RIG-I ligand in the in vitro transcription mix.

The notion that the hairpins used in our study are in equilibrium with duplex forms of the same RNA, at the low concentrations used for transfection, contradicts an entire literature on the thermodynamic stability of RNA. Hairpins of self-complementary duplexes have an enormous entropic stabilization relative to dimers of the same sequence and are always preferentially stabilized (see below). Furthermore, we used the stabilizing UUCG tetraloop to cap the hairpins, which was shown decades ago (see below) to result in extremely stable hairpins of even short RNA duplexes (4 bp).

Specifically:

(a) The successful NMR structural determination and thermodynamic investigations and dynamics studies of short hairpins (UUCG tetraloops) indicate they are exceptionally stable in solution even at very high RNA concentrations over very long time - up to 48 hours - of data recording (at least 1000 higher than the experimental concentrations used in our work) and low salt concentrations (12nt, 10mg/ml, in 10 mM phosphate buffer with a Tm of 65°C (see Cheong, below); 14nt, 0.7mM in 20 mM phosphate buffer with a Tm 74°C (see Nozinovic, below)). These same studies show that the hairpins are unable to hybridize into duplexes once formed. The Nakano paper cited by the reviewer involves totally self-complementary RNAs that lack stabilizing tetraloops. Two of the many seminal papers on this are: Cheong C, Varani G, Tinoco I. "Solution structure of an unusually stable RNA hairpin 5'GGAC(UUCG)GUCC" Nature (1990) 436, 680-682. Nozinovic S, Schwalbe H. et al. "High-resolution NMR structure of an RNA model system: the 14-mer cUUCGg tetraloop hairpin RNA." Nucleic Acids Res. 2010 Jan;38(2):683-94.

(b) The outstanding performance of shRNAs as RNAi triggers is due to the fact that they are exceptionally stable and function optimally in physiological environments. See Siolas, D. and Hannon, G. et al " Synthetic shRNAs as potent RNAi triggers." *Nature Biotechnology* **23**, 227 - 231 (2005)

(c) In our experiments, we took painstaking steps to ensure the homogeneity of the hairpin RNAs. All hairpin RNAs were PAGE purified; the re-annealing step was performed at low RNA concentrations (below 5 uM) by heating the RNA at 96°C for 2 mins and rapid cooling on ice; Native gel analysis indicated > 98% of the final products are in hairpin form and no traceable intermolecular duplex even for the shortest hairpin 5'ppp8L.

7) Comment #5. "GC-rich sequences could easily fold into higher order structures which were shown to be very potent RIG-I ligands even at very small concentrations (Binder et al., 2011)".

First of all, the Binder study does not contain any information on misfolding of GC sequences and did not even involve the use of duplexes that are unusually rich in GC content. Second, we were careful to use different duplexes of varying GC content in our study, and we get the same results in each case (the 5'pppCM12 and 5'ppp hairpins were mixed-sequence). Third, there is nothing in the RNA structure literature to suggest that short GC RNA oligomers form higher order structures. The reviewer is confusing this with polyG sequences, which can make four-stranded structures under certain ionic conditions.

8) Comment #6. "OH-dsRNA and ppp-dsRNA should be presented in the same figure. It is confusing and contradicts the whole literature that ppp-dsRNA should be as active as OH-dsRNA".

We have not contradicted the literature in stating that k_{cat} values are insensitive to the presence of the triphosphate group at RNA saturation. Nobody has ever reported a comparative dissection of k_{cat} and K_m values as a function of RNA triphosphorylation for RIG-I. The triphosphate enhances binding of RNA (K_m) but not the chemistry of ATP hydrolysis (k_{cat}). This makes sense, since the RNA triphosphate plays no role in the structure of the ATPase active site. We now elaborate on this point within the text (see subsection entitled "5'triphosphate enhances RNA binding, but not ATP hydrolysis", line 202).

Reviewer 2:

9) Comment #1a. The reason for the requirement of ATP binding for RIG-I activation (the K270A mutant does not induce interferon) could be discussed more, since several structures show an RNA bound form in the absence of nucleotide. The authors suggest that the ensemble of RIG-I structures now available show that the hinged Hel2i domain can reach a variable distance (up to 9-10bp) along the duplex from the CTD capped end and that only the ATP bound forms are extended enough along the duplex to sense the full 10bp and be able to release the CARDs. It would be very interesting to have a direct measure of CARD release to confirm this and the prediction that the K270A mutant can still bind RNA but does not release

the CARDs.

Although we agree that this would be a very interesting experiment, we currently do not have the tools to directly measure CARD release in solution. Future single molecule FRET studies would be ideal for addressing this question.

10) Comment #1b. Furthermore, whilst the movies are useful, Supp. Fig. 3 is too complicated to make out much. Indeed a more detailed, quantitative, structural analysis of the conformational differences between the ensemble of structures would be very useful, since it is not only the Hel2i that changes but also the contact of Hel2 with the RNA and the motifs (other than motif I and motif II) forming the ATP binding site.

We also agree that a more detailed analysis of the conformational differences between the ensemble of structures would be useful. However, given the scope our manuscript and the length limitations of EMBO Reports, we have removed Suppl. Fig3 and Suppl. mov2.

11) Comment #2. Much of the paper characterises the ATPase activity of RIG-I but skirts around discussion of the relevance of this activity for activation. Is a genuine and well characterised ATP hydrolysis mutant (that still binds ATP) active in interferon induction ? Also the notion of 'scanning' of Hel2i needs to be clarified. Is this envisaged as a stochastic process requiring ATP hydrolysis or does binding one ATP molecule induce a change to the extended binding mode that is sufficient to release the CARDS.

The influence of ATPase mutants on induction is a very important question, which we hope to address in the course of future work. Regarding the function of scanning and its relationship to stages of the ATPase cycle, we have opted to interpret our data on this relatively cautiously. Despite the fact that Hel2i appears to shift register as a function of occupancy in the ATPase active site, we hesitate to make firm conclusions yet because the results are based on a limited number of structures. With additional data, we will be able to differentiate stochastic behavior from highly coordinated motions.

12) Comment #3. It would be interesting to assess the relative stability of short RNA hairpins and duplexes in cells. Could this be done by transfecting RNAs with FRET labels at 5' and 3' end? This is biologically relevant since RIG-I is thought to detect panhandles of negative strand RNA viruses, which are often quite short and probably only marginally stable as duplexes in the absence of the viral polymerase and nucleoprotein forming an RNP; in which case how does RIG-I get access to the panhandle (c.f. recent paper of Weber et al., Cell Host Microbe. 2013, 13(3):336-46.)?

We agree that these are important questions, and we think it would be productive to address them as part of future studies using, as suggested by the reviewer, methods such as FRET. That said, we are confident that the hairpins used in this study are stable in the duplex form. Please see response #6, addressed to Reviewer 1.

13) Reviewer 2 Comment #4. The crystallographic statistics looks OK although the completeness and signal to noise in the outer shell of the conformation 2 is below standard.

Conformation 2 refers to the structure of RIG-I (ΔCARDs:1-229) : 5'OH-GC10 : SO_4^{2+}, which is already available under the accession code 2ykg and which was peer reviewed and published in Luo et al., 2011. We agree with the reviewer that the crystallographic data for conformation 2 was processed less conservatively. Nonetheless, to assure the referee that the conformation is accurately represented, we reprocessed the original diffraction data and refined the conformation 2 structure to 2.6 Å. The statistics indeed look slightly better: for the highest resolution shell (2.7 - 2.6 Å), R_{merge} is 53.9%, I /σ is 1.7, and the completeness is 94.7%. The refined structure using data cutoff at 2.6 Å, however, contains no detectable differences from 2ykg. The structure analysis in this manuscript and the previous publication (Luo, et al., 2011) based on 2ykg (conformation 2) is not affected by the resolution cutoff. Therefore we prefer to leave the original data for 2ykg within Supplementary Table 1.

Response to comments from the Advisor:

14) Comment #1. For example, the authors largely equate RIG-I activation with ATPase activity. That may be a biochemical use of the term but most people in the field would equate RIG-I activation with induction of type I IFN, which does not always correlate with ATPase activity as reviewer 1 points out.

Please see our response #5 to reviewer 1.

15) Comment #2. Another example is that the authors imply that their data would suggest that "that a multimeric form of RIG-I is not required for activation on RNA". Again, that may be true biochemically when there are no accessory factors and the readout is simply ATPase activity but may not be relevant in cells where oligomerisation may occur following polyubiquitination by TRIM25, which is clearly key for RIG-I function and IFN induction. In that regard, the authors could try to add to their assays lysates of RIG-I-deficient cells as a source of ancillary factors.

We have not contradicted the reviewer's point of view. We stated in our paper that, after RNA and ATP bind to RIG-I, the resultant complex is likely to multimerize by binding to cellular factors like polyubiquitin through the CARD domains. This has been suggested before, in a recent review by Kowalinski and Cusack, which we cite (RNA 2012). In fact, in **Supplementary Figure 3** of our initial manuscript, we actually included RIG-I polyubiquitin mediated oligomerization in our model of RIG-I activation. Our paper definitively shows that RIG-I does not form obligate protein-mediated oligomers on an individual RNA molecule, and that RIG-I has a strong affinity for the 5' ends of duplex RNA relative to the internal duplex stem region. Whether or not RIG-I subsequently oligomerizes in the presence of polyubiquitin before binding to MAVS is out of the scope of this paper, as is the addition of crude extract of unknown proteins to our *in vitro* assays. However, to clarify our point of view on activation, we now include a revised model of RIG-I activation (**Fig. 5**).

16) Comment #3. The last figure is an attempt to get at biological function but it is inadequate, with not even a positive control.

All positive controls were included in the original study. We provided poly I:C stimulation as a positive control (Figure 7 in our previous manuscript and Figure 4D of the revised manuscript). However, to make this more apparent, we have directly stated that poly I:C is the positive control for Figure 4D in the main text, and we also combined the poly I:C cell culture data with the short hairpin cell culture data. Furthermore, we include a mock experiment in Supplementary Figure 3 of the revised manuscript to confirm that our cell culture stimulation is dependent on RIG-I expression in 293T cells.

17) Comment #4. Finally, the paper is difficult for biologists to understand (e.g., KmATP vs. KmRNA) yet it appears to aim at a biological readership by trying to establish rules about the triggering of IFN responses.

The paper has been extensively rewritten so that it will be accessible to members of both the biochemistry and immunology community. But that said, investigators submitting manuscripts to the EMBO Journal should not be penalized or criticized for using standard forms of enzymology, and for employing well-validated kinetic parameters as tools to describe systems of biological interest. To do otherwise will ultimately reduce the rigor of papers published within the family of journals.

1st Editorial Decision 27 June 2013

We have now received the reports from the three referees that were asked to assess your study. Referee 1 is the former referee 2, referee 2 is an RNA biochemist that assessed the initial concerns of referee 1, and referee 3 was the advisor to the previous version of the study.

As you will see below, they now all support publication in EMBO reports, once a few minor revisions have been made. Importantly, further analysis of the structural changes that occur in the pincer domain, clarification of the figure 5 model and the three first points brought up by referee 3 need to be addressed. It would be also ideal to include a better positive control to experiment 4D, as suggested, given that virus RNA stimulates interferon response to a much higher extent than polyI:C, and the latter activates also MAD5. It would be interesting to see how the activation induced by the short hairpins compares. Nevertheless, we wouldn't make this a precondition for publication. However, at a minimum, a discussion of this issue and how the IFN activation you reports would compare with endogenous ligands such as virus RNA would need to be incorporated.

I have also noted that the number of independent experiments from which the error is calculated has not been specified for supplementary figure 3. Lastly, I do not seem to find the PDB access codes for the two new structures. These should be in the main body of the text when you first describe them in the results section, and in the respective figure legend (I apologize if I have missed them!).

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

REFEREE REPORTS:

Referee #1:

I originally reviewed the manuscript as submitted to EMBO J. I think the authors in their reply to the referees have largely addressed the issues raised by the 2 referees, notably that about hairpin stability in cells. The revised verison is improved in its clarification about what is claimed (e.g. that end binding on short hairpins is sufficient to obtain an interferon response)and what is not claimed (the paper does not address post-CARD release olgomerisation or the requirement or not for ATPase activity for inteferon). Nevertheless it would be better if the paper did include some more detaled analysis of the structural changes (point 1b (10 in the reply), of referee 2). Notably exactly what the pincer domain conformational changes are should be presented as revised Figure 5 misleadingly suggests that the angle between the two long helcies changes substantially.

Referee #2:

From a biochemical point of view, I think that the Authors' responses to the Reviewers' comments are reasonable. For example, the authors are correct in that, once formed, RNA hairpins with the tetraloop are extremely stable, and conversion into duplexes is virtually negligible at the normal experimental condition. The authors may want to include a more detailed protocol for their careful hairpin preparation as well as the validating data by native gel analysis in the Supplementary Information (as mentioned in the rebuttal letter). Overall, I support publication of this work in EMBO Reports.

Minor comment: The model shown in Fig. 5 appears as if the 'scanning' of HEL2i occurs independently of ATP binding and hydrolysis. Although I appreciate the authors' cautiousness as to the relationship between the HEL2i scanning and ATP binding/hydrolysis, the authors may want to slightly revise the cartoon and/or add an explanation in the Figure Legend to avoid potential misinterpretation.

Referee #3:

Kohlway et al. have addressed the reviewers' comments albeit somewhat belligerantly. The extensive editing has significantly improved the readability of the manuscript. A few minor details still need to be corrected in the text, including:

Results section for Figure 1: The authors should clearly describe the 5'OH-GC10 RNA.

Figure 1: The authors should indicate what they mean by pincer 1 and 2 as only one pincer domain has been described for RIG-I.

Figure 3a: the apparent size of the fraction A1 seems to be greater than 500nts and not around 500nts as stated in the text.

Figure 3b: By comparing the Km for RNA in (nM) from fractions A1 and A7 the authors concluded that the size of the poly(I:C) does not influence RIG-I affinity for RNA. However the Km for fractions A4 and A5 seem around 2-3 times lower. It would be nice if the authors could comment on this discrepancy in the text.

Fig. 4D: Are the poly I:C data in the figure the same as those in Fig. S4, in which case they are repeated? The stimulation appears very low and, as stated previously, it would have been nice if the authors had included for comparison a positive control that acts as a potent and selective RIG-I agonist for IFN induction (e.g., in vitro transcribed RNA, influenza virus RNA, etc). Poly I:C can additionally stimulate MDA5 and is not the agonist of choice for triggering RIG-I in cells.

1st Revision - authors' response 02 July 2013

We thank the referees and the Editor for their analysis of this paper, and for their helpful comments. Our responses are found below:

1) **Reviewer 1 Comment 1:** Nevertheless it would be better if the paper did include some more detailed analysis of the structural changes (point 1b (10 in the reply), of referee 2). Notably exactly what the pincer domain conformational changes are should be presented as revised Figure 5 misleadingly suggests that the angle between the two long helices changes substantially.

   To clarify the structural changes, we labeled the change in angle of pincer1 between all three conformations (**Figure 1C**). We also labeled the translational change in Angstroms of the HEL2i domain between all three conformations (**Figure 1B**). The figure captions were modified appropriately to highlight these changes.

   Figure 5 was modified to be less misleading regarding the pincer domain movements. Pincer1 was rotated less drastically upon ATP binding.

2) **Reviewer 2 Comment 1:** The authors may want to include a more detailed protocol for their careful hairpin preparation.

   We have included a detailed protocol for our hairpin preparations in the supplementary material which contains references to the existing research on UUCG hairpin stability (Supp. Methods under RNA synthesis and transcription).

3) **Reviewer 2 Comment 2:** The model shown in Fig. 5 appears as if the 'scanning' of HEL2i occurs independently of ATP binding and hydrolysis. Although I appreciate the authors' cautiousness as to the relationship between the HEL2i scanning and ATP binding/hydrolysis, the authors may want to slightly revise the cartoon and/or

add an explanation in the Figure Legend to avoid potential misinterpretation.

Figure 5 was modified appropriately to indicate HEL2i scanning upon ATP binding/hydrolysis (please see step 2 of Figure 5). We also modified the Figure 5 caption to help avoid misinterpretation ("HEL2i scanning may be directly linked to ATP binding and hydrolysis, or it may move stochastically.").

4) **Reviewer 3 Comment 1:** Results section for Figure 1: The authors should clearly describe the 5'OH-GC10 RNA.

We have modified the Figure 1 caption to clearly indicate the sequence and 5'character of our RNA for Figure 1 ("GC10 is a palindromic RNA duplex of repeating 'GC' with a 5'hydroxyl.").

5) **Reviewer 3 Comment 2:** The authors should indicate what they mean by pincer 1 and 2 as only one pincer domain has been described for RIG-I.

We have modified the figure 1 caption appropriately to describe what we mean by pincer1 and pincer2. ("Close-up view of the pincer domain, highlighting the motions of pincer1 (first α-helix). The change in angle between the pincer1 and pincer2 (second α-helix) is 11 degrees.").

6) **Reviewer 3 Comment 3:** Figure 3a: the apparent size of the fraction A1 seems to be greater than 500nts and not around 500nts as stated in the text.

We agree that there was some ambiguity in our estimation of sizes, especially given that the largest sizes did not migrate particularly well on a gel due to the inherent heterogeneity of poly I:C. LMW poly I:C is typically less than 1kb, and the very initial peak from the gel filtration column was not collected. Therefore, based on comparison with size standards, we interpreted the size of fraction A1 to be roughly 500 nts. Changing it to 600 or even 700 will not have a profound effect on our final conclusions. We also stated in the main text that "In fact, we observed an identical $K_{m,RNA}$ value of 20 nM for both fractions A1 and A7, which are at two extremes in terms of length, and the $K_{m,RNA}$ values for the other fractions were similar to this, within error."

7) **Reviewer 3 Comment 4:** Figure 3b: By comparing the Km for RNA in (nM) from fractions A1 and A7 the authors concluded that the size of the poly(I:C) does not influence RIG-I affinity for RNA. However the Km for fractions A4 and A5 seem around 2-3 times lower. It would be nice if the authors could comment on this discrepancy in the text.

We agree with the referee's observation. We believe that the ~2 fold change between fractions A1/A7 and A4/A5 is within the error of our experiment, and very likely may be directly attributable to the human error involved in estimating the size of each fractions (which was necessary in order to convert to nM). From an enzymological perspective, ~2-fold would be interpreted as a rather small change in Km and that the number of ends of polyI:C needed to stimulate RIG-I ATPase is approximately the same. The average Km,RNA for all of the fractions is 30 nM +/- 10 nM.

8) **Reviewer 3 Comment 5:** Fig. 4D: Are the poly I:C data in the figure the same as those in Fig. S4, in which case they are repeated? The stimulation appears very low and, as stated previously, it would have been nice if the authors had included for comparison a positive control that acts as a potent and selective RIG-I agonist for IFN induction (e.g., in vitro transcribed RNA, influenza virus RNA, etc). Poly I:C can additionally stimulate MDA5 and is not the agonist of choice for triggering RIG-I in cells.

The poly I:C data in **Figure 4D** is the same as **Figure S4**. The poly I:C data in **Figure S4** was done side by side with the RNA hairpins in **Figure 4D** and the poly I:C fraction A1-A7 data in **Figure S4**. We included it in both figures as a reference. The relative luciferase values can vary from experiment to experiment based on transfection efficiency/cell confluency, and therefore we felt it appropriate to include references from the same experiments. The stimulation we see in these experiments is approximately 10-fold above background. We added the following line to the Figure S4 caption "(note that the poly I:C data is the same as in Figure 4D and was done side by side with Fraction A1-A7 shown here)."

We understand the referee's preference for a more physiologically relevant RNA. We have extensive experience working with HCV RNA, and have in fact run several experiments stimulating RIG-I with HCV RNA, but in Huh 7.5 cells. Unfortunately, HCV RNA will not replicate and stimulate in 293T cells, and we do not have readily accessible influenza RNA. However, in regards to *in vitro* transcribed RNA, we included IFN-β stimulation by 5'pppGC22 in our earlier EMBO manuscript, and added it as a positive control in **Figure 4D** and referenced it in the main text and figure caption. We added the following to the main text, "Remarkably, we found that three of the four hairpins – 5'ppp10L, 5'ppp20L, and 5'ppp30L – stimulated an IFN-β response comparable to the positive controls, LMW poly I:C and 5'pppGC22 (mock control in **Supp. Fig. 3**). Both LMW poly I:C and short 19bp+ RNA duplexes have been shown to be good activators of RIG-I (Kato 2008, Schlee 2009)." Also note that in the seminal RIG-I paper (Yoneyama et al., Nature Immunology, 2004), poly I:C is used as a control and is a more effective stimulant than Newcastle disease viral RNA.

Additionally, we would like to point out that 293T cells have little to no RIG-I or MDA5 expression (http://www.proteinatlas.org/, Sabbah et al.,Virology Journal, 2009), and in our **Figure S3** we show that our IFN-β response is completely dependent on pUNO-RIGI overexpression. Furthermore, in Bamming et al. (JBC, 2009), the authors state "We and others (15,39) observe that HEK293T cells have a very low endogenous response to both poly(I-C) and Sendai virus in reporter gene assays. This response can be greatly elevated by ectopic expression of MDA5 and RIG-I." Thus, our LMW poly I:C controls represent exclusive stimulation of RIG-I and not MDA5, although in cells that express both proteins, there is overlap and LMW poly I:C has been shown to stimulate both proteins (See Figure 1 From Kato and Akira, J. Exp. Med. 2008).

9) **Editor's Comment 1:** I have also noted that the number of independent experiments from which the error is calculated has not been specified for supplementary figure 3.

This was added to supplementary figure 3 caption.

10) **Editor's Comment 2:** Lastly, I do not seem to find the PDB access codes for the two new structures. These should be in the main body of the text when you first describe them in the results section, and in the respective figure legend.

We included the PDB access codes under Accession Code in the Supp. Material. We added them to the main body of the text and the figure caption as well.

2nd Editorial Decision 02 July 2013

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

As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please let me know if you do NOT agree with any of the changes.

"Defining the functional determinants for RNA surveillance by RIG-I

Retinoic acid inducible gene-I (RIG-I) is an intracellular RNA sensor that activates the innate immune response in response to infection by RNA viruses. Here, we report the crystal structure of distinct conformations of a RIG-I:dsRNA complex, which reveal that HEL2i-mediated scanning allows RIG-I to sense the length of RNA targets. To understand the implications of such scanning for catalytic activity and signaling by RIG-I, we examined its ATPase activity when stimulated by duplex RNAs of varying lengths and 5' composition. A minimal RNA duplex that binds one RIG-I molecule can stimulate robust ATPase activity and elicit a RIG-I-mediated interferon response in cells. Our results show that the minimal functional unit of the RIG-I:RNA complex is a monomer that binds at the terminus of a duplex RNA substrate. This is markedly different from the RIG-I paralog MDA5, which forms cooperative filaments."

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