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MDA5 cooperatively forms dimers and ATP-sensitive filaments upon binding double-stranded RNA

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

1st Editorial Decision	24 November 2011

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

As you can see the referees appreciate the analysis and are overall supportive of publication here. However, both referee #1 and 2 raise a number of issues that have to be resolved and in particular further data supporting the dsRNA binding mode is needed. Should you be able to address the concerns raised in full then we would consider a revised manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision, and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The manuscript by Berke and Modis contains potentially very important new biophysical and biochemical data about the structure and behaviour of action of innate immune pattern recognition receptor MDA5, which has hitherto been rather mysterious. The results would imply a very different mode of action to the much better characterised RIG-I despite similar overall architectures and high sequence similarity.

The major results reported are as follows. MDA5 is monomeric in the absence of RNA. Co-operative binding of MDA5 to dsRNA (e.g. 2:1 complex of CARD-deleted MDA5 with AU20 by analytical ultracentrifugation). In general 10 fold lower affinity of MDA5 for dsRNA than RIG-I. The footprint of the dimer is 16-18 bp, 8-9 bp for monomer (like RIG-I). Coating of long dsRNA (form phage φ6) or poly(I:C) by MDA5 to form filaments is observed in negative stain EM. Filaments are protected from RNase degradation but not so in the presence of ATP, which seems to lead to a redistribution of the protein on the RNA. The crystal structure of MDA5 helicase insertion domain at 2 A resolution reveals a helical bundle similar to Hef and RIG-I. SAXS analysis of the CARDs alone is consistent with the tandem CARD structure described for RIG-I. However, unlike for RIG-I, no binding of the CARDs to the helicase domain are detected and they appear to be flexibly linked in the unbound state. Extensive SAXS analysis is performed of various other MDA5 constructs including the 2:1 complex of CARD-deleted MDA5 with AU20. Modelling suggests an intimate dimer with the dSRNA binding across both subunits and with the CTD forming dimer contacts rather than interacting with RNA. Finally the authors propose a model in which MDA5 co-operatively assembles on long dsRNAs forming a filament in which the externally projecting CARDs form a polymeric scaffold that can interact with the proposed 'prion'-like filaments of MAVS.

The various analyses are generally thorough and convincing and the results are generally supported by the data. The most problematic part is with the detailed SAXS analysis of the 2:1 complex which it is claimed supports a particular asymmetric arrangement of the two protein molecules with respect to the dsRNA (Figure 5F). Indeed I doubt whether such a precise model is justified. It is not at all clear what justifies presenting Figure 5E/F as 'the' model compared with the variety of models shown in SuppFig 3A apart from some unclear process of filtering and use of certain restraints to constrain the homology modelling. In particular no fits of the model scattering curve to the data are shown, no chi2s quoted, nor calculated model parameters such as Rg (in contrast Figure 4F is much more convincing). It is known from the recent crystal structure of RIG-I that RNA can be bound with or without ATP bound with the helicase in an open (flexible) state or closed (ordered) state. It would be important to show that models based on the known crystal structures of RIG-I were or were not excluded by the data. Furthermore, given that the resultant model for dsRNA binding is unexpected i.e. very different from that found by crystallography for RIG-I, some additional supporting data is needed. For instance the model suggests that neither the CTD nor Hel2i domains are involved in RNA binding but in inter-dimer protein-protein interactions. Is the CTD needed for this dimer formation? Does mutation of the putative RNA binding site of the CTD as mapped by NMR change the behaviour of MDA5. Is the SAXS model changed when (non-hydrolysable) ATP and RNA are both present?

Referee #2

Berke and Modis performed a variety of biophysical analyses of MDA5 and its complex with dsRNA. They solved the crystal structure of a unique helicase insertion (Hel2i) domain in MDA5, which is quite similar to the Hel2i domain of RIG-I. Although the structure of Hel2i does not provide much more insight than what is learnt from the RIG-I structures, which include full-length protein and RNA bound protein fragments, the authors of this paper are to be commended for having done a good job in characterizing MDA5:dsRNA complexes using biophysical techniques, including

AUC, SAXS and EM. They show quite clearly that MDA5 and short dsRNA forms a 2:1 complex with some degree of cooperativity. More interestingly, they found that MDA5 forms filaments on long dsRNA in an ATP dependent manner. Overall, the results provide new structural insight into MDA5 function, and may be appropriate for EMBO Journal after some revisions.

Specific Points

1. The authors stated several times that MDA5 CARDs cannot induce signaling, in contrast to RIG-I, citing Yoneyama et al paper. This is not correct. The Yoneyama paper shows clearly that both RIG-I and MDA5 CARDs induce IFNs.

2. The authors stated that there is no interaction between CARDs and helicase domains in MDA5, suggesting that MDA5 is regulated differently from RIG-I. This argument is not convincing. It is quite possible that in full-length MDA5 (not broken fragments) there is an interaction between CARDs and other parts of the molecules that keep the protein inactive in the absence of dsRNA.

3. The authors propose that MDA5 binds to dsRNA in an anti-parallel fashion (Figure 7). There is really no strong evidence for this for the full-length protein.

4. The ATP-dependent disassembly of MDA5 filament is counter-intuitive because it is known that ATP is required for MDA5 to induce IFN. The speculation about MDA5 regulation by metabolism in the Discussion seems way off base.

5. For the cooperativity analysis, the Kd changes from 187nM to 124nM is very mild, and it is better and more convincing if there is s.d. out of the analysis. The statement (page 7:line7) that "independent sites would predict Kd1/Kd2=4" is not intuitive.

6. In the EM images in Fig. 6A&B, it is better to have the insets in them showing the coated RNA filaments at a much higher magnification in order to show the even binding in A and the uncoated arrow-pointed regions in B. A control of uncoated dsRNAs would be helpful if it is possible to see them. The samples in Fig. 6D&E are very dense and make it difficult to discern the difference between them. Control panels of MDA5 alone and RNA alone should also be shown.

7. Page 13, line 2. "We note that RNA ligands smaller than 25-bp do not significantly activate RIG-I". This is not true. It has been shown that 19bp 5'-ppp-dsRNA can strongly activate RIG-I (Schlee et al., Immunity, 2009).

Referee #3

This is an excellent article describing biochemical and structural analysis of RNA recognition and signaling by MDA5, a viral RNA sensor in the same family as RIG-I and sharing structural similarity to the DNA repair helicase Hef. There are several key findings from the article, 1) cooperative binding, 2) comprehensive SAXS analysis on different forms and different complexes, and 3) formation of MDA5/RNA filaments. These studies have greatly advanced our understanding of MDA5-mediated signaling. In the context of the recent series of structural and mechanistic findings on RIG-I, this manuscript is timely and illustrates the differences in signaling mechanism by MDA5.

This reviewer only has two minor comments.
1. Could the authors derive a binding Hill coefficient of MDA5 for longer RNA? It is expected that this will be much larger than what is reported here for the short RNA fragments.
2. Given that the RIG-I structure is known and that MDA5 is more similar to RIG-I than to Hef, it might be useful to model the SAXS data based on models from RIG-I.

Referee #1:

1. *"The most problematic part is with the detailed SAXS analysis of the 2:1 complex which it is claimed supports a particular asymmetric arrangement of the two protein molecules with respect to the dsRNA (Figure 5F). Indeed I doubt whether such a precise model is justified. It is not at all clear what justifies presenting Figure 5E/F as 'the' model compared with the variety of models shown in SuppFig 3A apart from some unclear process of filtering and use of certain restraints to constrain the homology modelling. In particular no fits of the model scattering curve to the data are shown, no chi2s quoted, nor calculated model parameters such as Rg (in contrast Figure 4F is much more convincing)."*

We used a series of complementary analyses to obtain the clearest possible picture of the 2:1 MDA5:RNA complex. To obtain an unbiased low-resolution structure of the complex we used dummy atom modeling based on the SAXS data. The initial one-phase dummy atom model provided the overall shape of the complex using data to a maximum q value of 8/Rg (0.168 Å^{-1}). An average of 15 models is shown in Fig. 5D. The variability between models was low, with Normalized Spatial Discrepancy, NSD = 0.66 for the 15 models. To determine the distribution of protein and RNA within the structure, we performed three-phase dummy atom modeling combining SAXS data for each component and the complex to a maximum q value of 0.3 Å^{-1}. The eight three-phase models that were generated show a large degree of variability (NSD = 1.42), however in each of them the RNA phase is located at the core of the complex with the two protein phases on opposite sides surrounding the RNA (Supplementary Fig. 3A). The averaging of models was performed with the DAMAVER suite. Models with NSD to the other models greater than twice the variation from the overall average NSD were discarded during averaging by DAMAVER (one model was discarded). Fig. 5E simply represents the average of the highest occupancy positions of the seven remaining models. The software and cutoff levels for the q values and NSD are widely accepted and frequently used in the SAXS field. No other filtering or constraints were used in calculating the average model shown in Fig.5E.

The major conclusion we drew from the dummy atom modeling was that the two MDA5 molecules surround a central RNA duplex. We did not use the dummy atom models to select our final homology model of the MDA5-RNA complex. We have modified the text (p. 12, lines 4-9) to state more explicitly that, due to the observed variability of the three-phase models that the average model shown in Fig. 5E was derived from, it was not possible to "…interpret these models beyond the conclusion that two MDA5 molecules surround a central RNA duplex" and therefore "…we sought a complementary and independent modeling approach that explicitly exploited knowledge of helicase structure."

The final SAXS model of the 2:1 MDA5:RNA complex was obtained by fitting homology models of the MDA5 helicase domains and CTD to the SAXS data. As we now note in the text (p. 12, line 7), this approach was completely independent of the dummy atom modeling. Superfamily 2 helicases are known to bind RNA and nucleotide through eight conserved motifs, with the RecA-like Hel1 and Hel2 domains contacting the RNA 3' and 5' ends, respectively (Pyle, 2008, cited in the text). In particular, conserved threonine residues in motif Ib in Hel1 and in motif V in Hel2 play key roles in RNA recognition. Using proximity of these two threonines to phosphates at i and i+3 positions of the RNA as distance restraints (<8 Å), we placed helicase domain models in every possible position along a 20-bp poly(A:U) model dsRNA, generating 16 different clash-free molecular models of the MDA5-RNA complex. We then used the SAXS data to place the CTDs and score each of the models. The recent structures of Rig-I (Kowalinski et al 2011, Jiang et al 2011, Luo et al 2011; now cited in the text) validate the constraints that we used since in each structure, the threonines in motifs Ib and V are within 8 Å of the RNA (although in the structure by Luo et al, motif V is disordered). Model "[21,1]" had the lowest c score (c = 2.60 vs c = 2.76 for the next best model). This model was further refined by allowing small movements of each helicase domain while maintaining distance constraints. This final model is presented in Fig. 5F and had a c score of 1.35. This model differs from the dummy atom models in that it explicitly incorporates structural knowledge of helicase-RNA interactions. The overall shape and

arrangement of the protein and RNA within the complex is consistent in the dummy atom model and in the homology model. Since the two models were generated independently, and since the dummy atom model is free of model bias, we interpret this consistency as being mutually supportive of both models (Fig. 5E,F).

We have clarified these points in the text (p. 12, throughout the page, and in the legend to Fig. 5) and we now present in a new supplementary figure, Supplementary Fig. 5, the complete set of structural models that were generated and scored prior to selection of the final model for Fig. 5F. The fit of each model to the SAXS data is also given (as a c value). We have also included in Supplementary Fig. 5, as requested by the referee, the parameters for the fit of the final refined model to the data, including the calculated and experimental SAXS scattering curves, c value and calculated Rg and Dmax values.

2. *"It is known from the recent crystal structure of RIG-I that RNA can be bound with or without ATP bound with the helicase in an open (flexible) state or closed (ordered) state. It would be important to show that models based on the known crystal structures of RIG-I were or were not excluded by the data."*

We now show in Supplementary Fig. 5C a fit of a dimeric MDA5 homology model based on the RIG-I models determined by Kowalinski et al (2011) (helicase domain bound to 19 bp RNA) and Jiang et al (2011) (helicase domains + CTD bound to 14 bp RNA) both of which contained nucleotides. The dimeric MDA5 model was generated by superimposing the RNA ligand of each RIG-I structure onto both ends of an ideal 20 bp RNA duplex. In this configuration, the contacts of the helicase domain and CTD were identical to those in the crystal structures and the overall helicase dimer resembled the "[17,37]" MDA5 dimer model that was generated for refinement against the SAXS data (see Supplementary Fig. 5A and response to previous comment). Homology models of MDA5 domains were then superimposed onto the two RIG-I subunits. However, the structure of the model fit very poorly to SAXS data for MDA5 as evident from the extremely high c value of 22.3.

A calculated SAXS curve for the RIG-I-based model is now shown in Supplementary Fig. 4B, along with residuals for the fit to the experimental SAXS curve. Rg, Dmax and c value for the fit are now listed in Supplementary Fig. 4D. The poor fit of the RIG-I-based model to the MDA5 SAXS data is noted on p. 13 lines 11-14. We also added a paragraph in the Extended Materials and Methods (p. 16 lines 7-15) describing how the models based on the RIG-I crystal structures were generated.

An MDA5 homology model based the RIG-I structure from Luo et al (2011) resulted in a large number of steric clashes when it was assembled into possible dimeric configurations on a 20 bp RNA. This model was therefore not refined and is not shown in Supplementary Fig. 4.

3. *"Furthermore, given that the resultant model for dsRNA binding is unexpected i.e. very different from that found by crystallography for RIG-I, some additional supporting data is needed. For instance the model suggests that neither the CTD nor Hel2i domains are involved in RNA binding but in inter-dimer protein-protein interactions. Is the CTD needed for this dimer formation? Does mutation of the putative RNA binding site of the CTD as mapped by NMR change the behaviour of MDA5. Is the SAXS model changed when (non-hydrolysable) ATP and RNA are both present?"*

Indeed, the homology model of RNA:MDA5 complex refined against the SAXS data suggests that the CTD and Hel2i domains do not bind RNA directly but instead participate in protein:protein interactions, which may be responsible for the positive cooperativity of RNA binding. To test this hypothesis we now include as requested an analysis of the effect of the K983E mutation in the CTD on the affinity and cooperativity of RNA binding by MDA5. K983 is located in the center of the RNA binding surface of the MDA5 CTD as determined by NMR and binding studies in the work of Li et al (2009, now cited in the text). In electrophoretic mobility shift assays (EMSAs), MDA5 K983E had the same affinity for 20-bp RNA (K_d = 277 ± 14.8 nM) but the binding cooperativity was increased (Hill coefficient, n = 2.08 ± 0.2, versus n = 1.7 for wild type MDA5). In the study by Li et al (2009) the K983E mutation resulted in complete loss of RNA binding by the CTD (although with a Kd = 3 µM,

binding was weak to begin with). Our data suggests that in the context of full-length MDA5, the mutant CTD does not contribute significantly to the affinity of MDA5 binding to RNA. In contrast, EMSA with the MDA5 helicase domain only (CARD and CTD removed), resulted in a substantial loss of affinity and cooperativity of binding (K_d = 1450 ± 49.5 nM, n = 1.01 ± 0.03). These results are consistent with our SAXS model and suggest that the major function of the CTD in MDA5 signaling is to generate positive binding cooperativity through protein:protein contacts rather than direct RNA recognition. We do concede, however, that the SAXS models do not contain enough predictive power to exclude the possibility that either the CTD or Hel2i domain may still bind RNA directly. This is now noted in the text (p.12 lines 24-25).

The RNA binding curves for the K983E mutant and the helicase domain from which the affinity and cooperativity were calculated have been added to Fig. 1B, and representative gels are shown in Supplementary Fig. 1. The RNA binding parameters of the K983E mutant and helicase domain are now described on p. 5 lines 11-15.

SAXS analysis of the MDA5 helicase domain in the presence of the ATP analogs AMPPNP or ATPgS showed no significant differences in one dimensional scattering compared to solutions without the analogs. This suggests that, in the absence of RNA, there were no major changes in conformation of the unliganded helicase domain. In our EM analysis of MDA5 filaments on RNA, we also saw no changes in the morphology of the filaments in the presence of AMPPNP. Conformational changes that occur in RNA bound MDA5 upon adding ATP analogs may be too subtle to detect using low resolution methods such as SAXS and negative-stain EM. We therefore believe that further SAXS experiments would not readily yield additional structural insights on the effect of nucleotide on the MDA5-RNA complex or significantly alter the conclusions drawn in this paper. Since the addition of ATP analogs did not alter the appearance of SAXS models or EM data we have not included any additional figure panels. However, in response to this comment, we have added a note in the text that the presence of AMPPNP or ATPgS had no significant effect on the overall scattering and shape parameters of the helicase domain (p. 9 lines 4-6).

Referee #2:

Specific Points:

1. "*The authors stated several times that MDA5 CARDs cannot induce signaling, in contrast to RIG-I, citing Yoneyama et al paper. This is not correct. The Yoneyama paper shows clearly that both RIG-I and MDA5 CARDs induce IFNs.*"

    The text has been updated to correct this error in both of the instances that we could find (p. 10 line 17 and p. 16 line 6).

2. "*The authors stated that there is no interaction between CARDs and helicase domains in MDA5, suggesting that MDA5 is regulated differently from RIG-I. This argument is not convincing. It is quite possible that in full-length MDA5 (not broken fragments) there is an interaction between CARDs and other parts of the molecules that keep the protein inactive in the absence of dsRNA.*"

    Our SAXS analysis of full-length MDA5 shows that the protein is highly flexible with a significant population of the structural ensemble having a largely extended conformation (Fig. 5C). Since full-length MDA5 has significantly more conformational flexibility than CARD-deleted MDA5 based on SAXS data (in the absence of RNA), we conclude that the CARDs are unlikely to be tightly associated with other domains in the molecule. Still, we agree with the referee that we cannot completely rule out the possibility that the CARDs form stable interactions with other domains, or that there may be transient interactions between the CARDs and other domains in the context of full-length protein, which may keep the protein inactive in the absence of dsRNA. However, our assertion that MDA5 and RIG-I are regulated differently is based on the differences in the structures and behaviors of the RIG-I and MDA5 CARDs rather than on a lack of interaction between the CARDs and helicase domains *per se*.

Our data do demonstrate convincingly that differences exist between the CARD domains of MDA5 and RIG-I. For example, Phe540, which is important in the RIG-I CARD2-Hel2i interface, is an arginine residue in all MDA5 sequences. Mutation of Phe540 in duck RIG-I abrogates CARD-helicase interactions and increases basal signaling in overexpressing cells (Kowalinski et al, 2011). Moreover, the RIG-I CARDs and Hel2i interacted in a relatively stringent pulldown experiment (Kowalinski et al, 2011) while the MDA5 CARDs and Hel2i failed to interact in the less stringent size-exclusion experiment that we used in our study.

3. *"The authors propose that MDA5 binds to dsRNA in an anti-parallel fashion (Figure 7). There is really no strong evidence for this for the full-length protein."*

The schematic model of MDA5 filament assembly on dsRNA in Fig. 7 illustrates the prototypical MDA5 dimer as antiparallel based on our SAXS-based MDA5 homology model of a CARD-deleted MDA5 dimer bound to a 20 bp RNA. For the reasons outlined in the previous response, we have proposed that in full-length MDA5 the CARDs are flexibly attached to the helicase domains and located on the outside of the filaments. In further support of this notion, the binding affinities of full-length and CARD deleted MDA5 were similar and the full-length MDA5 filaments were thicker the CARD-deleted MDA5 filaments as judged from the EM images (Fig. 6 and Supplementary Fig. 6). We therefore expect that the RNA binding mode of full-length MDA5 is similar to that of CARD-deleted MDA5, with the CARDs only adding additional mass to the exterior of the filaments. Nevertheless, we agree with the referee that it cannot be ruled out the CARDs or the CARD-Hel1 linker may form stable interactions with each or other domains in the context of full-length protein bound to RNA, hence possibly resulting in a different RNA binding configuration. We have therefore added sentences in the legend to Fig. 7 stating clearly that the binding mode of full-length MDA5 may differ from that shown in Fig. 7.

4. *"The ATP-dependent disassembly of MDA5 filament is counter-intuitive because it is known that ATP is required for MDA5 to induce IFN."*

As we note in the text (p. 17, line 15) the role of ATP hydrolysis in MDA5 signaling is still controversial. Ectopic expression of an MDA5 mutant lacking ATPase activity was shown by Yoneyama et al. (2005) to neither augment nor suppress intrinsic IFN signaling when stimulated by Newcastle Disease Virus (NDV), while an analogous mutation in RIG-I showed a powerful dominant negative phenotype. In contrast however, Bamming & Horvath (2009) showed that two ATPase-deficient MDA5 mutants were constitutively active, based on IFN production upon stimulation with poly(I:C) RNA. The different results of the two studies results may be due to the cell lines or methods of stimulation used. We have added two sentences (p. 17 lines 15-19) to summarize these reported properties of ATPase-deficient MDA5 and RIG-I mutants.

5. *"The speculation about MDA5 regulation by metabolism in the Discussion seems way off base."*

We have significantly shortened the speculation about MDA5 regulation by metabolism (last 6 lines of the Discussion, p. 17-18). The remaining passage (6 lines) is clearly marked as speculation. Given the physiological range of ATP concentrations governing MDA5 filament formation and the manipulation of host metabolism by certain viruses, it is our view that this point is still worth mentioning.

6. *"For the cooperativity analysis, the Kd changes from 187nM to 124nM is very mild, and it is better and more convincing if there is s.d. out of the analysis. The statement (page 7:line7) that "independent sites would predict Kd1/Kd2=4" is not intuitive."*

We state in the text that independent, non-cooperative ligand binding sites would predict Kd2 / Kd1 = 4. We have added a paragraph in the Extended Materials and Methods (pages 12-13) showing the derivation of this relationship, which arises from the equations describing the microscopic and macroscopic dissociation constants for individual binding events to the two binding sites. Smaller numbers of this ratio indicate positive cooperativity (ie. binding affinity

of the second site is increased upon occupancy of the first site) whereas larger numbers indicate negative (ie. affinity of the second site is decreased upon occupancy of the first site).

To rigorously address the significance of our cooperativity analysis, we now include in a new supplementary figure, Supplementary Fig. 2, an error surface projection of the ratio of macroscopic dissociation constants, K_{d2}/K_{d1}, from the analytical ultracentrifugation analysis. In this analysis, F-statistics are used to determine a critical c^2 value for a fit above a confidence threshold (e.g. 68% or 1 s; 95% or 2 s; and 99% or 3 s). The parameter of interest is then fixed at a test value and other parameters are fitted to minimize the global c^2 to determine the confidence interval of the parameter of interest. The optimal value for K_{d2}/K_{d1} was 0.663 using this approach. A value of 4 (for the non-cooperative case) was outside the 95% confidence interval threshold. 1-s confidence intervals for K_{d1} and K_{d2} were also calculated with this method (K_{d1} = 92-368 nM; K_{d2} = 83-173 nM) and are now included in the text (p. 7 lines 10-12).

7. *"In the EM images in Fig. 6A&B, it is better to have the insets in them showing the coated RNA filaments at a much higher magnification in order to show the even binding in A and the uncoated arrow-pointed regions in B. A control of uncoated dsRNAs would be helpful if it is possible to see them. The samples in Fig. 6D&E are very dense and make it difficult to discern the difference between them. Control panels of MDA5 alone and RNA alone should also be shown."*

We have added insets to Fig. 6A and 6B showing the RNA filaments at higher magnification as requested. The original micrograph in Fig. 6B was replaced with a new micrograph that shows more clearly the uncoated regions of the RNA. The original micrographs in Fig. 6D and 6E were replaced with new images that were taken using more diluted preparations of the samples. The shorter size of the filaments upon addition of ATP is now clearly apparent. As requested, RNA and protein only controls are now shown in Supplementary Fig. 6, but neither images contains any interpretable features.

8. *"Page 13, line 2. "We note that RNA ligands smaller than 25-bp do not significantly activate RIG-I". This is not true. It has been shown that 19bp 5'-ppp-dsRNA can strongly activate RIG-I (Schlee et al., Immunity, 2009)."*

In the paper by Schlee et al (2009), strong signaling in more than one assay was only seen with ligands larger than 23 bp. Ligands shorter than 23 bp failed to activate signaling in some of the assays, which may have been due to the specific composition of the ligands and/or the assay conditions. Nevertheless, as the referee points out, in one experiment in the study by Schlee et al signaling was observed with a 19 bp 5'-triphosphate dsRNA. Accordingly, we now cite the Schlee et al study in the text, and we have changed the passage in question in the text (p. 13 lines 16-18) to read: "Moreover, the threshold length for 5'-triphosphate, blunt ended dsRNA to induce significant RIG-I-dependent signaling is 19-21 bp (Schlee et al, 2009; Takahasi et al, 2008)."

Referee #3:

Minor Comments:

1. *"Could the authors derive a binding Hill coefficient of MDA5 for longer RNA? It is expected that this will be much larger than what is reported here for the short RNA fragments."*

Longer RNA ligands should indeed have a larger Hill coefficient because of the larger number of available MDA5 binding sites per ligand. Using EMSAs as we did for the short RNA ligands, we measured the affinity and cooperativity of MDA5 binding to poly(I:C), which is a heterogeneous mix of dsRNA sizes from ~100-1000 bp. The derived binding affinity was similar to that for shorter RNA (K_d = 652 ± 34 nM versus 360 for a 24-bp dsRNA) and the Hill coefficient was modestly higher (n = 2.26 ± 0.25, versus n = 1.5 for the 24-bp dsRNA). We attribute the somewhat lower than expected Hill coefficient for poly(I:C) to the diffuse nature of the signal observed for poly(I:C) on the EMSA gel, which made it difficult to accurately

calculate the bound and unbound signal. The EMSA data for MDA5-poly(I:C) binding are now shown in Supplementary Fig. 1E,F and the binding parameters are listed in the text (p. 5 lines 15-17).

2. *"Given that the RIG-I structure is known and that MDA5 is more similar to RIG-I than to Hef, it might be useful to model the SAXS data based on models from RIG-I"*

We note that our homology modeling was actually performed incorporating the available structures of the Hel1, Hel2i and CTD domains of MDA5. Only the Hel2 domain was modeled based on Hef. The Hef structure was also used to initially position the domains. This positioning was then refined during the normal mode refinement using SAXS data. Nevertheless, based on this comment and on Comment #2 from Referee 1 we performed a fit of a dimeric MDA5 homology model based on the RIG-I models determined by Kowalinski et al (2011) (helicase domain bound to 19 bp RNA) and Jiang et al (2011) (helicase domains + CTD bound to 14 bp RNA). The structures of the individual domains were not significantly changed in the RIG-I-based model, however using a dimer generated from the RIG-I arrangement around RNA fit very poorly to SAXS data for CARD-deleted MDA5 bound to AU20. For additional information and a more detailed discussion, see our response to Comment #2 from Referee 1 and Supplementary Fig. 4B-D.

10 January 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #1 to review the revised version and I have now received the comments back. As you can see below, referee #1 appreciates the introduced changes and support publication here. I am therefore pleased to proceed with the acceptance of the paper. You will receive the formal acceptance letter shortly.

Editor
The EMBO Journal

REFEREE REPORT

Referee #1

I am satisfied that the authors have adequately addressed the referees concerns and the paper is now stronger and suitable for publication.