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Structural variability in the RLR-MAVS pathway and sensitive detection of viral RNAs

Qiu-Xing Jiang

Department of Microbiology and Cell Science, University of Florida 1355 Museum Drive, PO Box 110700, Gainesville, FL 32611, USA.

Abstract

Cells need high-sensitivity detection of nonself molecules in order to fight against pathogens. These cellular sensors are thus of significant importance to medicinal purposes, especially for treating novel emerging pathogens. RIG-I-like receptors (RLRs) are intracellular sensors for viral RNAs (vRNAs). Their active forms activate mitochondrial antiviral signaling protein (MAVS) and trigger downstream immune responses against viral infection. Functional and structural studies of the RLR-MAVS signaling pathway have revealed significant supramolecular variability in the past few years, which revealed different aspects of the functional signaling pathway. Here I will discuss the molecular events of RLR-MAVS pathway from the angle of detecting single copy or a very low copy number of vRNAs in the presence of non-specific competition from cytosolic RNAs, and review key structural variability in the RLR / vRNA complexes, the MAVS helical polymers, and the adapter-mediated interactions between the active RLR / vRNA complex and the inactive MAVS in triggering the initiation of the MAVS filaments. These structural variations may not be exclusive to each other, but instead may reflect the adaptation of the signaling pathways to different conditions or reach different levels of sensitivity in its response to exogenous vRNAs.

I. Introduction

For the purpose of drug development, many publications have demonstrated that various studies, both experimental and theoretical, can provide very useful information about potential drug targets, including their identification, validation and applications in drug discovery¹. Different druggable targets need investigations from various different angles and using different tools. For example, the five-step rules proposed by Chou in 2011² should be considered when various prediction methods for proteome / genome analysis are developed or applied^{3–20}. For structure determination of membrane proteins that are important for rational drug design, high-resolution NMR technique^{21–35}, X-ray crystallography^{36–53} and cryo-electron microscopy (cryoEM)^{54–71} are all quite useful nowadays. For structure-based drug design^{72–78}, structural bioinformatics is needed^{79, 80}. In order to make drugs specific for multiple targets, a new trend in pharmaceutical industry, strategies using multiple labels are desired^{81–88}. Intuitive graphical approach has greatly facilitated the studies of inhibitors against HIV (human immunodeficiency virus) reverse transcriptase and will be suitable for broader applications^{89–101}. For investigations of various posttranslational modifications

(PTM) in specific targets that are druggable for treatment of either cancers or other high-load diseases^{4, 5, 8, 11, 14, 81, 83, 88, 102–112}, tools like PseAAC^{113, 114} and PseKNC approach^{115–117} may generate unique insights. All these aspects and tools are suitable to identify which players of the signaling pathways in the immune responses are good druggable targets, obtain structural information to perform rational drug-design, especially multi-target drugs, combine both *in silico* optimization and *in vivo* testing of lead drugs, and identify key regulatory proteins of posttranslational modifications that regulate sensitivity or specificity of molecular signaling. Here we will focus on one of the key innate immunity pathways.

Innate immunity refers to the defensive processes cells launch against exogenous microorganisms, such as viruses, bacteria, fungi, etc.^{118, 119} To reliably detect exogenous microorganisms, cells have evolved a set of intracellular and extracellular sensors. The retinoic acid-inducible gene I (RIG-I, for the coded protein)-like receptors (RLRs) are main intracellular sensors for at least two different types of pathogen-associated molecular patterns (PAMPs) that are derived from exogenous viral RNAs --- either the stem-loop dsRNAs with 5'-triphosphates or 5'-diphosphates, or the poly U/UC sequences^{120–124}. RLRs, including RIG-I and melanoma differentiation-associated gene 5 (MDA5; the coded protein), thus form a family of intracellular RNA sensors. The protein coded by the Laboratory of Genetics and Physiology 2 gene (LGP2) is a different RLR because it does not have N-terminal tandem caspase recruitment domains (CARDs; top of Fig. 1). It seems to play a negative regulatory role by competing with RLRs. Alternatively, in *Lgp2* knockout or mutated mice and cells, LGP2 acts upstream of RIG-I and MDA5 as a positive regulator by facilitating RLR-based recognition of vRNAs¹²⁵. Binding of RIG-I and MDA5 to viral RNA fragments signals to the mitochondrial antiviral signaling protein (MAVS; also known as IPS-1, VISA or CARDIF) on the surface of mitochondria, which, once activated, forms linear helical filaments and allows better exposure of the long-unstructured region between its N-terminal CARD domains and the single transmembrane segment (alpha-helix) at its very C-terminal end (Fig. 1)^{126–133}. So far, the mechanistic understandings of the RLR-MAVS functions in time and space remain incomplete^{134, 135}, which, nonetheless, does not mitigate the importance of applying systematic tools to searching suitable druggable targets, but instead, emphasizes the necessity of such investigations.

Multiple factors are involved in the RLR-MAVS signaling. Poly-ubiquitins are known to be important for efficient activation of RIG-I^{135–140}. Deubiquitinases may deactivate the active RIG-I and suppress cellular immune response^{141–143}. ISGylation, phosphorylation, autophagic clearance, protein degradation and ER-mitochondria contact and their exchange of materials and signals may be involved in signal regulation and contribute to the homeostatic balance in the RLR/MAVS pathway^{143–170}. A new adaptor protein, zyxin, was recently discovered to be important for bridging individual RLR / vRNA complexes and MAVS together in order to enhance successful RLR-MAVS interaction and the subsequent formation of MAVS filaments¹⁷¹. A long non-coding RNA (lnc-Lsm3b) was recently discovered to be a competitor against viral RNAs in inactivating the active RIG-I/RNA complex and exert a negative feedback in the RIG-I response, suggesting an important role of the RNA sensors in maintaining a fine balance between self and non-self RNAs^{172–177}. Various proteinaceous factors that are involved in maintaining healthy mitochondria or

removing unhealthy ones may also participate in regulating MAVS functions^{178, 179}. RLR-MAVS signaling may thus play a key role in metabolic balance between self-sustainment and defensive responses in different cells, which might be important for identification of druggable targets in the antiviral responses in a broader scope^{180–182}. Bioinformatic studies at different levels of the RLR-MAVS signaling are therefore justified.

There have been multiple reviews on the detection of various types of viruses by RLRs and the possible structural and regulatory mechanisms for signal transduction from the active RLR / vRNA complexes to MAVS^{124, 153, 178, 179, 183}. We will therefore focus on how the molecular events are likely to occur in a more or less physiological context and how this may achieve the detection of vRNA with high sensitivity and specificity. Structural studies using different methods have revealed variability in both the RLR-vRNA interactions and the engagement of active RLR/vRNA complexes with silent MAVS. Heterogeneity in composition and conformational states makes it difficult to achieve a self-consistent model that can explain all observations in literature^{183–189}. We will discuss mainly the structural diversity and corresponding functional variations in the RLR/RNA complexes, the interactions between the RLR / vRNA complexes and inactive MAVS, and the possible aggregation of MAVS filaments. Some of the key questions considered here include whether the RLR/MAVS is sufficiently sensitive for detecting single copy of a viral-RNA, the contradiction between high-sensitivity detection and the relatively low expression level of RLRs, the differences between the native minimal ligands and the minimal ligands in testtubes, the different states of the inactive full-length MAVS that is kept inactive by associating with other factors and the potential cost of failing to subdue MAVS in silence, the potential role of zyxin in the signaling^{171, 190, 191}, and the structural ambiguity in the MAVS CARD filaments. In order to gain more metaphysical insights, we will consider the problem from the expected (or desired) sensitivity of detecting one copy of viral RNA in the cytosol, and will move on to consider the variation in the length of the vRNAs, and the potential increase in copy numbers. When possible, thermodynamic considerations will be used to discuss the physiological significance of biophysical observations. These considerations are purposed to view the RLR/MAVS signaling from a physiological setting whose homeostatic regulation and high-sensitivity in detecting non-self pathogenic molecular patterns are fundamentally important. From a systems standing point, the key players in RLR/MAVS pathway and the regulatory factors for striking such a physiological balance will constitute a good collection of targets for medicinal chemistry and it is foreseeable that a systematic analysis using bioinformatic tools may cast the signaling pathway into a large network of intracellular signaling events and reveal new connections.

2. Structural heterogeneity in the RLR-RNA interactions and its functional implications

Depending on the vRNAs or synthetic vRNA-mimicking oligos, the RLR/RNA complexes can be monomers, dimers, tetramers, or oligomeric aggregates that may be inactive or may be capable of signaling to MAVS (Fig. 2)^{137, 187}. Currently data from different sources seem to suggest that monomers, dimers or tetramers might all be able to activate MAVS signaling *in vitro*, in cells, and / or in model animals. Analysis of viral RNAs in forming the 5'-pan

handle structures suggested that the pan-handle dsRNA segments are often too short to accommodate multiple copies of RIG-I. For example, the flu vRNA has a pan-handle of 12–13 bp in length¹⁹². Our cell-based assays showed that 22-mer dsRNA and 51-mer stem-loop RNAs are sufficient to form active RIG-I dimers (Fig. 3) and activate the RIG-I/MAVS pathway (Fig. 4)^{131, 193}. With synthetic ligands, we did not observe activities from dsRNAs that are shorter than 19 bp, which is the minimal length for the binding to two RIG-I monomers (Fig. 4). Single particle EM studies found that 51-mer RNA / RIG-I complex appears to be a parallel dimer (Fig. 3). Parallel dimers of RIG-I / RNA complexes were proposed by others before¹⁸⁸. We have unpublished data showing short 5'-ppp-ssRNA (~10 nucleotides) can induce the formation of parallel RIG-I dimers that apparently were in an inactive state, consistent with the RNA-seq data from native RIG-I¹⁹⁴. These observations suggest that structural studies might catch the RLR-RNA complexes in either inactive or active states, which sometimes might be difficult to distinguish. Practically, structures of RIG-Is in complex with active RNA ligands are generally assumed to be in an active state. Because of potential biophysical artifacts from *in vitro* studies, it might be cautious to note that cell-based reporter assays might not necessarily reflect the property of the most abundant species of the RIG-I/RNA complexes sequestered for structure determination, or different cell lines used for the assays might differ in RIG-I expression level (see next), which might lead to cell-specific variations in the assay results and could account for the different minimal lengths of dsRNAs identified by different labs (see the following). With the recent progress in cryoEM and chemically functionalized carbon film (ChemiC) for EM imaging^{195, 196}, it will be feasible to selectively enrich RLRs onto RNA-presenting ChemiC films, characterize the structures of the RLR in the capping position, and then add ATP to initiate RLR transition into the active conformation along the dsRNA. Conformation-specific study by cryoEM single particle analysis of monomers, dimers and tetramers of full-length RLRs will likely provide key insights into the differences between the inactive RLR/RNA complexes and the active ones.

Under physiological conditions, the intracellular concentration of RIG-I may be fairly low. In a typical cell, if RIG-I is expressed at ~30,000 copies, pretty abundant for a signaling molecule, it will give rise to a concentration of ~100 nM. For reliable detection of single copy or a few copies of viral RNAs in the cytosol, high binding affinity (~10 nM or better) would be required. However, at the initial bound state, the capping position, the C-terminal domain of RIG-I (CTD; also called the regulatory domain -- RD) binds to the 5'-ppp only with an affinity of ~200 nM^{197, 198}, which would not be suitable for high-affinity binding and sensitive detection of vRNAs. To overcome this thermodynamic limit, RIG-I may utilize the enzyme activity of its helicase domain to move it away from the capping position^{184, 199}, leading to a more stable state driven by the energetic input from ATP hydrolysis. In this sense, the helicase activity appears necessary to prevent the RIG-I / 5'-ppp-vRNA complex from falling apart in the capping position, which would likely serve a reliable positive mechanism for detecting low copies of viral RNAs in cytosol. To test this mechanism, experimental evidence will be needed to determine the basal copy number of RIG-I or MDA5 proteins at the resting state, the binding affinity between full-length RLRs and 5'-ppp-RNAs, the reaction rate of the RLR helicase in moving along the dsRNA, and the threshold numbers of viral RNAs that are sufficient to trigger successful RLR binding

and activation in cells. Alternatively, from a kinetic standing point, adapter molecules that can sequester RLRs into a smaller volume or help orient RLRs and viral RNAs for productive collisional interactions are expected to enhance RLR sensitivity and efficiency in detecting vRNAs. Similarly, an adapter protein that stabilizes the RLR / vRNA complex may achieve similar effects. So far, no such adapter molecule is known yet.

Even though biochemical and structural studies of tandem CARDS have seemingly favored the proposal that the tetrameric RLR / RNA complexes might be the minimal unit (with a minimal length of ~36 bp dsRNA) in using the RLR CARD octamer to nucleate the formation of the MAVS CARD filaments, experimental evidence has showed that the MAVS CARD filaments grew out of spheroid RLR/RNA aggregates, instead laterally as branches from the well-prepared RLR / RNA filaments¹³⁷. Testtube data added further controversy on the requirements for polyubiquitin (poly-ub) for successful RIG-I activation and signaling to MAVS because the RLR / RNA complexes *in vitro* were able to induce MAVS filaments without poly-ub^{137, 138}, indicating that tetramers or high-order filamentous oligomers might not be physiological. When 5'-ppp-stem-loop RNAs of a 10-bp stem (SLR10) or of different stem lengths were introduced into cultured cells, SLR10 was the shortest active form, consistent with the panhandle length in the flu viral RNAs¹⁹². When SLR10 RNAs were injected intravenously into live mice, they were able to induce potent immune response in 5 hours²⁰⁰. SLR10 has a length that is equivalent to the footprint of only a RIG-I monomer. It was not observed that at high concentrations the RIG-I / 10-bp dsRNA complexes were able to stack linearly with each other in order to form the proposed tetramers; nor is it expected that the 10-bp dsRNAs would be concatenated inside cells for making longer dsRNAs to facilitate filament formation and activate RLRs. In my laboratory, the 51-mer stem-loop 5'-ppp-RNA has a segment of ~22 bp dsRNA, and it is sufficient to interact with two RIG-I to form stable dimers, which in the presence of poly-ubiquitins (poly-ub), forms disordered aggregates, instead of stacking together in a head-to-tail fashion into linear filaments. Further, biochemical reconstitution or biophysical studies in the past have all used large amounts of materials that are probably not physiological, adding extra difficulty in making direct application of the biophysical analysis of RLR / RNA complexes to *in vivo* mechanistic events. Together, available evidence suggests that the disordered amorphous aggregates might be the functional state of the RIG-I / RNA / poly-ub complex, instead of the linear filaments. The aggregates might contain active monomers, dimers or tetramers. This parsimonious explanation raises the question on whether the biophysical analysis so far has revealed a truly physiologically relevant mechanism or not.

3. Potential structural variability in inactive MAVS

MAVS is inserted in the outer membrane of the mitochondria and can interact with many different partners. In the resting state, MAVS is kept silent. It is known that multiple proteins interact with MAVS on the surface of mitochondria and suppress its activity¹⁷⁸. NLRX1 presumably interacts with RIG-I and keeps it from interacting with silent MAVS^{201, 202}. Mitofusin 2 (MFN2), which is involved in fusion of mitochondria, inhibits RIG-I/MAVS signaling, probably by stabilizing silent MAVS^{203, 204}. MFN2 might promote the MAVS signaling after activation by promoting mitochondrial fusion, which appears to be necessary for forming long MAVS filaments and redistributing MAVS among mitochondria. The

anchorage of MAVS on mitochondria reduces the entropy of individual molecules such that reactions based on mass action may gain energetic advantage by ~ two orders of magnitude. Truncated forms of MAVS, including mini-MAVS, may be important for keeping the individual MAVS from being spontaneously activated²⁰⁵. So is the separation of silent MAVS among a large number of mitochondria in order to keep the copy numbers low in each mitochondrion. Other proteins may also interact with MAVS in the absence of viral infection. The general needs for keeping the effector-binding sites in the extended random-coil region of MAVS may be easy to satisfy because the probability for a long random-coil segment to keep these sites spontaneously in a fully-extended state would be very low, if not nil. So far there is no structural detail on how the inactive MAVS is maintained, even though it is expected that different binders to MAVS may together stabilize the random coil regions, which may be intrinsically disordered, and prevent them from presenting the binding motifs, and that there might be different structural complexes and different conformations of the inactive MAVS. Zyxin was recently found to be an adaptor protein for RIG-I interactions with MAVS, and appears to be capable of bringing 2–3 copies of MAVS together with one copy of RIG-I / vRNA complex, which might favor either monomer or dimeric RIG-I/vRNA complex as the minimally active form of RIG-I¹⁷¹. MDA-5 might follow the same principles, except that it might be more stable after oligomerization on longer dsRNA segments.

4. Structural complexity for the active MAVS filaments

Once MAVS is activated after viral infection, it forms short filaments on the surface of mitochondria (Fig. 5)¹³¹. In principle, the MAVS on the peroxisomes can do the same if there is a way to bring the molecules together. Fusion and fission of mitochondria is known to be important because the filaments grow by bringing inactive MAVS molecules from different mitochondria together, which eventually allows the formation of a small number of longer filaments (Fig. 5A). In a native MAVS filament, the N-terminal CARD domains form a rigid helical assembly and the MAVS transmembrane domains are retained inside the mitochondrial membranes such that the random-coiled region in the middle region of full-length MAVS will have a good chance to become fully extended. The fact that many of these middle segments aligned together might allow multi-dentate interactions between active MAVS and the oligomers of the downstream effectors, which will potentially increase the apparent affinity of such interactions by 2–3 orders of magnitude. Currently, it is not known whether there are strong tensions between the CARD filaments and the MAVS transmembrane segments that may extend the middle regions because the mitochondria may adapt themselves to the tension by changing their membrane shapes. There lacks good evidence to support that the RLRs or the adapter proteins (zyxin) stay bound with the active MAVS filaments. Both RLRs and zyxin are soluble and could easily leave the MAVS filaments. They may together engage more inactive MAVS to form more active filaments, which is another way to amplify the signal from RLR / vRNA to MAVS and may help enhance the sensitivity of vRNA detection. To better study this tri-partite system, it is better to reconstitute the inactive MAVS and its accessory proteins into an membrane system that facilitates oriented protein insertion²⁰⁶, where the interaction of zyxin with inactive MAVS and the recruitment of active RLR / vRNA complexes can be quantitatively analyzed.

Structural variability of activated MAVS filaments may exist in at least three areas: 1) The CARD filaments and their interaction with RLR / vRNA complexes; 2) Distribution and redistribution of the MAVS transmembrane segments and their possible effects on remodeling the mitochondrial membranes; and 3) Arrangements of multiple MAVS random-coil segments that are stretched from the CARD filaments toward the mitochondrial membrane. We don't have much data to discuss the second and third points, and will thus discuss the first point only.

MAVS CARD domains when in high concentration can self-interact and form helical filaments without the induction by the RLR/RNA complexes, which was thus named as a prion-like filament due to self-perpetuation¹³¹. In the filaments, there appear to be at least two different types of symmetry groups: C1 and C3 (Fig. 6)¹³². The C1 and C3 filaments differ in the selection rules for their helical symmetry: 9 turns per 32 units for C1 and 4 turns per 27 units for C3. The two groups working on the MAVS filaments started with different specimens^{131, 207}. Wu *et al* prepared the MAVS CARD filaments from refolded proteins out of denatured CARDS in inclusion bodies in bacteria. Xu *et al* formed the filaments from His-tagged MAVS CARD proteins purified from cultured cells, where the CARDS were properly folded in cytosol and filaments were formed when CARD protein in either normal high-salt or low-salt conditions^{131, 132}. Inside the cultured cells, the concentration of recombinant CARD protein was low and thus did not produce severe aggregates as seen in the inclusion bodies in bacteria. Upon purification, MAVS CARD started to form filaments after being concentrated. In the frozen specimens, detailed analysis by parallel classification found that more than 83% of the C1 filaments or more than 86% of the C3 filaments had an out-of-plane tilt angles smaller than 13.5 degrees. Among them, those with out-of-plane tilt angles smaller than 7.5 degrees were 64.3% for C1 filaments and 62.5% for C3 filaments¹³². It means that the heterogeneity between the two symmetry groups might probably not be due to high out-of-plane tilt angles, but more likely be caused by intrinsic symmetry ambiguity in forming the helical assemblies. We also found that once the C1 and C3 filaments were well separated and divided into two independent halves, perturbation of symmetry parameters in both rotational angles and axial rises allowed convergence to the stable symmetry parameters, which is a reasonable requirement for a convergent refinement to produce a stable solution of high likelihood^{208, 209}. It is worth noting that the conditions used by both groups to produce CARD filaments in testtubes were non-physiological to different degrees, which raises the question of whether the biophysical studies in testtubes or of overexpressed proteins reveal truly the filaments that are formed on the surface of mitochondria in cells. In practice, cryoEM analysis has an intrinsic limitation due to the fact that the final 3D reconstructions are usually derived from a small fraction of raw data, and the resulted structural models are then extended to a larger scope in order to represent a significant fraction of the structural assemblies. Such an experimental approach might carry intrinsic uncertainty, and thus the resulted structural models need to be cautiously evaluated. For the MAVS filament, both C1 and C3 filaments could account for nearly all point mutations introduced into the interaction interfaces between individual units¹³¹, which are still insufficient to exclude other alternative structural solutions. For example, *cross-seeding of MAVS CARD* filaments by other death domain filaments or prion proteins might be due to mass actions of releasing multiple MAVS CARDS, instead of molecular imprinting²¹⁰.

Further biophysical evidence will be needed to examine the symmetry properties of native, full-length MAVS filaments. High-resolution electron tomography that can reach ~1 nm resolution via sub-tomogram averaging may be pursued to resolve the structural features of native MAVS filaments in the future.

For the molecular imprinting hypothesis, the RLR / RNA tetramers fit well with one end of the C1 MAVS CARD filaments in order to initiate the filament formation autonomously¹⁸³. However, the MAVS CARD domains can spontaneously form filaments when there are enough of them. With zyxin as an adapter to bring one RLR / RNA complex and three MAVS CARD together, it might be possible to allow the initiation of a three-stranded MAVS filament from one copy of the active RLR/RNA complex, which could be a monomer or a dimer, suggesting that the RIG-I filaments on linear RNAs, or the tetrameric forms may not be essential, especially in cases where native vRNAs only have a short dsRNA segment (Fig. 2)¹⁹². Further, after the MAVS filament forms, the RIG-I/RNA complex could fall apart from the adapter so that the active RIG-I/RNA complex and the MAVS filaments would not be co-localized, differing from what was suggested by the molecular imprinting mechanism. This could explain weak or negative data in co-localizing RIG-I and MAVS on mitochondria after viral infection [for example, see data in reference²¹¹]. It may also explain why a low expression level of RLRs inside a cell can convey sufficient sensitivity in detecting the invasion by single or a small number of viruses. The filaments on the surface of mitochondria could take either the poorly structured C3 forms or the better-ordered C1 forms, depending on the native conditions (Figs 6 & 7). High-resolution electron tomography of native filaments formed on the surface of mitochondria after viral infection may be the best way to resolve this point^{212, 213}. Because of the fast growth of native MAVS filaments after initiation, it might be necessary to use a reconstituted membrane system, such as a highly-stable bead-supported unilamellar vesicles (bSUM) or a supported planar membrane^{206, 214}, to limit the number of silent full-length MAVS, and study the initiation of MAVS filaments in a controlled manner.

5. Structural variations in the interactions between RLR/RNA complexes and silent MAVS.

For detection of single vRNA, the RLRs would form only one RLR/RNA complex (Fig. 7). The low expression level of RLRs may not be sufficient to form RLR filaments along one vRNA. Further, there are abundant 5'-OH-RNAs, such as short dsRNAs, hairpin RNAs, 5S ribosomal RNA (RNA5SP141), noncoding (nc) RNAs or long-ncRNAs^{215–222}, in cytosol (called cRNAs here) that may nonspecifically interact with RLRs such that the RLR/vRNA must be distinguished from the RLR/cRNA complex^{223, 224, 225}. The importance of the adapter protein, such as zyxin, becomes apparent in this scenario because it adds another layer of selection for the active RLR/vRNA complex via direct interaction with the N-terminal region of zyxin, which probably interacts with three silent MAVS molecules and promotes three-stranded MAVS filaments¹³¹. In accord with this mechanism (Fig. 7), overexpressing zyxin was found to promote the MAVS-mediated activation of the interferon beta-1 promoter and knockdown of zyxin expression showed the opposite effect. Recently it was recognized that the competitive recognition of cytosolic RNAs and vRNAs by RLRs

(MDA5) may contribute to distinguishing self RNAs from non-self ones inside the cells²²⁵ and a long non-coding RNA (lnc-Lsm3b) was found to be a negative regulator of RIG-I. Mutations in RLRs might lead to their activation by cytosolic RNAs, and cause auto-immune responses.

In order to detect a single copy of a vRNA, a short vRNA fragment might allow the formation of one active RLR/RNA complex at a time, which, if remaining active, may interact with multiple copies of zyxin molecules, and in turn initiate multiple three-stranded MAVS filaments on the surfaces of different mitochondria. The prion-like self-perpetuation of the MAVS filaments will allow another level of amplification by recruiting a major fraction of MAVS molecules from different mitochondria into the growing filaments.

6. Relevance of the structural variations to medicinal chemistry.

Signaling from RLR to MAVS involves multiple steps of molecular recognition --- the vRNA recognition against the abundant cytosolic RNAs, the potential signal amplification by forming multiple active RLRs (monomers, dimers or oligomers) with one vRNA, the likely engagement of one active RLR/vRNA complex with two or three copies of inactive MAVS via one adapter protein (zyxin), the potential repetitive use of one RLR/vRNA complex to initiate the formation of multiple MAVS filaments, the formation of long MAVS filaments via dynamic fusion and fission of mitochondria and redistribution of MAVS among mitochondria, the recruitment of multiple effector molecules onto a condensed phase made of multiple extended random-coil regions peripheral to the MAVS CARD filaments, and the shutdown of signaling by inactivating the RLR/vRNA complex or by removing MAVS filaments through autophagy or degradation. These processes suggest that there are many different ways to manipulate and interfere with the antiviral immune response. We will discuss a few possibilities next.

The detection of viral RNAs may be manipulated by finding chemical or biological reagents to stabilize or destabilize different RLR/RNA complexes. The RLRs and cytosolic RNA may form inactive complex as we observed in the RIG-I/short 5'-ppp-ssRNA. Stabilizing the inactive RLR/cRNA complexes can down-regulate the immune response against RNA viruses in order to prevent over-responsiveness. Destabilizing the active RLR/cRNA complex may suppress auto-immune responses against self-RNAs. Similarly, elongating the lifespan of the active RLR/vRNA complex will boost the immune response against invading viruses. For example, a 5'-ppp-RNA mimicking the vRNAs, as an agonist of RLRs, was found to enhance the antiviral response in model animals of influenza infection²²⁶. The structural model in Fig. 2A, suggests that short stem-loop 5'-ppp-RNA, such as SLR5, may serve as a potent antagonist of RLRs and suppress the over-sensitized immune system or autoimmune response through the RLR-MAVS pathway.

The engagements of RLR/vRNA and the inactive MAVS via the adapter protein may be targeted for regulating the anti-viral responses. This step makes it possible to select the active RLR/vRNA complex, and utilize even one copy of the active RLR/vRNA complex to engage multiple MAVS molecules such that the MAVS CARD domains may be relieved from its inhibited state via interactions with RLR tandem CARDS, and spontaneously form

filaments due to local mass action and /or possible molecular imprinting. In cells, the active RLR/vRNA complex could be released from the adaptor protein once a new MAVS filament is initiated (without molecular imprinting) such that the colocalization study of RLR and MAVS, or mutual co-IP of RLR and MAVS would have led to weak or negative signals. Chemically or biochemically manipulating the recognition of the active RLR/vRNA complex by the adapter proteins and its interaction with inactive MAVS through the adapter proteins may enhance the antiviral responses, and suppress non-specific response from nonspecific RLR/cRNA complexes. Without a detailed understanding of how the adapter protein (e.g. zyxin) binds the active RLR/vRNA complex and recruits multiple inactive MAVS molecules, a general prediction is that activators for the zyxin-mediated interaction between RLR/vRNA and inactive MAVS will significantly enhance the antiviral response.

The self-promoting formation of MAVS filaments requires the dynamics of healthy mitochondria, which is an energy-consuming process. ATP production from healthy mitochondria is thus required. The effective clearance of the MAVS filaments through autophagy regulates the duration of the antiviral response. The mid-regions of MAVS appear to resemble an intrinsically disordered protein (IDP). As a linear polymer, it is probably recoiled into different conformational states that, when bound by other proteins, hide the binding motifs for downstream effectors, and prevent the MAVS CARD from becoming spontaneously available to form filaments. At another level, the active MAVS filaments may be blocked by specific reagents that occupy the effector-binding sites in the condensed middle regions. Promoters of autophagic clearance of MAVS filaments are expected to down-regulate the antiviral responses ¹⁴⁷.

Other processes, such as phosphorylation of MAVS, poly-ub binding or linkage to RLRs, MAVS interaction with other mitochondrial proteins in outer or inner membranes, MAVS presence at the contact site between ER and mitochondrial membranes, etc. may all be intervened in order to affect the specificity, intensity and temporal and spatial spreading of the antiviral response. Detailed quantitative analysis of these processes might be ultimately needed in order to identify key rate-limiting steps as druggable targets for effective chemical intervention.

7. Conclusions and future directions

The structural models for the RLR/vRNA interactions, the adapter-mediated engagement of the active RLR/vRNA with inactive MAVS and the formation MAVS filaments reveal structural and functional variability for the cytosolic vRNA-triggered innate immune response. Some of the variability may be required for high-sensitivity in vRNA detection, substrate specificity in RLR-vRNA interactions, and the effective coupling between the RLR activation by vRNAs and the MAVS filament formation. The nearly all-or-none response for detecting one or a small number of viral RNAs may be a native requirement for effective and immediate response. Specific steps in vRNA recognition, MAVS activation and clearance of both MAVS filaments and RLR/vRNA complexes may be suitable targets for developing specific chemical or biochemical reagents that can enhance or suppress the antiviral responses.

Due to the variability of vRNA signature features for RLR recognition and the interaction partners of RLR and MAVS, a webserver with various vRNA signatures sequences, interactions between RLR and MAVS with their partners would be useful for providing a real-time resource for RLR/MAVS signaling. As pointed out by Chou and Shen²²⁷ and implemented in a series of recent publications (e.g.^{9, 19, 20, 81, 83, 228–232}), a user-friendly web-server will be an excellent direction for future development and for presenting new findings in this area. Indeed, many useful webserver have been created for different topics and are generating strong impacts on medical sciences⁸⁹, and potentially will revolutionize medicinal chemistry²³³. It is hence worth the efforts to develop a web-server for detecting signature patterns of new viral RNAs in the future.

Multiple fundamental questions remain open and deter fully quantitative understandings in the RLR-MAVS signaling. Future studies need to evaluate the properties of minimally functional RLR/vRNA complexes (RIG-I or MDA5), determine whether the RLR/vRNA filaments themselves can support the lateral growth of multiple MAVS filaments, reveal a mechanistic model for zyxin-induced engagement of the RLR/vRNA complex and inactive MAVS monomers, define how the inactive MAVS is kept silent, determine the symmetry properties of native MAVS filaments and elucidate the molecular processes for the clearance of MAVS filaments.

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Fig. 1: RLR-MAVS signaling for sensing cytosolic viral RNAs.

Viral invasion into the cells leads to the appearance of viral RNA fragments in cytosol, which are detected by RLR monomers. Either binding or linkage of polyubiquitin would stabilize the active RLR / vRNA complex, which with the support of zyxin, is able to interact with inactive MAVS on mitochondria, freeing the MAVS CARD domains for forming helical filaments. The helical filaments of MAVS serve as a platform for bringing downstream effectors together and activating IRF₃ or NFκB, and inducing the production of IFNs. Adapted from Dr. Hui Xu's PhD dissertation in reference 234.

Fig. 2. Structural variations in RIG-I/RNA complexes. Monomers:

Full-length RIG-I and its CARD-less fragment may form complexes with short non-functional 5'-ppp-RNA, suggesting a possible functional state after the helicase domain enables the movement. In the structural model (PDB code: 3ZD6; Luo D. et.al (2011)), the CTD (red) caps the 5'-ppp, which represents an initial state. The helicase is in yellow and RNA in blue. **Dimers** are not shown here. **Tetramers:** tandem CARDs of four RLRs form tetramers in testtubes. The structural model (PDB code: 4NQK; Peisley et al. (2014)) on the right is colored with four tandem CARDs in green, yellow, cyan and blue, respectively, with multiple ubiquitin molecules (in red, brown or gold) attached at the periphery. But full-length RIG-I, viral RNA and poly-ubiquitins tend to form amorphous aggregates.

Filaments: RLRs are assumed to be able to form stacked helical filaments along dsRNAs. The helicase domains appear nonessential for such filaments. The proposed tetrameric structures on the RIG-I/RNA filaments have not been shown to be capable of initiating

MAVS filaments. Figures were modified by using structural models and diagrams with permission of Springer Nature and Cell Press for open access.

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Fig. 3. Dimeric RIG-I/RNA complex.

A 51 mer-5'-ppp ssRNA is designed to form a stem-loop structure, and its ds-stem is long enough for binding of two RIG-I helicase domains. Data from negatively stained specimens were used for 3D reconstruction. The structure of the CARD-less human RIG-I fragments (245–922; PDB code: 3ZD6; Luo D. et.al (2011)) was docked into the map with the central hole sufficient for a dsRNA helix. RNA is in blue, RD in red and helicase in yellow. The C2 symmetry suggests a relative rotation of one monomer so that the two undergo head-to-tail interactions. The bottom density is not enough to accommodate two double-CARDs, suggesting possible flexibility between the two tandem CARDs.

Fig. 4. Relative activity of different RNA ligands in activating the RIG-I/MAVS pathway and inducing IFN production.

Different RNAs were introduced into cultured cells. Relative transcription activity of IFN-promoter was measured and compared. Details are available in ¹³¹.

Fig. 5. MAVS activation by changing from inactive form to activated filament form.

A) Flag-tagged MAVS could be detected by antibody labeling (green) on the surface mitochondria (red as labeled by mitotracker). After viral infection, the FLAG-tagged MAVS molecules were concentrated in elongated puncta on mitochondrial surfaces (merged panel and the expanded view below it). The length of the puncta follows roughly a normal distribution (bottom bar graph). B). Model of inactive MAVS with the coiled middle region and the activated MAVS filaments. The middle regions become extended and suitable for the binding downstream factors, such as TRAFs. Figures were modified from those published in ¹³¹ with permission of eLife Sciences Publications, Ltd for open access.

Fig. 6. Two different symmetry groups of the MAVS CARD filaments.

The C1 and C3 data have their own selection rules: 9 turns per 32 units for C1 and 4 turns per 27 units for C3. **Top row:** the C1 data and the C3 data show difference in the relative intensity of layer lines 4 and 5 (LI= 4 & 5). For C1, the layerline 5 is very weak and its first peaks are almost invisible in the power spectrum. First peaks of layer line 5 are pretty strong for C3 data. The power spectra from Wu et al (2014) are showed for comparison in the rightmost panel. Their C1 model also showed very weak first peaks in layer line 5, suggesting that the data for the CARD filaments formed by denatured and refolded MAVS CARDS were seemingly dominated by the C1 form, but also contain the C3 form. There are only near-atomic resolution structures for the C1 forms, not for the C3, suggesting possible structural heterogeneity. The purified MAVS CARDS from cultured cells formed filaments at quasi-physiological conditions, which appeared to be dominated by the C3 forms. Low salt (≤ 50 mM) was found to favor more C1 forms for the MAVS CARDS purified from cultured cells. Figures were modified from those published in ¹³² and Wu et al (2014) with permission of eLife Sciences Publications, Ltd and Cell Press.

Fig. 7. RLR/vRNA complexes activate MAVS through an adaptor to enhance specificity. Because the minimal functional forms of vRNAs are short fragments that are 10–20 nucleotides long, it is likely that both monomers and dimers are functional. The half-turn helical arrangement of 8 CARD domains in the tetramers has not been showed to induce the MAVS filament yet, but is assumed to be active here. The adapter protein allows the selective engagement of one active RLR/vRNA complex and 3 copies of silent MAVS, likely a good platform to promote a three-stranded MAVS filament. When the active RLR/vRNA complex dissociates from the zyxin/MAVS filament, it may interact with another zyxin and trigger more MAVS filaments. Alternatively, multiple active adaptors may cooperatively induce the formation of active MAVS filaments. Diagrams were prepared from modeled parts used with permission of eLife Sciences Publications, Ltd, Cell Press and Springer Nature.