Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus

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RIG-I is a cytosolic pathogen recognition receptor that engages viral RNA in infected cells to trigger innate immune defenses through its adaptor protein MAVS. MAVS resides on mitochondria and peroxisomes, but how its signaling is coordinated among these organelles has not been defined. Here we show that a major site of MAVS signaling is the mitochondrial-associated membrane (MAM), a distinct membrane compartment that links the endoplasmic reticulum to mitochondria. During RNA virus infection, RIG-I is recruited to the MAM to bind MAVS. Dynamic MAM tethering to mitochondria and peroxisomes then coordinates MAVS localization to form a signaling synapse between membranes. Importantly, the hepatitis C virus NS3/4A protease, which cleaves MAVS to support persistent infection, targets this synapse for MAVS proteolysis from the MAM, but not from mitochondria, to ablate RIG-I signaling of immune defenses. Thus, the MAM mediates an intracellular immune synapse that directs antiviral innate immunity.

Results

We assessed MAVS cellular distribution by confocal microscopy of human hepatoma (Huh7) cells expressing MAVS-mEGFP. In addition to the previously described mitochondrial and peroxisomal localization of MAVS (5, 6), our higher-resolution analyses also revealed MAVS staining directly adjacent to and around mitochondria (Fig. 1A). The MAM is known to link the ER to mitochondria (14), and we assessed the possibility that it also could make contacts with peroxisomes, which are derived from ER membranes (17). Indeed, confocal microscopy of Huh7 cells expressing mEGFP-PSS-1, a MAM-enriched marker protein (18, 19), revealed an interconnected MAM network, distinct from the ER, between mitochondria and peroxisomes (Fig. S1A and B). Thus, we biochemically assessed the cellular distribution of MAVS and various intracellular organelle marker proteins by fractionating Huh7 cells to isolate the MAM from mitochondria and microsomes, the latter of which contains light membranes of the ER and plasma membrane (Fig. S2A) (18). MAVS was present in Huh7 cells within both MAM and mitochondria, as well as in “F1”, a membrane fraction of lighter density than MAM alone that contains both MAM and peroxisomes (Fig. 1B), but not in microsomal fractions (Fig. S2B). The MAM-localized MAVS cofractionated with a previously described MAM-enriched marker, FACL4 (Fig. 1B and Fig. S2 B–D) (15, 18). The MAM fractions were largely devoid of both peroxisomal (Pex19) and mitochondrial markers (Coxl and VDAC1) (Fig. 1B and Fig. S2B), whereas standard mitochondrial fractions contained all of these markers (Fig. S2B). In addition, MFN2, a MAM- and mitochondrial-localized protein that tethers the ER to mitochondria and interacts with MAVS (20, 21), cofractionated with MAVS and was present in both MAM and mitochondria (Fig. S2C).

To verify biochemical identification of the MAM localization of MAVS, we performed confocal microscopy of endogenous MAVS in Huh7 cells. This revealed MAVS staining directly adjacent to mitochondria and partially overlapping with three different MAM-enriched proteins: FACL4, sigma-1 receptor (Sig1R), and mEGFP-PSS-1 (Fig. 1C and Figs. S2D and S3) (15, 18). This MAVS distribution pattern at the MAM–mitochondrial axis was apparent in cells that were either mock-infected or infected with Sendai virus (SenV), which activates RIG-I signaling and accurately models HCV activation of the RIG-I pathway (Fig. S3) (7, 10). Thus, MAVS is localized to the MAM, peroxisomes, and mitochondria, likely residing at the junctions between these organelles.

The HCV NS3/4A protease complex is localized within infected cells to intracellular membranes and regions near mi-
tochondria by membrane-anchor domains within NS3 and the NS4A cofactor (22), suggesting that MAM localization of NS3/4A could govern its dual distribution between the ER and mitochondria. Therefore, we assessed the distribution of NS3/4A transiently expressed in HeLa cells that coexpress mEGFP-PS521. We found that NS3 and NS4A fractionated with MAM, F1 (representing peroxisomes with MAM), and microsomes (representing the ER and HCV replication complex; described in ref. 12), with a small amount of NS4A also detected in the mitochondria (Fig. 2 A). Confocal microscopy confirmed NS3/4A localization adjacent to peroxisomes and mitochondria, both of which are sites of MAVS localization (Fig. 2 C) (5, 6). In addition, confocal microscopy revealed that in cells propagating a self-replicating HCV RNA genome (a model of persistent HCV infection; Huh7-HCV K2040 cells) (3), both NS3 and NS4A were present at MAM–mitochondrial junctions (Fig. 2 B). In HCV-infected Huh7.5 cells (3), both NS3 and NS4A were associated with the MAM either (i) at junctions with mitochondria (Fig. 2 D and E, large arrows) or (ii) independent of mitochondria (Fig. 2 D and E, small arrows), similar to what was observed in Huh7-HCV K2040 cells (compare Fig. 2 B and E). Thus, biochemical fractionation and direct imaging of cells establish that NS3/NS4A localizes to the junctions among MAM, mitochondria, and peroxisomes during HCV infection and persistent viral RNA replication.

In cells replicating HCV RNA, NS3 was found in both MAM and microsomal fractions (Fig. 3 A and B and Fig. S2C). MAVS was cleaved during HCV replication by NS3/4A and accumulated within the cytosolic fraction (Fig. 3 B, lane 8 and Fig. S2C), as described previously (10). Importantly, we detected cleaved MAVS in the MAM, but not in the mitochondrial fraction, during HCV RNA replication (Fig. 3 B, compare lanes 4, 6, and 8; Fig. S2C). The residual cleaved MAVS present in the MAM likely reflects the homotypic interaction of MAVS with uncleaved protein and/or binding to other MAM-associated signaling cofactors, with the cleaved MAVS in the cytosol likely representing both MAM and peroxisomal-MAVS cleavage by NS3/4A. Surprisingly, neither the mass nor the relative amount of mitochondrial MAVS was affected by the presence of HCV proteins (Fig. 3 B, lanes 5 and 6, with markers of these samples shown in Fig. 3 A; see also Fig. S2C). We note that the lack of MAVS cleavage from mitochondria by NS3/4A differs from findings in previous studies by us and others in which analysis of crude mitochondrial fractions led to the conclusion that MAVS was cleaved from the OMM (10, 23). We have now found that such mitochondrial fractions indeed contain MAM, explaining these differences (Fig. S2B). Moreover, confocal microscopy analysis of cells expressing NS3/4A identified residual mitochondrial MAVS staining, thus defining a pool of uncleaved mitochondrial-associated MAVS (Fig. S4). Therefore, MAVS is cleaved by NS3/4A on the MAM, but not on mitochondria, during HCV infection.

Because MAM, but not mitochondrial MAVS, is the proteolytic target of NS3/4A, we evaluated the possibility that the MAM-localized MAVS is capable of transducing RIG-I signaling. We assessed MAM fractions from Huh7 and Huh7-HCV K2040 cells for the presence of RIG-I and found that a portion of RIG-I was redistributed from cytosol into the MAM, but not into the mitochondria, in cells chronically replicating HCV RNA (Fig. 3 A and B and Fig. S2C). This redistribution was not due to cytosol contamination of these fractions or to changes in MAM marker levels during HCV RNA replication, because the cytosol marker tubulin was absent from the MAM and microsomes, and MAM marker levels were unchanged by HCV RNA replication (Fig. 3 A and C and Fig. S2C). RIG-I levels are known to increase by membrane anchor domains within NS3 and the NS4A cofactor (22), suggesting that MAM localization of NS3/4A could govern its dual distribution between the ER and mitochondria. Therefore, we assessed the distribution of NS3/4A transiently expressed in HeLa cells that coexpress mEGFP-PS521. We found that NS3 and NS4A fractionated with MAM, F1 (representing peroxisomes with MAM), and microsomes (representing the ER and HCV replication complex; described in ref. 12), with a small amount of NS4A also detected in the mitochondria (Fig. 2 A). Confocal microscopy confirmed NS3/4A localization adjacent to peroxisomes and mitochondria, both of which are sites of MAVS localization (Fig. 2 C) (5, 6). In addition, confocal microscopy revealed that in cells propagating a self-replicating HCV RNA genome (a model of persistent HCV infection; Huh7-HCV K2040 cells) (3), both NS3 and NS4A were present at MAM–mitochondrial junctions (Fig. 2 B). In HCV-infected Huh7.5 cells (3), both NS3 and NS4A were associated with the MAM either (i) at junctions with mitochondria (Fig. 2 D and E, large arrows) or (ii) independent of mitochondria (Fig. 2 D and E, small arrows), similar to what was observed in Huh7-HCV K2040 cells (compare Fig. 2 B and E). Thus, biochemical fractionation and direct imaging of cells establish that NS3/NS4A localizes to the junctions among MAM, mitochondria, and peroxisomes during HCV infection and persistent viral RNA replication.

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Because MAM, but not mitochondrial MAVS, is the proteolytic target of NS3/4A, we evaluated the possibility that the MAM-localized MAVS is capable of transducing RIG-I signaling. We assessed MAM fractions from Huh7 and Huh7-HCV K2040 cells for the presence of RIG-I and found that a portion of RIG-I was redistributed from cytosol into the MAM, but not into the mitochondria, in cells chronically replicating HCV RNA (Fig. 3 A and B and Fig. S2C). This redistribution was not due to cytosol contamination of these fractions or to changes in MAM marker levels during HCV RNA replication, because the cytosol marker tubulin was absent from the MAM and microsomes, and MAM marker levels were unchanged by HCV RNA replication (Fig. 3 A and C and Fig. S2C). RIG-I levels are known to increase after RIG-I pathway activation (9) and were accordingly increased during the course of SenV infection (Fig. 3 C). RIG-I levels also
were moderately increased within Huh7-K2040 cells, reflecting the cellular compensation due to MAVS cleavage and RIG-I signaling suppression (9, 10). We also conducted confocal microscopy analysis and confirmed that a portion of RIG-I was redistributed into the MAM near sites of MAVS localization (Fig. S5). Furthermore, immunoprecipitation analyses of the MAM fraction from PH5CH8 hepatocytes revealed that the portion of RIG-I that redistributed to the MAM during acute RNA virus infection interacted with both MAVS and TRAF3 (Fig. 3C). This indicates that RIG-I and its signaling cofactor TRAF3 are distributed to the MAM during RNA virus infection for interaction with MAVS.

Contacts between the MAM and mitochondria are maintained by the mitochondrial fusion protein MFN2 (20). To assess the role of MAM contacts in coordinating MAVS localization and signaling during virus infection, we used siRNA technology to knock down MFN2 expression in Huh7 cells. Although MFN2 is the MAM tethering protein, it shares homology with the related MFN1 protein, and both are involved in mitochondrial fusion and bind to MAVS (21, 24). Therefore, we also used siRNA to knock down MFN1 to control for the shared functions of these proteins and to specifically isolate the MAM–mitochondrial tethering role of MFN2. Manders’ colocalization coefficient analysis (25) of confocal micrographs confirmed that loss of MFN2 expression, but not of MFN1 expression, disrupted the ER–mitochondria interaction (Fig. 5A) (20). Colocalization analysis of MAVS and TOM20 in confocal micrographs through an object-based overlap approach (26) revealed that knockdown of MFN2, but not of MFN1, resulted in a slight but significant increase in nonmitochondrial MAVS during SenV infection (Fig. 4A and B and Fig. S7B). Knockdown data are provided in Fig. 5A. Colocalization analysis with
the peroxisomal marker PMP70 established that this increase in nonmitochondrial MAVS corresponded with its enhanced association with peroxisomes (Fig. 4C and D). In addition, it appeared that detection of peroxisomes by immunofluorescence was easier because of an increased number or a reduced association with mitochondria when MFN2 was knocked down (Fig. 4E), perhaps explaining the increased MAVS association with peroxisomes on MFN2 knockdown.

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**Fig. 3.** The MAM is an innate immune signaling platform. (A and B) Immunoblot analysis of fractionated Huh7 (−) and Huh7-HCV K2040 (+) cells. Arrows indicate full-length (FL) and cleaved (C) MAVS. (C) Immunoblot analysis of fractions immunoprecipitated with anti-RIG-I from mock- or SenV-infected (for 8 h) PH5CH8 cells and input. *Nonspecific band. Fractionation markers: calnexin, ER; Cox-1, mitochondria; FACL4, MAM; tubulin, cytosol; Pex19, peroxisomes.

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**Fig. 4.** Mitochondrial-ER contacts regulate MAVS localization and signaling. (A) Immunostain of endogenous MAVS and TOM20 (OMM). Arrows indicate MAVS localized independent of TOM20. (C) Immunostain of cells expressing MAVS-mEGFP for PMP70 (peroxisomes) and TOM20. (B, D, and E) Values (mean ± SD; n = 3–4 cells) of MAVS localization to mitochondria (B) or peroxisomes (D) (object-based overlap approach) (26), or overlap between peroxisomes and mitochondria (E) (Pearson’s coefficient) (26). *P ≤ 0.05; **P ≤ 0.005; ***P ≤ 0.0005, by unpaired Student t test. Single-cell images are representative of two experiments and >10 cells analyzed per experiment. (Scale bar: 10 μm.)
In addition to increasing MAVS localization with peroxisomes, treatment of cells with siRNA specific to MFN2 or with methyl-β-cyclodextrin, which disrupts MAM-mitochondria contacts through distinct mechanisms (27), enhanced RIG-I pathway signaling as defined by an increase in phosphorylated active IRF-3, enhanced IFN-β promoter activity, and ISG56 expression after SenV infection (Fig. 5A and B). We note that knockdown of MFN2 enhanced RIG-I signaling despite an attenuation of the previously described virus-induced mitochondrial elongation (Fig. S7A), demonstrating that its role in the antiviral response is actually independent of a role in mitochondrial elongation (28). MFN1 knockdown attenuated SenV-induced RIG-I signaling, as described previously (24, 28), while maintaining ER-mitochondria contacts (Fig. S6). When infected with HCV, MFN2 knockdown of cells resulted in a moderate but significant reduction in HCV RNA early after infection up to the point of MAVS cleavage by NS3/4A, consistent with acute enhancement of innate immune defenses induced by RIG-I–HCV RNA interaction due to deregulated RIG-I pathway signaling (Fig. 5C) (7, 29). Indeed, MFN2 knockdown reduced the permissiveness of cells for infection by HCV and vesicular stomatitis virus (VSV), consistent with enhanced innate immune defenses (Fig. 5D). Therefore, MAVS localization and antiviral signaling are supported by the MAM–mitochondrial tethering function of MFN2, which may regulate MAVS distribution among mitochondria, MAM, and peroxisomes.

We also found that during SenV infection, the interaction between peroxisomes and mitochondria increased in an MFN2-dependent manner (Fig. 4C and E). This observation suggests that MAM–mitochondrial tethering mediates an increased interaction between these organelles during RIG-I pathway activation. Indeed, confocal microscopy of SenV-infected Huh7 cells revealed that the MAM forms a synapse between peroxisomes and mitochondria (Fig. 5E). An apparent synapse between peroxisomes and mitochondria anchored by MAVS also can be detected on MAVS overexpression (Fig. 5A). Moreover, we found that this synapse is targeted by the NS4A subunit of the HCV protease complex (Fig. 5F). These observations raise the possibility that NS3/4A targeting of this MAM-anchored synapse may regulate innate immune signaling by MAVS from MAM, peroxisomes, and mitochondria.

**Discussion**

Our results identify an innate immune signaling synapse in which the MAM serves as the central scaffold that coordinates MAVS-dependent signaling of the RIG-I pathway between mitochondria and peroxisomes. This model implicates the MAM in immune signaling to a variety of viruses in addition to HCV that are engaged by RIG-I–like receptors to trigger the immune response (2). The MAM-anchored synapse contains both MAVS and RIG-I pathway factors that physically interact in RNA virus-infected cells to drive the immune actions of an MAVS signalosome. STING, a putative RIG-I signaling cofactor and IPS-1 binding protein, also may have MAM localization, but this localization is likely specific to cell type and context (30). Because the viral NS3/4A protease targets the MAM-anchored synapse and cleaves MAVS from the MAM, but not from mitochondria, to ablate innate immune signaling during HCV infection, the MAM-resident MAVS likely mediates the RIG-I pathway function against HCV. This notion is further supported by previous studies showing that cells supporting chronic HCV RNA replication (which, as we show here, contain intact mitochondrial-localized MAVS but not intact MAM-localized MAVS) are unable to signal IRS-3 activation and the expression of IRS-3 target genes despite the presence of MAVS on the mitochondria (9). Although MAM-localized MAVS directs innate immunity against HCV, our observations do not exclude a role for mitochondrial MAVS in innate immune signaling. In this respect, mitochondrial MAVS supports apoptotic signaling of innate immune actions against virus infection (31, 32), and also could serve as a local source of MAVS for distribution among peroxisomes and MAM through dynamic membrane trafficking regulated by MFN2. This latter
idea is supported by previous work showing that peroxisomes are not required for populating mitochondria with MAVS (6), suggesting that peroxisomes may receive MAVS by membrane trafficking through MAM–mitochondrial interactions.

We found that when MAM–mitochondrial contacts were disrupted, there was increased localization of endogenous native MAVS to peroxisomes, with a concomitant increase in signaling to IFN-β compared with cells maintaining MAM–mitochondria contacts and harboring less peroxisomal MAVS. In contrast, previous work has shown that ectopic MAVS targeted solely to peroxisomes through replacement of the native MAVS transmembrane domain with a peroxisome-targeting domain does not signal to IFN-β (6). In that case, the presence of the nonnative MAVS transmembrane domain likely precluded essential membrane interactions with signaling factors that drive IFN-β production, thus explaining the divergent findings. In the present study, peroxisomal MAVS enhanced signaling to IFN-β, possibly because when localized to peroxisomes, MAVS no longer interacts with mitochondrial negative regulatory proteins of innate immune signaling, including NLRX1 (33). In support of this, NLRX1 has been shown to be unable to suppress signaling by peroxisomal MAVS (6). Placement of NLRX1 or other negative regulators of RIG-I signaling on mitochondria also might explain why MAVS must be redistributed to MAM to initiate innate immune signaling. In this case, the mitochondria-to-MAM-to-peroxisome redistribution of MAVS would release it from signaling inhibition by a negative regulator, and the MAM would provide the platform for recruiting positive signaling effector proteins to interact with MAVS for innate immune signaling.

Our study and others indicate that dynamic immune signaling is coordinated by the MAM through membrane–protein interactions governed by MFN2, with membrane tethering sites acting as signaling microdomains that contain localized sources of MAVS and other signaling factors (15). This organization is analogous to the immunologic synapse that forms between a T cell and an antigen-presenting cell in which adhesion molecules tether opposing plasma membranes to establish T-cell receptor signaling microdomains supplied with key signaling factors through membrane interactions (34). Taken together with the role of the MAM in NLRP3 inflammasome signaling (16), our findings indicate that the MAM plays a central role in initiating both the innate immune and inflammatory responses to infection. Therapeutic strategies directed toward regulating MAM processes could serve to preserve and enhance antiviral immunity.

Materials and Methods

Additional information is provided in SI Materials and Methods.

Cell Culture and Viruses.

Huh7, Huh7.5, and Huh7-HCV K2040 replication cells that propagate culture-adapted variants of the Con1 HCV (genotype 1B) subgenomic replicon RNA (3), along with immortalized human hepatocyte PHSCH8 cells, were cultured according to standard techniques. SenV strain Cantell was obtained from Charles River Laboratory. Cell culture adapted HCV JFH1 genotype 2A strain was propagated and infectivity titrated, as described previously (35). HSV-GFP infections were done on MOI 1 for 16 h. Methyl-β-cyclodextrin treatment used a concentration of 150 μM in DMEM.

Subcellular Fractionation.

MAM, mitochondria, and microsomes were isolated from cells using Percoll gradient fractionation (18). Equivalent amounts of protein from each fraction were analyzed by SDS/PAGE and immunoblot.

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Supporting Information

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SI Materials and Methods

Immunofluorescence and Confocal Microscopy. Where indicated, cells were stained with 500 nM Mitotracker Orange CMTMRos or Deep Red (Invitrogen), according to the manufacturer’s protocol, before fixation. Cells were fixed in 4% paraformaldehyde, permeabilized, and immunostained as described previously (1). Antibody binding was detected using conjugated Alexa Fluor 488, 546, or 633 secondary antibodies (Molecular Probes). Samples were imaged on either a Nikon TE2000-E microscope equipped with a laser-scanning confocal imaging system or a Zeiss 510 META confocal microscope with a 63x oil immersion lens with a numerical aperture of 1.4 at the Nyquist limit. Images were collected as Z stacks with a focal step size ranging from 0.2 to 0.43 μm and processed with Nikon Elements or ImageJ. In some cases, Z stacks were deconvolved using AutoDeblur (Media Cybernetics), and Imaris software (Bitplane) was used to volume surface render confocal stacks.

Image Analysis. Organelle interactions were analyzed in deconvolved Z stacks by Manders’ colocalization coefficient, as noted in the text, and by Pearson’s coefficient for mitochondria-peroxisome interaction (2, 3). MAVS interaction with organelles in deconvolved Z stacks was analyzed using the objects-overlap approach with the JACoP plug-in of ImageJ (2) to give a colocalization index measuring the overlap in the center of mass of MAVS staining within the area of the organelle staining.

Antibodies. The following antibodies were used: anti-HCV NS3 (Novocastra); polyclonal anti-HCV 2A NS3 and NS4A (custom antibody; Genscript); anti-HCV NS3 1B6 (a gift from D. Moradpour, University of Lausanne); anti–HCV-NS4A (Virogen); anti–NS5A 9E10 (a gift from C. Rice, Rockefeller University); anti-Cardf (for MAVS, AT107, and Adri-1; Axxora); anti-Calnexin (Stressgen); anti–Cox-1 (1D6) and anti-GFP (Invitrogen); anti–FACL4 (Abgent); anti-Pex19, and anti-tubulin (Sigma-Aldrich); anti–IKK-ε (Imgenex); anti–IRF-3 (a gift from M. David, University of California at San Diego); anti-ISG56 (a gift from G. Sen, Cleveland Clinic); anti–MFN1, anti–OPRS1 (Sig-1R), anti–PMP70, and anti–VDAC1 (Abcam); anti–Mfn2, anti–Tom20, and anti–TRAF3 (Santa Cruz Biotechnology); anti–P–IRF-3 (Cell Signaling); anti–RIG-I (Alme-1; Alexis); and anti–SenV (Biodesign International).

Plasmids and Transfections. The following plasmids have been described previously: pEF-HCV NS3 and pEF-HCV NS3/4A (4), pmEGFP-huPSS-1 (5) (a gift from A. Colberg-Poley, Children’s National Medical Center), pYFP-RIG-I (a gift from A. Miyawaki, RIKEN), and pCMV/myc/ER/GFP (Invitrogen). pmEGFP-N1 was created using site-directed mutagenesis to make the A206K mutation that inactivates GFP oligomerization (6). The plasmid encoding mEGFP-NS4A was generated by inserting oligonucleotide duplexes into the AgeI and KpnI sites of pmEGFP-N1. The plasmid encoding pEFTak-mEGFP-MAVS was created by cloning MAVS into the NotI and Pmel sites of pEFTak-mEGFP. DNA transfections were done using FuGENE 6 (Roche). pmCherry-huPSS-1 was created by cloning PSS-1 into the NotI and Pmel sites of pEFTak-mCherry. IFN-β promoter luciferase assays were conducted as described previously (7). siRNA pools (ON-TARGETplus SMARTpool; Dharmaco) directed toward MFN1 (NM_017927), MFN2 (NM_014874), or nontargeting (CTRL) were transfected using Lipofectamine RNAiMAX (Invitrogen).

RNA Analysis. For cultured cells, total RNA was extracted using the Qiagen RNeasy kit. HCV RNA copy number was measured by quantitative RT-PCR as described previously (8), with triplicate reactions analyzed using an Applied Biosystems 7300 RT-PCR system.

Immunoblotting and Immunoprecipitation. Cells were lysed in modified RIPA buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, and 1% Triton X-100] supplemented with protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitor mixture II (Calbiochem). After harvest, protein was subjected to SDS/PAGE, transferred to nitrocellulose membranes in a 25 mM Tris-192 mM glycine and 0.01% SDS buffer, and blocked in 5% milk-PBS with 0.1% Tween-20 buffer. For NS4A immunoblotting, transfer onto Immobilon-P membranes (Millipore) was done in buffer without SDS. After washing, membranes were incubated with species-specific HRP-conjugated antibodies (Jackson ImmunoResearch), treated with ECL+ (GE Healthcare), and imaged on X-ray film. For immunoprecipitation, 50 μg of subcellular fractions were incubated overnight with an anti–RIG-I antibody, followed by washing with RIPA and then immunoblotting.

Statistical Analysis. P values were calculated using the unpaired Student t test.


Fig. S1. MAMs are distinct from the membranes of the ER and link mitochondria and peroxisomes. (A) Confocal microscopy of Huh7 cells expressing mEGFP-PSS-1 (MAM) and stained for PMP70 (peroxisomes) and TOM20 (mitochondria). (B) Confocal microscopy analysis of Huh7 cells preloaded with Mitotracker (blue) expressing the MAM marker mCherry-PSS-1 (red) and ER-GFP (green). Zoom views 1 and 2 are taken from within the corresponding white frame, and 3D reconstructions of the corresponding Z stacks are shown in the far right panels. Arrows indicate MAM staining enriched near mitochondria. (Scale bar: 10 μm.) Colocalization analysis (Manders’ coefficient) represents the overlap between mitochondria and the indicated membrane. Values are mean ± SD; n = 3.
Fig. S2. Subcellular fractionation to isolate the MAM. (A) MAM fractionation scheme. F1 contains MAM and peroxisomes. (B) Immunoblot analysis of subcellular fractions from Huh7 cells. (C) Immunoblot analysis of subcellular fractions from Huh7 (-) or Huh7-HCV K2040 (+) cells. Full length (FL) and cleaved (C) MAVS are indicated by arrows. Fractionation markers: calnexin, ER; Cox-1, mitochondria; VDAC1, OMM; FACL4, MAM; Pex19, peroxisomes. (D) Immunoblot analysis of subcellular fractions from PH5CH8 cells. The table displays quantitation of the immunoblot signal for Sig-1R (MAM), FACL4 (MAM), and calnexin (ER/microsomes), as well as fold enrichment of these proteins in the MAM relative to calnexin.

Fig. S3. Confocal microscopy reveals that MAVS is localized to MAM. (A) Confocal microscopy analysis of immunostaining for endogenous MAVS and the MAM marker Sig-1R in Huh7 cells preloaded with Mitotracker. (B) Immunostaining for endogenous MAVS and TOM20 (OMM) in mock- or SenV-infected Huh7 cells expressing mEGFP-PSS-1 (MAM). (Lower) 3D reconstructions of the Z stacks from the zoom regions of the SenV-infected Huh7 cells in B. Single-cell images are representative of at least 10 cells analyzed. Histograms display measured fluorescence intensity along the line in the merge panels. (Scale bar: 10 μm.)
Fig. S4. Residual MAVS staining after HCV NS3/4A expression. Confocal microscopy analysis was conducted on Huh7 cells immunostained for endogenous MAVS (green) and TOM20 (red, OMM) after expression of NS3/4A. The cell on the left, marked by the arrow, expresses NS3/4A, as demonstrated by loss of MAVS staining. The cell on the right does not express NS3/4A. (Bottom) Signal-enhanced images highlighting residual MAVS staining in the cell expressing NS3/4A.

Fig. S5. RIG-I and MAVS are detected on the MAM after SenV infection. (A) Confocal microscopy of Huh7 cells expressing YFP-RIG-I (green) and the MAM marker mCherry-PSS-1 (red), immunostained for MAVS (blue), and mock- or SenV-infected for 18 h. (Scale bar: 10 μm.) (B) Zoom views of the white frame in A. Arrows indicate contact points of RIG-I, MAM, and MAVS staining. Intensity plots represent the relative fluorescence intensity of the green, red, and blue pixels along the white line in the zoom panel, with gray bars indicating the peaks of RIG-I staining. (C) 3D reconstruction of the Z stack from the zoom region. (D) Colocalization analysis of Z stacks using Manders’ colocalization coefficient, with a higher value representing more colocalization. Values are mean ± SD; n = 3.
Fig. S6. MFN2 knockdown disrupts the contacts between ER and mitochondria. Confocal microscopy analysis of Huh7 cells transfected with ER-GFP, subjected to treatment with nontargeting control (CTRL), MFN1, or MFN2 siRNA pools and then SenV-infected and immunostained for TOM20 (red, OMM). Colocalization analysis was carried out on Z stacks (single planes shown) using Manders’ colocalization coefficient, with a higher value representing more colocalization. Zoom views are from the area within the white frame. (Scale bar: 10 μm.)

Fig. S7. Mitochondrial morphology and MAVS localization during RNA virus infection. (A) Confocal microscopy analysis of Huh7 cells treated with siRNA pools with nontargeting control (CTRL), MFN1, or MFN2 siRNA pools and then mock- or SenV-infected for 18 h. Cells were immunostained with anti-TOM20 (red, OMM). The white arrow denotes representative cells with elongated mitochondria. Images are representative of >250 cells analyzed per experiment in three independent experiments, and the resulting data are quantified and given as mean ± SD. *P ≤ 0.05 **P ≤ 0.005, by unpaired Student t test. (B and C) Confocal analysis of Huh7 cells treated with MFN1 siRNA pools and virus infection as in A and immunostained for MAVS (green) and TOM20 (red) (B) or TOM20 (blue) and PMP70 (peroxisomes, red) in cells expressing MAVS-mEGFP (C). (Scale bar: 10 μm.) Arrows denote MAVS that does not overlap with TOM20 and are considered to represent MAM or peroxisomal MAVS.