The small GTPase RAB1B promotes antiviral innate immunity by interacting with TNF receptor–associated factor 3 (TRAF3)

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Innate immune detection of viral nucleic acids during viral infection activates a signaling cascade that induces type I and type III IFNs as well as other cytokines, to generate an antiviral response. This signaling is initiated by pattern recognition receptors, such as the RNA helicase retinoic acid-inducible gene I (RIG-I), that sense viral RNA. These sensors then interact with the adaptor protein mitochondrial antiviral signaling protein (MAVS), which recruits additional signaling proteins, including TNF receptor–associated factor 3 (TRAF3) and TANK-binding kinase 1 (TBK1), to form a signaling complex that activates IFN regulatory factor 3 (IRF3) for transcriptional induction of type I IFNs. Here, using several immunological and biochemical approaches in multiple human cell types, we show that the GTPase-trafficking protein RAB1B up-regulates RIG-I pathway signaling and thereby promotes IFN-β induction and the antiviral response. We observed that RAB1B overexpression increases RIG-I–mediated signaling to IFN-β and that RAB1B deletion reduces signaling of this pathway. Additionally, loss of RAB1B dampened the antiviral response, indicated by enhanced Zika virus infection of cells depleted of RAB1B. Importantly, we identified the mechanism of RAB1B action in the antiviral response, finding that it forms a protein complex with TRAF3 to facilitate the interaction of TRAF3 with mitochondrial antiviral signaling protein. We conclude that RAB1B regulates TRAF3 and promotes the formation of innate immune signaling complexes in response to nucleic acid sensing during RNA virus infection.

Viruses are detected in the infected cell by the antiviral innate immune system. This system is activated when specific pattern recognition receptors sense pathogen-associated molecular patterns that are unique to viruses. These viral pathogen-associated molecular patterns include cytosolic nucleic acids derived from either RNA or DNA viruses. Specifically, RIG-I and MDA5 sense viral RNA in the cytoplasm, whereas cGAS and IFI16 sense viral DNA (1, 2). Upon sensing of viral nucleic acids, these sensor proteins become activated, allowing them to signal to their respective adaptor proteins, MAVS2 and STING (3, 4). These adaptors then recruit the downstream signaling molecules that ultimately drive the transcriptional induction of type I and type III IFNs (5), leading to production of hundreds of IFN-stimulated genes (ISGs), many of which have antiviral functions (6).

The antiviral innate immune response is carefully regulated to inhibit viral infection without causing excessive inflammation in host tissues. This regulation can occur in several ways, including posttranslational modification of signaling proteins, unique protein–protein interactions between signaling proteins and their regulators, and through changes in localization of signaling proteins and regulators to different subcellular compartments. The RNA sensor RIG-I is regulated by all three of these mechanisms. Before viral infection, RIG-I resides in the cytoplasm in an inactive state (7). During RNA virus infection, both Riplet and TRIM25 ubiquitinate RIG-I with Lys63-linked ubiquitin chains (8–10). This ubiquitination allows RIG-I to interact with the chaperone protein 14-3-3-ε. Subsequently, 14-3-3-ε translocates RIG-I into membranes to interact with MAVS and induce downstream signaling (11). In addition to RIG-I, other antiviral signaling proteins are regulated by changes in their localization during antiviral innate immune induction. For example, both the serine/threonine kinase TBK1 and the E3 ubiquitin ligase TRAF3 have been shown to relocalize upon nucleic acid sensing (12, 13) to interact with the MAVS signaling complex at mitochondria and ER contact sites, otherwise known as mitochondrion-associated ER membranes (MAMs) (14). The mechanism(s) by which these proteins relocalize upon nucleic acid sensing and the consequences of this relocalization are not fully understood.

Previously, we have found that, during viral infection, many cellular proteins change their localization to and from MAMs (15). One of these proteins is the GTPase RAB1B, which is recruited into MAMs to interact with the MAVS signaling complex.
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Previously, we found that, in response to RIG-I signaling, the GTPase protein RAB1B localizes to the MAM and is in a complex with MAVS (15). To determine whether this MAVS-interacting protein also regulates RIG-I/MAVS pathway signaling to IFN-β, we measured Sendai virus (SenV)-mediated signaling to the IFN-β promoter following overexpression of RAB1B in 293T cells. SenV is a negative-strand RNA virus that potently induces RIG-I/MAVS signaling to IFN-β (18, 19). We found that although overexpression of RAB1B on its own did not significantly induce signaling to IFN-β, it did augment SenV-mediated signaling to IFN-β, as measured by an IFN-β promoter luciferase assay and by real-time quantitative PCR (RT-qPCR) for IFNB1 (Fig. 1, A and B). Importantly, overexpression of RAB1B also enhanced the levels of IFNB1 transcripts following transfection of a RIG-I agonist (the hepatitis C virus 5’ppp–containing polyU/UC RNA) (20) in both 293T cells and HuH7 cells (Fig. 1C), suggesting that RAB1B directly regulates RIG-I pathway signaling. We also found that RAB1B overexpression enhanced SenV-mediated induction of IFNB1 transcripts in human monocyte THP1 cells (Fig. 1D). Collectively, these data suggest that although RAB1B does not induce signaling on its own, it does enhance RIG-I pathway signaling to IFN-β.

To determine whether loss of RAB1B decreased RIG-I pathway signaling to IFN-β, we depleted RAB1B in 293T cells using an siRNA. Depletion of RAB1B resulted in a decrease in the induction of IFNB1 transcripts following SenV infection (Fig. 1A). To confirm this result, we generated two cell lines deficient in RAB1B KO-1 and RAB1B KO-2. Then we measured SenV-induced signaling to IFN-β and IFNB1 transcripts following overexpression of a RIG-I agonist (the hepatitis C virus 5’ppp–containing polyU/UC RNA) (20) in both 293T cells and HuH7 cells (Fig. 1C), suggesting that RAB1B directly regulates RIG-I pathway signaling. We also found that RAB1B overexpression enhanced SenV-mediated induction of IFNB1 transcripts in human monocyte THP1 cells (Fig. 1D). Collectively, these data suggest that although RAB1B does not induce signaling on its own, it does enhance RIG-I pathway signaling to IFN-β.

Results

RAB1B positively regulates RIG-I pathway signaling to IFN-β and the antiviral response

Figure 1. RAB1B positively regulates RIG-I pathway signaling to IFN-β. A, IFN-β promoter reporter luciferase expression from 293T cells transfected with the indicated plasmids and then mock- or SenV-infected (16 h). Shown is a representative experiment of three independent repeats. B–D, RT-qPCR analysis of RNA from 293T (B and C, left panels), HuH7 (C, right panel), or THP1 (D) cells expressing FLAG-RAB1B or vector and stimulated with SenV (12 h) or a RIG-I agonist (8 h). IFNB1 transcript levels were measured relative to GAPDH and normalized to the vector (mock) condition. RAB1B expression was verified by immunoblot analysis of extracts from paired samples in A–C and by RT-qPCR for RAB1B relative to GAPDH in D. Individual dots represent technical replicates, with error bars displaying the mean ± S.D. (n = 3) of one of three representative experiments. *, p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, by unpaired Student’s t test comparing the activated samples.
IFN-β induction (Fig. 2B). Next, to determine whether RAB1B also regulates activation of NF-κB, we measured the mRNA levels of A20, a known NF-κB target gene, in the presence and absence of RAB1B (21). We found that A20 transcripts were induced in response to SenV at similar levels in both the WT and RAB1B KO cells (Fig. 2, C and D), revealing that loss of RAB1B does not prevent induction of the NF-κB signaling pathway.

Because RAB1B positively regulated induction of IFN-β, we hypothesized that it would also be required for antiviral responses driven by IFN. Therefore, we tested whether loss of RAB1B resulted in higher levels of viral infection by Zika virus (ZIKV), a virus known to be susceptible to type I IFN (22). To determine whether RAB1B is required for phosphorylation of IRF3 and TBK1, we measured phosphorylation of IRF3 at Ser396 (p-IRF3) in response to SenV (18 h). We found that phosphorylation of IRF3 in parental and RAB1B KO cells was not altered by depletion of RAB1B (Fig. 2E). This, along with the fact that the Huh7-STAT1 KO cells do not produce ISGs in response to IFN-β (Fig. 2F), suggests that during ZIKV infection, the loss of RAB1B in Huh7 WT cells results in decreased levels of IFN signaling and ISG induction, leading to increased levels of infectious ZIKV. Collectively, these data reveal that RAB1B positively regulates the antiviral response.

**RAB1B is required for phosphorylation of IRF3 and TBK1**

The induction of IFN-β in response to viral infection requires the kinase TBK1. Upon activation, TBK1 becomes autophosphorylated and phosphorylates the transcription factor IRF3 at multiple residues, resulting in IRF3 translocation to the nucleus where it cooperates with NF-κB to transcriptionally induce IFN-β (5, 23, 24). To determine whether RAB1B is required to activate this signaling cascade, we first measured phosphorylation of IRF3 at Ser396 (p-IRF3) in response to SenV infection in parental and RAB1B KO-1 293T cells by immunoblot analysis. We found that phosphorylation of IRF3 in response to SenV was decreased in RAB1B KO-1 cells compared with parental cells, revealing that RAB1B activates innate immune signaling upstream of IRF3 phosphorylation (Fig. 3A). Next, to test whether RAB1B is required for activation and autophosphorylation of TBK1, we measured phosphorylation of TBK1 at Ser172 (p-TBK1) after SenV infection in parental and RAB1B KO-1 293T cells by immunoblot analysis. We found
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This suggests that RAB1B can form a complex with either MAVS–TRAF3 or MAVS–TRAF2 but not with TRAF6, suggesting that RAB1B could facilitate formation of the MAVS–TRAF2/3 complexes for downstream signaling.

RAB1B is required for phosphorylation of IRF3 and TBK1 in response to RIG-I pathway activation by SenV

As MAVS interaction with TRAF proteins has been shown to be required for its interaction with TBK1 and downstream signaling (27, 28), we next tested whether RAB1B was required to promote the interaction of either TRAF2 or TRAF3 with MAVS in response to RIG-I signaling. We measured the interaction of endogenous MAVS and TRAF3 or MAVS and TRAF2 during a time course of SenV infection by coimmunoprecipitation. We found that MAVS and TRAF3 interacted at 6 h post-SenV infection in 293T cells; however, this interaction was reduced in RAB1B KO-1 293T cells (Fig. 4E). TRAF2 also interacted with MAVS at 6 h post-SenV infection in WT cells (Fig. 4F). However, in RAB1B-KO-1 cells, TRAF2 interacted with MAVS both in the presence and absence of SenV (Fig. 4F). These data demonstrate that, although RAB1B can interact with both TRAF2 and TRAF3 during overexpression, RAB1B facilitates the interaction of TRAF3, but not TRAF2, with MAVS in response to activation of innate immune signaling.

Discussion

Although many of the signaling proteins that function in cytosolic nucleic acid–sensing pathways have been well-studied, the molecular mechanisms by which these proteins are turned on and off in response to nucleic acid sensing are less defined. Previously, we identified proteins that may regulate assembly of the MAVS signaling complex at membrane contact sites between the ER, mitochondria, and peroxisomes by using proteomics to identify the proteins that relocalize into these contact sites during RIG-I activation (15). Some of these proteins with differential membrane association upon RIG-I signaling included GTPase proteins, such as RAB1B. Here we have shown that RAB1B positively regulates RNA sensing by promoting RIG-I signaling to IFN-β through interactions with the E3 ubiquitin ligase TRAF3. This interaction facilitates TRAF3 recruitment to MAVS, leading to phosphorylation of TBK1 and IRF3 for transcriptional induction of IFN-β. In summary, our work reveals that the known cellular trafficking protein RAB1B interacts with TRAF3 to facilitate assembly of the MAVS signaling complex, providing a new example of a trafficking or chaperone protein that is repurposed to regulate the host response to virus infection (see model in Fig. 5).

Our results suggest that the interaction of RAB1B with TRAF3, but not TRAF2 or TRAF6, is important for IFN induction. Although TRAF2, TRAF3, and TRAF6 have all been shown to interact with MAVS and have roles in regulating IFN induction in response to viral infection, they do have distinct functions that likely mediate each of their regulatory roles in IFN induction (25). In response to viral infection, TRAF3 and TRAF2 have been shown to activate TBK1–IRF3 signaling, whereas TRAF2 and TRAF6, but not TRAF3, have been shown to activate NF-κB signaling (25, 27-29). We found that overexpressed RAB1B interacts with TRAF2 and TRAF3 but not TRAF6 (Fig. 4). However, as we found that RAB1B is not
required for induction of the NF-κB pathway (Fig. 2C) and the fact that, of these TRAFs, only TRAF3 is localized to the Golgi (13), where RAB1B can be localized, we conclude that the function of RAB1B in promoting IFN induction is a result of the interaction (either direct or, more likely, indirect) with TRAF3. It is interesting that loss of RAB1B resulted in TRAF2 interaction with MAVS in the absence of a stimulus. As we did find that overexpressed RAB1B can bind to TRAF2 (Fig. 4F), it is possible that some population of RAB1B binds to TRAF2 to prevent it from interacting with MAVS in the absence of a stimulus. Although RAB1B regulates TRAF3, other RAB or GTPase proteins may regulate TRAF2 or TRAF6 to promote IFN-induction.

It is unclear whether RAB1B directly regulates TRAF3 or whether this regulation is indirect and mediated by RAB1B effector proteins. We know that small GTPase proteins such as RAB1B regulate intracellular membrane trafficking by interacting with effector proteins (16). RAB1B, similar to other GTPase proteins, cycles between an inactive GDP-bound state and an active GTP-bound state that associates with intracellular membranes (16). Importantly, this GTP-bound, membrane-associated RAB1B facilitates COPI recruitment and cargo transport between the ER and the Golgi by interactions with specific effector proteins (30). These effector proteins include tethering factors that link organelles and vesicles prior to fusion, adaptors for motor proteins that direct organelle trafficking, and regulators of other GTPases that are recruited to the specific subcellular compartments of those GTPases (31). Therefore, RAB1B effector proteins are critical for RAB1B trafficking functions and are likely important for mediating the role of RAB1B in antiviral innate immunity. Indeed, the RAB1B effector protein p115 has been shown previously to interact with TRAF3 and is required for TRAF3 recruitment to the MAVS signaling complex (13). Thus, it is likely that RAB1B interacts with p115 or another effector protein to facilitate movement of TRAF3 on vesicles from the Golgi apparatus to sites of MAVS signaling (see model in Fig. 5, steps 3–5). There is evidence that the Golgi and other intracellular membranes rearrange during MAVS signaling to bring MAVS in contact with the Golgi-associated TRAF3 (13, 14). As we were unable to detect relocalization of RAB1B to MAVS signaling sites (data not shown), this suggests that only a small proportion of RAB1B, likely the p115-bound form, interacts with TRAF3 at any given time. Indeed, RAB1B has multiple effector proteins (16, 32), which suggests that different pools of RAB1B in the cell may have different functions that could be activated by interactions with specific effector proteins.

The mechanisms by which RAB1B would interact with a unique set of effectors during cytosolic nucleic acid sensing are not entirely clear. Although the overall localization of RAB1B does not change in the cell during innate immune signaling, it is possible that specific posttranslational modifications are altered (either added or removed) on RAB1B, which may facilitate interactions with a new set of effectors. Indeed, posttrans-
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![Diagram of RAB1B regulation of IFN-β induction](image)

The regulation of innate immune responses by small GTPase proteins is a general cellular mechanism to repurpose these proteins into signaling or trafficking proteins. For example, the GTPase RALB is ubiquitinated by Lys63–ubiquitin linkages to switch from its role in autophagy to a role in innate immune signaling (33, 34). This ubiquitination prevents RALB from binding to its autophagy effector protein EXO84 and instead allows RALB to bind to the Sec5 effector protein for interaction with TBK1 to regulate innate immunity. Similarly, ubiquitination of the ARF domain of TRIM23 activates the GTP hydrolysis activity of TRIM23 to regulate the function of TBK1 in autophagy (35). Therefore, it is possible that changes in the posttranslational modification of RAB1B allow it to interact with TRAF3 for innate immune signaling. Other innate immune signaling proteins are also known to be activated by specific posttranslational modifications (36). The addition and removal of these posttranslational modifications on signaling chaperone or trafficking proteins may be a general cellular mechanism to repurpose these proteins into innate immune signaling regulators, and future studies will examine this possibility for RAB1B.

Our work adds RAB1B to a growing list of GTPase proteins that have been shown to regulate innate immune signaling, either by regulating trafficking of innate immune signaling proteins or specific effector protein interactions. For example, cytosolic DNA-induced innate immune responses mediated by cGAS have been shown to be regulated by RAB2B and its effector protein GARIL5 (37). Signaling from the bacterial LPS sensor TLR4 is also regulated by RAB proteins (38–41). Specifically, both RAB10 and RAB11A positively regulate TLR4 signaling (38), with RAB10 promoting TLR4 recycling to the plasma membrane and RAB11B promoting TLR4 localization to the Escherichia coli phagosome (40, 41). Conversely, RAB7B negatively regulates TLR4 signaling by promoting lysosomal degradation of TLR4 (39). As intracellular innate immunity is often regulated at the cell biological level (42), it is likely that other RAB proteins will control specific intracellular trafficking events that regulate nucleic acid–induced innate immunity and viral infection.

**Experimental procedures**

**Cell culture**

293T, Huh7, T-REx-293, and Vero cells were grown in DMEM (Mediatech) supplemented with 10% FBS (HyClone) and 25 mM HEPES (Thermo Fisher). THP1 cells (a gift from Dr. Dennis Ko, Duke University, who obtained them from the ATCC) were grown in RPMI 1600 medium (Thermo Fisher) supplemented with 10% FBS (HyClone) and 25 mM HEPES (Thermo Fisher). The identity of the Huh7 cells in this study was verified using the GenePrint STR Kit (Promega, Duke University DNA Analysis Facility). The 293T and Vero (CRL-3216 and CCL-81) cells were obtained from the ATCC. The Huh7 cells were a gift from Dr. Michael Gale (University of Washington). The T-REx-293 cells were a gift from Dr. Matthias Gromeier (Duke University, Thermo Fisher). Cells were verified as mycoplasma-free using the LookOut PCR Detection Kit (Sigma).

**Viruses**

SenV Cantell strain was obtained from Charles River Laboratories and used at 200 hemagglutination units/ml. SenV infections were performed in serum-free medium for 1 h, after which complete medium was replenished. ZIKV-Dakar (Zika virus/A. africanus-tc/SEN/1984/41525-DAK) (GenBank accession number KU955591) was provided by Dr. Scott Weaver (University of Texas Medical Branch). Stocks were prepared as described previously (43). ZIKV infections were performed at an m.o.i. of 0.01 for 48 h in Huh7 cells.

**Focus forming assay for ZIKV titer**

Supernatants were harvested from ZIKV-infected cells 48 h after infection, serially diluted, and used to infect naive Vero cells in triplicate wells of a 48-well plate for 2 h before overlay with methylcellulose. After 48 h, plates were fixed in methanol acetone. Cells were blocked (10% FBS in PBS) and then immunostained with a mouse anti-4G2 antibody, generated from the D1-4G2-4-15 hybridoma cell line against the flavivirus envelope protein (ATCC). Infected cells were visualized following incubation with a horseradish peroxidase– conjugated secondary antibody (1:500, Jackson ImmunoResearch Laboratories) and the VIP Peroxidase Substrate Kit (Vector Laboratories). The titer (focus-forming units (FFUs) per milliliter) was calculated from the average number of 4G2-positive foci at ×10 magnification, relative to the amount and dilution of virus used.

**Plasmids**

The following plasmids have been described previously: pEF-TAK-FLAG (44), pEF-TAK-FLAG-RAB1B (15), pEF-BOS-
Table 1

| Primers used for cloning and RT-qPCR | Primer name | Primer sequence |
|-------------------------------------|-------------|----------------|
| HA-RAB1B F | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| HA-RAB1B R | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| Myc-TRAFL F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| Myc-TRAFL R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| Myc-TRAFC F | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| Myc-TRAFC R | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| Myc-TRAFL F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| Myc-TRAFL R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| Myc-TRAFC F | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| Myc-TRAFC R | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| Myc-TRAFL F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| Myc-TRAFL R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E1 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E1 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| STAT1 F | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| STAT1 R | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| RAB1B E1 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E1 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| STAT1 F | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| STAT1 R | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| RAB1B E1 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E1 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| STAT1 F | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| STAT1 R | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| RAB1B E1 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E1 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| STAT1 F | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| STAT1 R | 5'-GCTGGAATTCTAGGCTACATGCACTGCGACAGC-3' |
| RAB1B E1 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E1 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 F | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |
| RAB1B E2 R | 5'-GGACCGCAGATCGTACCTGAGCTGACAGCTGAACTG-3' |

Flag-FLAG-RIG-I-N (26), pIFN-β-luc (45), pGL4.74 [hRLuc/TK] (Promega), pcDNA-Blast (46), pX459 (Addgene), pEF-TAK-Myc-MAVS (47), pEF-TAK-FLAG-MAVS (47), and pEGFP-N1-TFAR (a gift from Dr. Soman Abraham, Duke University) (48). The following plasmids were generated during this study: pEF-TAK-FLAG-RAB1B, pcDNA-Myc-TRAFL3, pEF-TAK-Myc-TRAFL2, pEF-TAK-Myc-TRAFL4, pX330-sgRAB1B, pX459-sgRAB1B-2, pX459-sgRAB1B-3, and pX330-sgSTAT1. pEF-TAK-FLAG-RAB1B was generated by PCR amplification of RAB1B from pEF-TAK-FLAG-RAB1B and insertion into the EcoRI-XbaI-digested pEGF vector using InFusion cloning (Clontech). The pcDNA-Myc-TRAFL3 plasmid was generated by cloning PCR-amplified TRAF3 (pEGFP-N1-TFAR) into pcDNA-Myc using Sall and Kpnl. The pEF-TAK-Myc-TRAFL2 and pEF-TAK-Myc-TRAFL4 plasmids were generated by cloning PCR-amplified TRAF2 or TRAF6 (pcDNA-Myc-TRAFL2 or pcDNA-Myc-TRAFL6, a gift from Dr. Zhijie Chang at Tsinghua University) into pEF-TAK-Myc using Agel and Pmcl. To generate the CRISPR guide RNA plasmids, sgRNA oligonucleotides were annealed and inserted into the BbsI-digested pX330 or pX459 (49, 50). The oligonucleotide sequences used for cloning are listed in Table 1. The plasmid sequences were verified by DNA sequencing and are available upon request.

Generation of RIG-I agonist
Annealed oligonucleotides containing the sequence of the hepatitis C virus 5′ppp poly-U/UC region (Table 1) (20) were in vitro transcribed using the Megashortscript T7 Transcription Kit (Ambion) followed by ethanol precipitation, with the resulting RNA resuspended at 1 μg/μl for use in experiments.

Transfection
DNA transfections were performed using FuGEN6 (Promega) or Lipofectamine 3000 (Invitrogen). RIG-I agonist transfections were done using the TransIT-mRNA Transfection Kit (Mirus Bio). The siRNA transfections were done using Lipofectamine RNAiMax (Invitrogen). The IFN-β promoter luciferase assays were performed as described previously 12 or 16 h after mock or SenV infection and normalized to the renilla luciferase transfection control (18).

Generation of KO cells lines
RAB1B KO cells were generated by CRISPR/Cas9 using a single guide targeting exon 4 designed using the MIT CRISPR design tool or two guides targeting exon 1 using the UCSC Genome Browser (51). STAT1 KO cells were generated using a single guide targeting exon 2 (52). pX330-sgRAB1B-1 (RAB1B KO-1) or pX330-sgSTAT1 was transfected into 293T or Huh7 cells, respectively, along with pcDNA containing blasticidin resistance (pcDNA-Blast) for 24 h. pX459-sgRAB1B-2 and pX459-sgRAB1B-3 (RAB1B KO-2) were cotransfected into T-REx-293 cells for 24 h. Cells were then replated at limiting dilutions into 15-cm dishes with 10 μg/ml blasticidin (pcDNA-KO-1 and STAT1 KO) or 2 μg/ml puromycin (RAB1B KO-2) treatment for 3 days. Single cell colonies were then amplified and screened for RAB1B or STAT1 expression by immunoblotting. Genomic DNA was extracted from candidate RAB1B KO or STAT1 KO cells using QuickExtract DNA Extraction Solution (Lucigen) according to the manufacturer’s instructions. This genomic DNA was then amplified using primers across RAB1B exon 4 (RAB1B KO-1), RAB1B exon 1 (RAB1B KO-2), or STAT1 exon 2 (Table 1). For RAB1B KO-2, the PCR-amplified genomic DNA was subjected to agarose gel electrophoresis to identify a size shift from a product size of 509 bp to a size of 258 bp, the expected size if both guides targeted this exon. RAB1B KO-1 and STAT1 KO genomic DNA was cloned into the pCR4-TOPO TA vector (Invitrogen) and Sanger-sequenced. For RAB1B KO-1, 11 resulting genomic DNA subclones were sequenced, and nine clones had a 27-bp deletion at the end of exon 4 that extended to the intron junction, and two clones had a 1-bp insertion in exon 4 that resulted in a frameshift that lead to a premature stop codon within exon 5. For STAT1 KO, seven resulting genomic DNA subclones were sequenced, and all had a 1-bp insertion in exon 2 that resulted in a premature stop codon four amino acids downstream. STAT1 KO cells were validated by immunoblotting after treatment with 50 units/ml human IFN-β (PBL Assay Science).

RNA analysis
Total cellular RNA was extracted using the Purelink RNA Mini Kit (Life Technologies). RNA was then reverse-transcribed using the Megashortscript Kit (Ambion) followed by ethanol precipitation, with the resulting RNA resuspended at 1 μg/μl for use in experiments.
scribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. The resulting complementary DNA was diluted 1:3 in double-distilled H₂O. RT-qPCR was performed in triplicate using Power SYBR Green PCR Master Mix (Thermo Fisher) and the Applied Biosystems StepOnePlus or QuantStudio 6 Flex RT-PCR system. The oligonucleotide sequences used are listed in Table 1.

**Immunoblotting**

Cells were lysed in a modified radioimmune precipitation assay (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) and Halt phosphatase inhibitor (Thermo Fisher), and post-nuclear lysates were isolated by centrifugation. Quantified protein (between 5–15 μg) was resolved by SDS-PAGE, transferred to nitrocellulose or PVDF membranes in buffer containing 25 mM Tris, 192 mM glycine, and 0.01% SDS and blocked in StartingBlock (Thermo Fisher) buffer, 5% milk in PBS containing 0.01% Tween 20 (PBS-T), or 3% BSA in TBS containing 0.01% Tween 20 (TBS-T). After washing with PBS-T or TBS-T (for phosphoproteins) buffer, membranes were incubated with species-specific horse-radish peroxidase–conjugated antibodies (Jackson ImmunoResearch Laboratories, 1:5000), followed by treatment of the membrane with ECL+ (GE Healthcare) or Clarity Western ECL substrate (Bio-Rad) and imaging on X-ray film or a LICOR Odyssey FC. The following antibodies were used for immunoblotting: anti-RAB1B (rabbit, Santa Cruz Biotechnology, 1:1000 or mouse, Sigma, 1:500), rabbit anti-SenV (MBL, 1:1000), mouse anti-FLAG M2 (Sigma, 1:5000), rabbit anti-GFP (Thermo Fisher, 1:1000), mouse anti-tubulin (Sigma, 1:5000), mouse anti-STAT1 (BD Biosciences, 1:1000), mouse anti-ISG15 (Santa Cruz Biotechnology, 1:1000), rabbit anti-p-IRF3 (Cell Signaling Technology, 1:1000), mouse anti-IRF3 (53) (1:1000), rabbit anti-p-TBK1 (Cell Signaling Technology, 1:1000), rabbit anti-TBK1 (Cell Signaling Technology, 1:1000), anti-HA (mouse, Abcam and rabbit, Sigma, 1:1000), anti-TRAF3 (mouse, Santa Cruz Biotechnology, or rabbit, Cell Signaling Technology, 1:1000), rabbit anti-TRAF2 (Abcam, 1:1000), mouse anti-MAVS (mouse, AdipoGen, or rabbit, Bethyl Laboratories, 1:1000), rabbit anti-GAPDH (Cell Signaling Technology, 1:1000), and anti-Myc (mouse, Santa Cruz Biotechnology, or rabbit, Cell Signaling Technology, 1:1000).

**Quantification of immunoblot**

Immunoblots imaged using the LICOR Odyssey FC were quantified by ImageStudio software, and raw values were normalized to relevant controls for each antibody. Quantified values were normalized to relevant controls for each antibody. Quantified protein (between 200–500 μg) was incubated with protein-specific or isotype control antibody (Cell Signaling Technology) in lysis buffer at 4 °C overnight with head-over-tail rotation. The lysate/antibody mixture was then incubated with either protein A or G Dynabeads (Invitrogen) for 2 h. Beads were washed three times in PBS or modified RIPA buffer for immunoprecipitation and eluted in 2X Laemmli buffer (Bio-Rad) supplemented with 5% 2-mercaptoethanol at 95 °C for 10 min. Proteins were resolved by SDS-PAGE and immunoblotting as above.

**Statistical analysis**

Student’s unpaired t test and two-way ANOVA with Tukey’s post hoc test were used for statistical analysis of the data using GraphPad Prism software. Graphed values are presented as mean ± S.D. or S.E. (n = 3 or as indicated); *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

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