Supporting Information

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SI Materials and Methods

Viruses and Cell Lines. RV-1B was obtained from ATCC (VR-1645) and amplified in H1-HeLa cells (a generous gift from W. M. Lee, University of Wisconsin, Madison, WI). RV-1BM was obtained by serial passage of RV-1B 27 times in the LA-4 mouse lung cell line (ATCC; CCL-196). In brief, cells were inoculated with RV-1B and allowed to incubate for 2–3 d at 33 °C, and then 0.5 mL of the cell lysate was used to reinfect a new LA-4 monolayer. In later passages, lysate titers were determined and monolayers were reinfected with an MOI of 0.01. A cytopathic effect in LA-4 cells became visible by passage 9. After 27 passages, the mouse-adapted virus was amplified in LA-4 cells to obtain virus stock for the experiments. To concentrate and purify virus, virus was pelleted from clarified lysates of infected H1-HeLa (for RV-1B) or LA-4 (for RV-1BM) cells by ultracentrifugation through a 30% (vol/vol) sucrose cushion (Beckman Optima LE-80K ultracentrifuge, SW28 rotor, 25,000 rpm, 5 h) and resuspended in PBS. H1-HeLa cells, LA-4 cells, and mouse embryonic fibroblasts (MEFs) were maintained in MEM, F12K, and DMEM media with standard supplements (Gibco). Infection media were as follows: for HeLa cells, MEM (Gibco; 11095) containing 5% heat-inactivated newborn calf serum, nonessential amino acids, penicillin/streptomycin, and 30 mM $MgCl_2$; for LA-4 cells, F12K medium (Mediatech; 10-025-CV) containing 5% heat-inactivated FBS, penicillin/streptomycin, and 30 mM $MgCl_2$.

Sequencing. For RV-1B and RV-1BM sequencing, RNA was extracted from cell lysates of RV-1B, RV-1B passage 10, and RV-1B passage 27 (RV-1BM) using TRIzol and reverse-transcribed using SuperScript II Transcriptase (Invitrogen) reverse transcriptase (Invitrogen) and random hexamer primers (Applied Biosystems). Consensus PCR primers were constructed from HRV-1B [GenBank accession no. D00239 (1)]. The genomes for both parent and experimental strains were amplified using touchdown PCR using primers to generate seven fragments ∼2 kb in length. PCR fragments were then sequenced using these PCR primers and additional sequencing primers to generate sequences ∼300–500 bp in length with two- to threefold coverage of each genome. PCR fragments were analyzed using CLC DNA Workbench (CLC bio; version 6). Primer sequences are available upon request.

Growth Curves and Plaque Assay. H1-HeLa cells and LA-4 cells were plated the day before infection in 6- or 12-well plates at 80% confluence and infected at an MOI of 0.001. Cell lysates were prepared at the indicated time points by freeze/thaw, and titer was determined by plaque assay on H1-HeLa cells using a procedure modified from Fiala and Kenny (2). Briefly, for the plaque assay, HeLa cells in six-well plates were incubated with 200 μL virus diluted in PBS + 0.1% BSA at 33 °C for 1 h with rocking and then overlaid with plaque assay medium (1× MEM, 5% FBS, 0.3% agarose, 30 mM $MgCl_2$, 30 μg/mL DEAE-dextran) and incubated at 33 °C for 3 d before staining with crystal violet.

Primary Cell Culture and Infection. Primary cells were isolated from WT or KO B6 mice as indicated below.

Primary mouse airway epithelial cells. Primary mouse AECs were cultured from mouse tracheas based on a previously described protocol (3). Cells were maintained in Millipore airway medium plus supplements (SCML, SCML002-S). After primary amplification for ∼1 wk, airway cells were plated on collagen-coated 12-well plates at 5×10^4 to 1×10^5 per well (Becton Dickinson; BioCoat). Cells were infected or stimulated 2–8 d postplating when cells appeared >80% confluent. Replicate wells were counted at the time of infection to calculate the MOI. To infect, medium was removed and cells were exposed to virus diluted in PBS + 0.1% BSA for 1 h at 33 °C. Next, the inoculum was removed and cells were washed with 1–2× warm PBS and then overlaid with medium. Cells were incubated at 33 °C and/or 37 °C until the indicated time points. For low MOI growth curves (>24 h), medium was supplemented with 30 mM $MgCl_2$ to enhance viral growth.

DCs. WT and KO BM cells were plated at 10^6 per well in a 24-well plate and cultured in complete RPMI medium (containing 10% heat-inactivated FBS, 10 mM Hepes, penicillin/streptomycin) containing GM-CSF for 5 d. Cells were washed with PBS before inoculation with 100 μL virus in PBS + 0.1% BSA (1 h, 33 °C, with rocking). After inoculation, 300 μL medium was added and cells were incubated at 33 °C and/or 37 °C for 20–24 h, at which time supernatants were collected for ELISA.

pDCs. BM cells were cultured in complete RPMI containing 0.1 μg/mL FLT3L (Gemini Bio-Products; 300-118P) at a density of 2.25×10^6/mL. On day 7, pDCs were collected and plated at 10^6 per well in a 48-well plate just before mixing with virus diluted in 0.1 μg/mL FLT3L-containing medium. Cells were incubated at 33 °C and/or 37 °C for 20–24 h, at which time supernatants were collected for ELISA. Positive control for cell viability was IFN-α expression in response to the TLR9 agonist CpG2216 (TriLink).

ELISA. To assess mouse IFN-β levels in AEC supernatants, ELISA was performed using a PBL kit according to the manufacturer's instructions. To assess mouse IFN-α levels in dendritic cell supernatants, ELISA was performed using coating antibody (Novus Biologicals; NB100-64387) and detection antibody (PBL InterferonSource; 32100-1).

Quantitative RT-PCR. To assess viral RNA levels and cellular IFN mRNA levels, cellular RNA was extracted from cultured cells using an RNeasy Kit (Qiagen) and reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using SYBR Green (Qiagen; QuantiTect SYBR Green Kit 204145) or iTaq Universal SYBR (Bio-Rad) with primers as follows.

Rhinovirus. RV-1BM 3C [forward (F): 5′-CAATGGAAAATTTACAGGTCTTGG-3′; reverse (R): 5′-ATGCCGTTGACTTGAACCTC-3′].

Mouse.

IFN-α4 (F: 5′-CTGCTACTTGGAATGCAACTC-3′; R: 5′-CAGTCTTGCCAGCAAGTTGG-3′).

IFN-β (F: 5′-GCACTGGGTGGAATGAGACTATTG-3′; R: 5′-TTCTGAGGCATCAACTGACAGGTC-3′).

IFN-$\lambda_{2,3}$ (F: 5′-AGCTGCAGGCCTTCAAAAAG-3′; R: 5′-TGGGAGTGAATGTGGCTCAG-3′).

Oas1a (F: 5′-ATTACCTCCTTCCCGACACC-3′; R: 5′-ATTACCTCCTTCCCGACACC-3′).

Oasl2 (F: 5′-GGGAGGTCGTCATCAGCTTC-3′; R: 5′-CCCTTTTGCCCTCTCTGTGG-3′).

Eif2ak2/*PKR* (F: 5′-GATGGAAAATCCCGAACAAGGAG-3′; R: 5′-AGGCCCAAAGCAAAGATGTCCAC-3′).

Stat-1 (F: 5′-CGCGCATGCAACTGGCATATAACT-3′; R: 5′-ATGCTTCCGTTCCCACGTAGACTT-3′).

Isg15 (F: 5′-TCCATGACGGTGTCAGAACT-3′; R: 5′-TACCCTTTCCAGTCTGGGTC-3′).

Hprt mRNA (F: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; R: 5′-GAGGGTAGGCTGGCCTATTGGCT-3′).

Mx1 (F: 5′-AGTCCTTTCCACAGGCAGAA-3′; R: 5′-CATTGAGAGAAACTCACCTAAGAAC-3′).

Mouse cDNA amplicons of interest were normalized to levels of Hprt. Normalized amplicon levels are graphed as fold change relative to the indicated control.

RNA-Seq. RNA was isolated from RV-1BM–infected mouse primary airway epithelial cells using a Qiagen RNeasy Kit per the manufacturer's instructions. Libraries were generated and sequenced on the Illumina HiSeq 2000 using paired-end sequencing at the Yale Center for Genomic Analysis. Raw reads were mapped to the mouse reference genome (mm10) with the TopHat (version 2.0.6) algorithm (4), and mapped to the virus RNA sequence (HRV-1BM) with the bwa (version 0.6.2) algorithm (5). RNA abundance of both was calculated by Cufflinks (version 2.0.2) (6). Gene set enrichment of differentially expressed genes in mouse in the Gene Ontology database was performed by the online tool DAVID (7, 8).

Poly I:C Treatment. Stimulation with poly I:C (Sigma; P9582) was performed as follows. Medium was aspirated and cells were incubated with poly I:C complexed with Lipofectamine 2000 (Invitrogen; 1168-019) for 1 h at 33 °C at the indicated concentrations or Lipofectamine only for mock treatment. After this time, the poly I:C mixture was removed and complete medium was added. Cells were incubated at 33 °C or 37 °C until the indicated time points before assessment.

Malachite Green ATP Hydrolysis Assay. To determine the steady-state kinetic parameter, k_{cat}, of ATP hydrolysis for RIG-I and MDA5 at various temperatures, an established malachite green assay was used (9). RIG-I or MDA5 (50 nM) was first preincubated with an excess of RNA (15 ng/μL low-molecular weight poly I:C) for 1–2 h at the desired temperature in ATP hydrolysis buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM DTT). The reactions were then initiated by the addition of a 1:1 ATP–$MgCl_2$ complex for a final concentration of 2 mM. For initial velocity measurements, aliquots of the reaction were quenched at six time points between 15 s and 10 min by the addition of 5× quench buffer (250 mM EDTA). Malachite green reagent was added (9:1 malachite green:reaction volume) and allowed to age for 30 min at room temperature. The A_{650} was then measured using a Synergy 2 plate reader (BioTek) and resulted in the following k_{cat} values at the indicated temperatures for MDA5: $5.39 \pm 0.14\ s^{-1}$ (30 °C), $9.55 \pm 0.67\ s^{-1}$ (33 °C), $11.35 \pm 0.55\ s^{-1}$ (37 °C), and $12.14 \pm 0.34\ s^{-1}$ (42 °C); and for RIG-I: $1.04 \pm 0.12\ s^{-1}$ (30 °C), $2.78 \pm 0.15\ s^{-1}$ (33 °C), $4.59 \pm 0.22\ s^{-1}$ (37 °C), and $1.08 \pm 0.07\ s^{-1}$ (42 °C).

IFN-β Treatment. Recombinant mouse IFN-β was obtained from PBL InterferonSource (product 12400-1) and used to assess inhibition of RV-1BM replication in LA-4 cells and MEFs and to assess ISG induction in LA-4 cells. Briefly, cells were plated at $1–2 \times 10^5$ per well in six-well plates the day before the experiment. At the start of the experiment, growth medium was replaced with medium containing the indicated concentration(s) of IFN-β. For the IFN-β stock used in these experiments, 10 U/mL is equivalent to 1 ng/mL.

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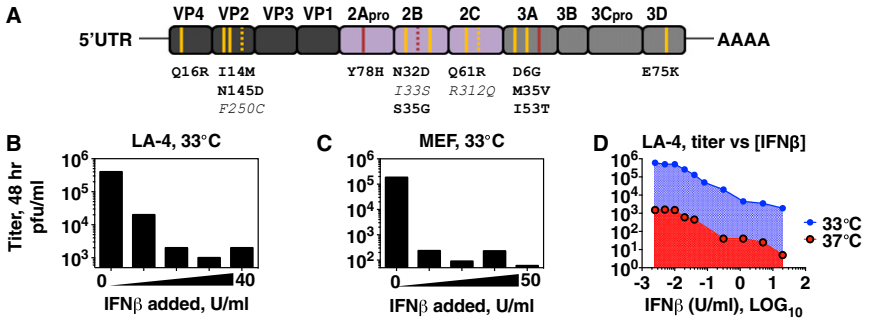

Fig. S1. Amino acid changes and inhibition by IFN-β of mouse adapted RV-1B (RV-1B*M*). (*A*) Amino acid changes acquired by RV-1BM during serial passage in LA-4 cells. Yellow vertical lines indicate amino acid changes detected in RV-1BM compared with RV-1B by passage (P)10, whereas mutations detected between P10 and P27 are indicated by red lines. Dashed lines and italicized text indicate an RV-1BM sequence that differs from our RV-1B parent sequence but converges with the published sequence (GenBank accession no. D00239). (*B*) LA-4 cells were inoculated with RV-1BM for 1 h at 33 °C, and then medium containing serial fivefold dilutions of recombinant mouse IFN-β (40, 8, 1.6, 0.32 U/mL) was added postinoculation as indicated. After incubation at 33 °C for 48 h, cell lysates were prepared and titer was determined by plaque assay on HeLa cells. (*C*) Mouse embryonic fibroblasts were preincubated with serial fourfold dilutions of IFN-β–containing medium for 6 h (50, 12.5, 3.1, 0.8 U/mL) and then inoculated with RV-1BM for 1 h, after which IFN-containing medium was added back. Virus was allowed to amplify for 48 h, after which lysates were assessed for viral titer. (*D*) Dose–response curve for IFN-β–mediated inhibition of RV growth in LA-4 cells incubated at 33 °C or 37 °C for 48 h, using serial fourfold dilutions of IFN-β ranging from 20 to 0.0025 U/mL.

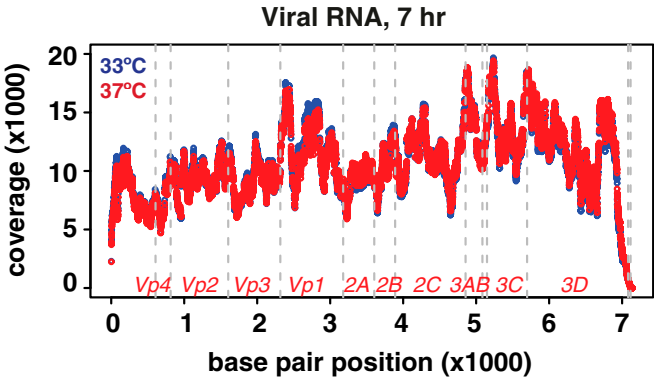

Fig. S2. Normalized reads across the viral genome in mouse primary airway cells incubated at 33 °C or 37 °C. RNA-Seq was performed on RNA obtained from primary mouse airway epithelial cells infected with RV-1BM and incubated at 33 °C (blue) or 37 °C (red) for 7 h. In this plot, the *x* axis indicates the base pair position within the RV genome, and the *y* axis value represents the coverage at each position: (number of viral reads mapping to each position/total number of viral reads present in the sample) × 10^6. This plot normalizes for the quantity of viral RNA in each sample and thereby allows direct comparison of the relative representation of the viral genome in cells supporting viral replication at 33 °C vs. 37 °C at 7 h postinoculation.

overnight	inoculation	incubation
33°C	33°C	33°C
37°C	33°C	33°C
37°C	37°C	33°C
37°C	33°C	37°C
37°C	37°C	37°C

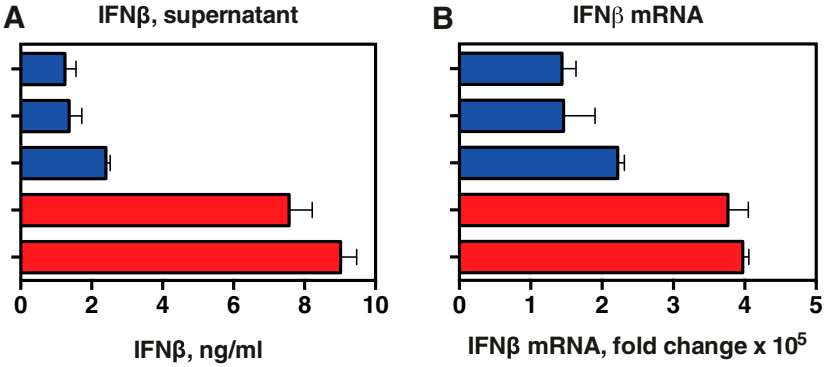

Fig. S3. Effect of preincubation temperature and inoculation temperature on temperature-dependent induction of IFN-β by airway epithelial cells during RV-1BM replication. Primary AECs were grown to confluence in 12-well plates and then incubated overnight at 33 °C or 37 °C and counted before inoculation. Overnight incubation temperature did not affect cell number (2.5 × 10^5 cells per well). Next, cells were inoculated with RV-1BM, MOI 10, for 1 h at either 33 °C or 37 °C. Following the inoculation period, the inoculum was removed and medium was added and plates were allowed to incubate for 9 additional hours at either 33 °C (blue bars) or 37 °C (red bars), at which time supernatants were collected for IFN-β ELISA (*A*) and cells were collected for RNA isolation, followed by RT-qPCR for IFN-β transcripts (*B*). IFN-β mRNA levels are plotted relative to the level in mock-treated cells that were preincubated and incubated for 10 h at 33 °C. Bars represent the mean and SD of three or four replicates per condition. No IFN-β was detected at 10 h in the supernatants of mock-treated cells in any of the five incubation temperature combinations. Data are representative of two replicate experiments.

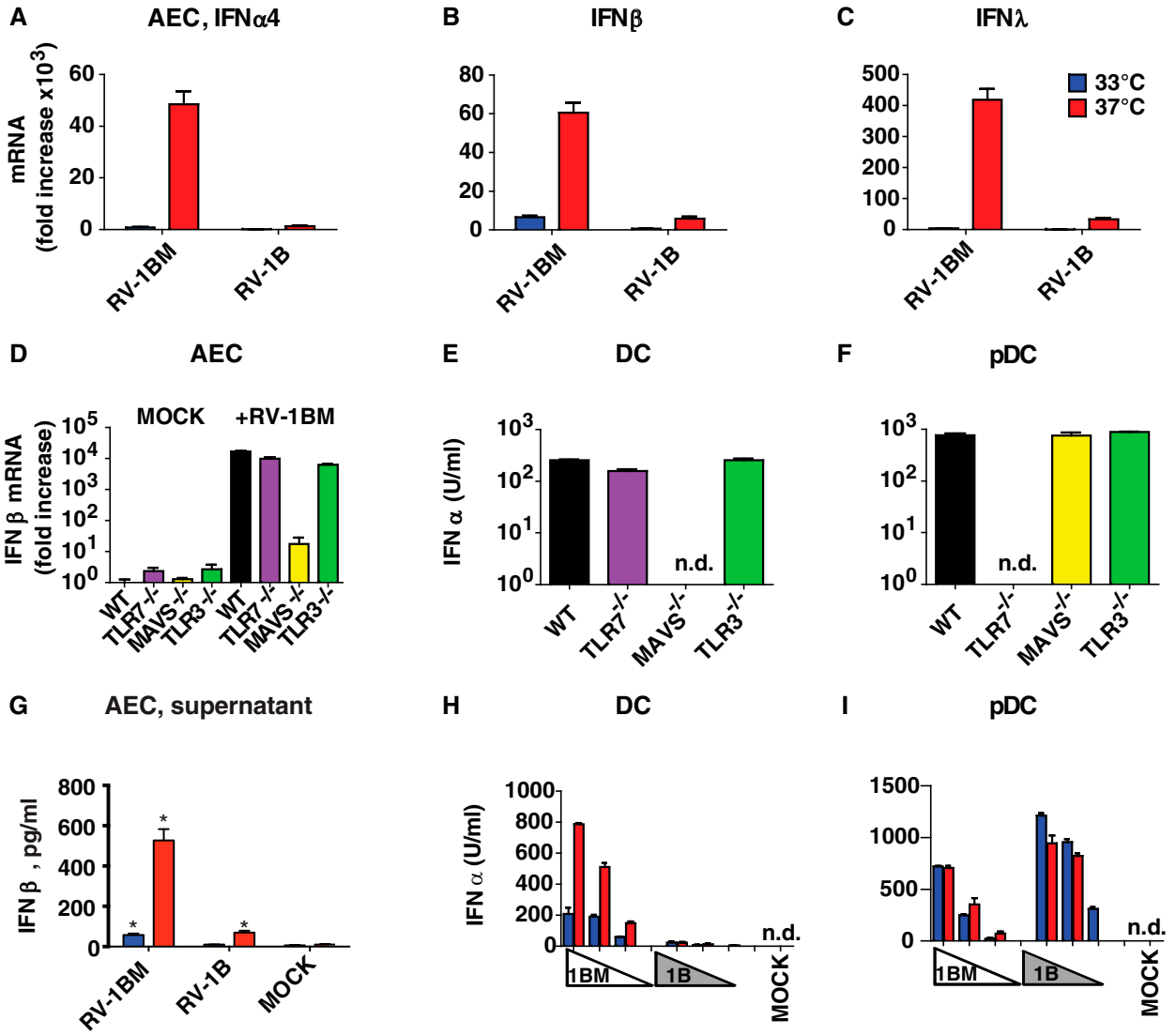

Fig. S4. RLR-, but not TLR7-, dependent induction of IFN is enhanced at core body temperature during RV infection. (*A*–*C*) Fold induction in IFN mRNA 8 h after infection of mouse primary AECs with RV-1B or RV-1BM at an MOI of 5. After inoculation, cells were incubated at 33 °C (blue bars) or 37 °C (red bars). (*D*) IFN-β mRNA level in WT or KO primary AECs 8 h postinfection with RV-1BM (MOI 5), at 37 °C, relative to mock WT level. (*E* and *F*) IFN-α levels in supernatants of WT and KO mouse DCs or pDCs infected with RV-1BM, MOI 2.5, 22 h postinoculation and incubated at 37 °C. (*G*) IFN-β protein (picograms/ml) detected in the supernatant of primary AECs 8 h after exposure to RV-1BM or RV-1B, MOI 5, incubated at 33 °C or 37 °C. (*H* and *I*) IFN-α levels in mouse DC and pDC supernatants 20–24 h after infection with serial twofold dilutions of RV-1B or RV-1BM, beginning at an MOI of 5 (*H*) or MOI of 2 (*I*), at 33 °C or 37 °C. Bars show the mean ± SEM of three replicates. Each graph is representative of at least three independent experiments. n.d., not detected.

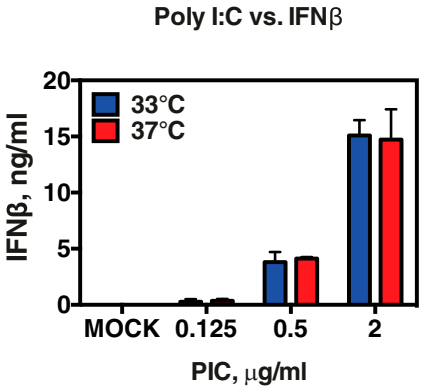

Fig. S5. IFN secretion by primary mouse airway epithelial cells in response to transfected poly I:C at 8 h poststimulation. AECs were transfected with serial fourfold dilutions of PIC for 1 h at 33 °C and then kept at 33 °C or shifted to 37 °C, and supernatants were collected at 9 h for IFN-β ELISA. The mean and SD of three replicates are indicated.

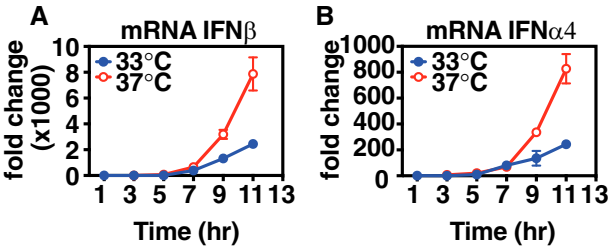

Fig. S6. Induction of type I interferons IFN-β (*A*), or IFN-α4 (*B*), during single-step RV-1BM replication in IFN-αβR–deficient mouse primary airway cells. Primary AECs from *Ifnar1*$^{-/-}$ mice were infected with RV-1BM, MOI 20, for 1 h at 33 °C, and then medium was added and cells were kept at 33 °C or shifted to 37 °C. Cells were collected for RNA at the indicated time points, and levels of IFN mRNA were determined by qPCR and are graphed relative to the level at $t = 1$ h (postinoculation). Error bars represent mean and SEM of 2-3 replicates per condition.

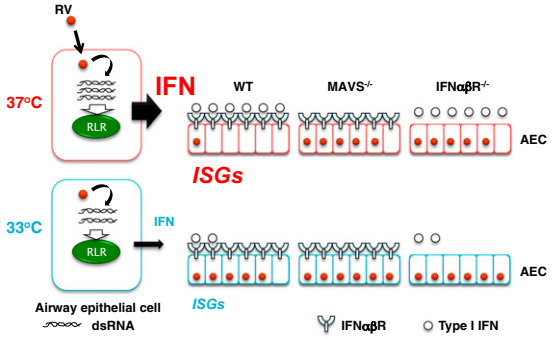

Fig. S7. Model. Role of differential innate signaling in the temperature-dependent replication of the common cold virus. In this model, RV enters an airway epithelial cell (red or blue box; *Left*) and begins to replicate. At 37 °C, viral replication triggers more RLR-dependent secretion of type I IFNs. IFN (circles) binds to the type I IFN receptor IFN-αβR (symbols) and blocks viral replication in adjacent WT cells via induction of ISGs, a process that is more robust at 37 °C relative to 33 °C. Cells lacking the MAVS protein in the RLR signaling pathway fail to induce IFN production, and cells lacking IFN-αβR fail to respond to IFNs, allowing virus to replicate and spread. Because RLR stimulation and signaling as well as ISG induction are more robust at 37 °C, the growth advantage conferred by these innate immune deficiencies is more pronounced at this temperature.

Table S1. RNA-Seq alignment summary: Rhinovirus-infected mouse airway epithelial cells

Temperature	Total reads (mouse + virus)	No. of reads after quality control (mouse + virus)	Mapping rate (mouse + virus), %	Mapped to virus (%)
33 °C	130,840,838	90,783,012	88.07	2,313,534 (2.55)
37 °C	156,009,116	108,373,889	88.17	4,159,101 (3.84)