Ifit1 regulates norovirus infection and enhances the interferon response in murine macrophage-like cells [version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

Background: Norovirus, also known as the winter vomiting bug, is the predominant cause of non-bacterial gastroenteritis worldwide. Disease control is predicated on a robust innate immune response during the early stages of infection. Double-stranded RNA intermediates generated during viral genome replication are recognised by host innate immune sensors in the cytoplasm, activating the strongly antiviral interferon gene programme. Ifit proteins (interferon induced proteins with tetratricopeptide repeats), which are highly expressed during the interferon response, have been shown to directly inhibit viral protein synthesis as well as regulate innate immune signalling pathways. Ifit1 is well-characterised to inhibit viral translation by sequestration of eukaryotic initiation factors or by directly binding to the 5' terminus of foreign RNA, particularly those with non-self cap structures. However, noroviruses have a viral protein, VPg, covalently linked to the 5' end of the genomic RNA, which acts as a cap substitute to recruit the translation initiation machinery.

Methods: Ifit1 knockout RAW264.7 murine macrophage-like cells were generated using CRISPR-Cas9 gene editing. These cells were analysed for their ability to support murine norovirus infection, determined by virus yield, and respond to different immune stimuli, assayed by quantitative PCR. The effect of Ifit proteins on norovirus translation was also tested in vitro.

Results: Here, we show that VPg-dependent translation is completely refractory to Ifit1-mediated translation inhibition in vitro and Ifit1 cannot bind the 5' end of VPg-linked RNA. Nevertheless, knockout of Ifit1 promoted viral replication in murine norovirus infected cells. We then demonstrate that Ifit1 promoted interferon-beta expression following transfection of synthetic double-stranded RNA but had little effect on toll-like receptor 3 and 4 signalling.

Conclusions: Ifit1 is an antiviral factor during norovirus infection but cannot directly inhibit viral translation. Instead, Ifit1 stimulates the antiviral state following cytoplasmic RNA sensing, contributing to restriction of norovirus replication.
Keywords
IFIT, norovirus, innate immunity, interferon

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Author roles: Mears HV: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Emmott E: Formal Analysis, Investigation, Methodology, Writing – Review & Editing; Chaudhry Y: Investigation, Methodology, Writing – Review & Editing; Hosmillo M: Investigation, Methodology, Writing – Review & Editing; Goodfellow IG: Formal Analysis, Funding Acquisition, Investigation, Supervision, Writing – Review & Editing; Sweeney TR: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Writing – Original Draft Preparation

Competing interests: No competing interests were disclosed.

Grant information: This research was funded by a Wellcome Trust/Royal Society Sir Henry Dale Fellowship to T.S. (202471) and a Wellcome Trust Senior Fellowship to I.G. (207498). H.M. is supported by a Department of Pathology PhD studentship.

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How to cite this article: Mears HV, Emmott E, Chaudhry Y et al. Ifit1 regulates norovirus infection and enhances the interferon response in murine macrophage-like cells [version 1; peer review: 1 approved, 2 approved with reservations] Wellcome Open Research 2019, 4:82 (https://doi.org/10.12688/wellcomeopenres.15223.1)

First published: 15 May 2019, 4:82 (https://doi.org/10.12688/wellcomeopenres.15223.1)
Introduction
The Caliciviridae family of small positive-sense RNA viruses comprises 11 genera, including Norovirus and Sapovirus. Noroviruses are the leading cause of non-bacterial gastroenteritis in humans, accounting for 18% of acute gastroenteric disease worldwide. While recent advancements in human intestinal organoids have made it possible to study human noroviruses in culture, murine norovirus (MNV) remains a valuable model for dissecting interactions between noroviruses and their host, owing to readily cultivable permissive cell lines and a flexible reverse genetics system.

The innate immune response to viral infection is essential for the control of norovirus replication and clearance. Sensing of calicivirus infection is predominately mediated by cytoplasmic double-stranded RNA sensors; both RIG-I and MDA5 have been implicated in controlling the innate immune response at different stages of infection. By contrast, TLR3, an endosomal dsRNA sensor, has little effect on norovirus replication. RIG-I and MDA5 signalling converge on the activation of the antiviral signalling complex MAVS, which recruits TBK1 to induce the phosphorylation of interferon regulatory factor (IRF)-3. Activated IRF-3 dimerises and translocates into the nucleus where it promotes the transcription of type I interferon (IFN) and early antiviral genes.

During the antiviral response, among the most strongly upregulated IFN-stimulated genes are the IFIT family of RNA-binding proteins. In humans, IFIT1 directly inhibits the translation of non-self RNA viruses at the initiation stage, by binding over the 5' terminus, occluding the recruitment of eukaryotic translation initiation factor (eIF)-4F. IFIT1 binding is highly specific for capped mRNA which lacks methylation at the first or second cap-proximal nucleotides (cap0). Murine Ifit1 similarly binds cap0 RNA and mediates the inhibition of cap0 viruses in vivo. It is important to note, however, that murine Ifit1 and human IFIT1, which share 52% sequence identity, have distinct evolutionary origins, with murine Ifit1 being more closely related to another gene family member, IFIT1B.

However, IFIT1 may have antiviral activity independent of its RNA-binding capability. IFIT1 was reported to inhibit hepatitis C virus replication by binding to eIF3 to prevent viral translation initiation. Additionally, direct binding to the human papilloma virus DNA helicase, E1, was reported to inhibit viral DNA replication. IFIT1 also modulates different stages of the host innate immune response during both viral and bacterial infection and may regulate the inflammatory response in human astrocytes. MNV can antagonise innate immune sensing and was consequently shown to inhibit the expression of a number of interferon-stimulated genes, including Ifit2. However, associations between noroviruses and other members of the Ifit family have not been established.

We investigated whether Ifit1 played a role in the antiviral response to calicivirus infection. We show that Ifit1 knockout promoted MNV replication in a macrophage cell line. However, calicivirus translation was not inhibited by Ifit1. Instead, we show that Ifit1 knockout cells have impaired cytoplasmic double-stranded RNA sensing, resulting in a weaker type I IFN response, which permits increased viral replication.

Methods
Cells, viruses and plasmids
Murine macrophage RAW264.7, microglial BV2 and Crandell-Rees feline kidney cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) foetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). LLC-PK cells, expressing bovine viral diarrhoea virus NPro to render them IFN-deficient, were cultured in Eagle’s minimal essential medium (EMEM) supplemented with 200 μM glycochenodeoxycholic acid (GCDA; Sigma), 2.5% FCS, and 1% P/S. MNV-1 strain CW.1 was recovered from the pT7:MNV-G 3'Rz plasmid as described. Feline calicivirus (FCV) strain Urbana was recovered from the pQ14 full length infectious clone. The porcine sapovirus (PSaV) Cowden tissue culture adapted strain was obtained from K. O. Chang (Kansas State University) and recovered from the full-length infectious clone pCV4A. For lentivirus generation, psPAX2 (Addgene plasmid # 12259) and pMD2.G (Addgene plasmid # 12259) were gifts from Didier Trono. For bacterial expression, murine Ifit1 (NM_008331.3), Ifit2 (NM_008332.3) and Ifit3 (NM_010501.2) were cloned between Ncol and Xhol sites in pTriEx1.1, to contain a C-terminal His tag.

Knockout cells
First, five guide RNAs designed against the 5' end of the second exon of Ifit1 (Table 1), were cloned into lentiCRISPR v2. Next, 3 μg guide RNA plasmid was cotransfected into 5 × 10⁶ HEK293T cells with 3 μg psPAX2 packaging vector and 1.5 μg pMD2.G VSV-G envelope vector using lipofectamine 2000 (Invitrogen). Supernatants were harvested over 72 hours, pooled and used directly for transduction of subconfluent RAW264.7 cells. After 3 days, transduced cells were selected with puromycin for one week, before single cell clones were generated by dilution in 96-well plates. Knockout was verified by western blotting, as described below, after treatment with murine IFNβ for 12 hours and harvesting in passive lysis buffer (Promega).

Table 1. Guide RNA sequences for CRISPR-Cas9 knockout of Ifit1. Guide RNAs were generated using crispr.mit.edu and cloned into LentiCRISPRv2. The 3’ protospacer adjacent motif (PAM) sequences are underlined in bold.

| Guide RNA sequence | PAM     |
|--------------------|---------|
| GGAAGGGTTGAGCATCCCACGTTGG | TGG    |
| ATTCGATCGATGCAGACACCTCTG | TGG    |
| CTTGACATCGAAGACCCCTGTTGG | TGG    |
| GAAGCGAGATTCCTCCATGACCTTGG | TGG    |
| AAATAATGACATACCTGATT | TGG    |

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**Table 1.** Guide RNA sequences for CRISPR-Cas9 knockout of Ifit1. Guide RNAs were generated using crispr.mit.edu and cloned into LentiCRISPRv2. The 3’ protospacer adjacent motif (PAM) sequences are underlined in bold.
Infections
Cells were infected for 1 hour at 37°C at the multiplicity of infection (MOI) indicated in the legend to Figure 1. Cells were harvested by freezing at the indicated times and titres were determined by 50% tissue culture infectious dose (TCID\textsubscript{50}) in BV2 cells, as described\textsuperscript{4}, performed in technical quadruplicate. Plates were scored by cytopathic effect after 5 days and titres were calculated by the Reed and Muench method\textsuperscript{5}.

Stimulation of RAW264.7 cells
Cells were treated with 10 ng/mL LPS (Sigma) or 1 μg/mL polyI:C (Sigma), or transfected with 2 μg polyI:C using lipofectamine 2000 (Invitrogen). Cells were harvested by washing twice in PBS before lysis in passive lysis buffer (Promega) and RNA was extracted using TRIreagent (Sigma).

Reverse transcription-quantitative PCR (RT-qPCR)
For RT-qPCR analysis, cDNA was generated using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) with random hexamer primers. qPCR was performed on cDNA using primers for murine IFN\textbeta\textsuperscript{37}, TNF\textalpha\textsuperscript{38} and GAPDH, using the qPCR core kit for SYBR green I with low ROX passive reference (Eurogentec), using the manufacturer’s recommended parameters: 95°C for 15 seconds then 60°C for 1 minute, for 50 cycles. Data were normalised against GAPDH, expressed as fold change over mock (2\textsuperscript{-ΔΔCq}).

Western blotting
Cell lysates were separated in 12.5% SDS-PAGE and transferred to 0.45-μm nitrocellulose membrane by semi-dry blotting. Membranes were blocked in 5% milk phosphate buffered saline with 0.1% tween-20 (PBS-T) and primary antibodies were incubated in 5% BSA PBS-T at 4°C overnight. Anti-Ifit1 (Santa Cruz, sc-134949, rabbit polyclonal) was used at 1:500, anti-Ifit2/3 (ProteinTech, 12604-1-AP, rabbit polyclonal) was used at 1:800 and anti-GAPDH (Invitrogen, AM4300, mouse monoclonal) was used at 1:8000. Blots were incubated with IRDye 680LT Goat anti-Mouse IgG (Li-Cor, 926-68020) and IRDye 800CW Donkey anti-Rabbit IgG (Li-Cor, 926-32213) secondary antibodies at 1:10000 in PBS-T, for 1 hour at room temperature, then imaged on an Odyssey CLx Imaging System (Li-Cor).

RNA extraction and in vitro transcription
Preparation of VPg-linked RNA from MNV, FCV\textsuperscript{39,40} and PSaV\textsuperscript{32} infected cells was performed as described using the GenElute total RNA extraction kit (Sigma). In vitro transcribed RNAs were generated with T7 polymerase (New England Biosciences) from linearised plasmids and subsequently capped using the ScriptCap Capping System (CellScript).

Recombinant protein purification
Recombinant Ifit1, Ifit2 and Ifit3 were expressed in BL21 (DE3) Star Escherichia coli (Invitrogen). Cells were grown to an OD\textsubscript{600} of ~1.0 in 2x TY media at 37°C. Expression was induced with 1 mM isopropyl b-D-1-thogalactopyranoside at 22°C for 16 hours. Cells were harvested in a lysis buffer containing 400 mM KCl, 40 mM Tris pH 7.5, 5% glycerol, 2 mM DTT and 0.5 mM phenylmethylsophonyl fluoride with 1 mg/mL lysozyme. Proteins were purified by affinity chromatography on NiNTA agarose (Qiagen), followed by FPLC on MonoQ (GE Healthcare) as described\textsuperscript{41}.

In vitro translation
Using the Flexi Rabbit Reticulocyte Lysate system (Promega), 8 nM cap0 or 20 ng/μL VPg-linked RNA was translated in the presence or absence of 1.5 μM Ifit proteins, including 5 uCi EasyTag\textsuperscript{TM} L-\textsuperscript{[35S]}-Methionine (Perkin-Elmer). After 90 min at 30°C, reactions were terminated by addition of 50 mM EDTA and 0.5 μg/μL RNaseA. Labelled proteins were separated by 12.5% PAGE and detected by autoradiography using an FLA7000 Typhoon Scanner (GE).

Primer extension inhibition
Primer extension inhibition assays were performed as described\textsuperscript{12}. Briefly, 1 nM cap0 or VPg-linked RNA were incubated with 1.5 μM Ifit proteins for 10 minutes at 37°C in reactions containing

\textbf{Figure 1.} Ifit1 decreases MNV infection in RAW264.7 cells. (A) Ifit1 knockout RAW264.7 cells were generated by CRISPR-Cas9 gene editing. Cells were stimulated with IFN\textbeta for 12 hours then analysed by western blotting against Ifit1 and Ifit2/Ifit3. GAPDH was included as a loading control for each membrane. (B, C) Infection of wild-type (WT) and Ifit1 knockout (KO) RAW264.7 cells at (B) high or (C) low multiplicity of infection (MOI) with murine norovirus (MNV-1). Viral titres were determined by 50% tissue culture infectious dose (TCID\textsubscript{50}) in BV2 cells and expressed as log\textsubscript{10}-transformed values. At late time points, indicated, severe cytopathic effect (cpe) was visible. Graphs show the mean and the standard error of three biological replicates. Titres were compared between WT and KO cells for each time point by two-tailed Student’s t-test. Asterisks indicate that a statistically significant difference (p < 0.05) was observed for both KO cell lines.
20 mM Tris pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 1 mM ATP, 0.2 mM GTP, 1 mM DTT and 0.25 mM spermidine. RT was carried out using 2.5 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega) and a 32P-labelled primer in the presence of 4 mM MgCl₂ and 0.5 mM dNTPs. Primer sequences used for RT were CCTGCTCAGGAGGGTCATG (MNV-1), GTCATAACTGCAACAAGAAG (FCV) and GTCTGTCGGGTGCCAGAATA (PSaV). Sequencing reactions were performed using the Sequenase Version 2.0 DNA Sequencing Kit (ThermoFisher) in the presence of β³S-labelled ATP. cDNA products were resolved on 6% denaturing PAGE and detected by autoradiography using an FLA7000 Typhoon Scanner (GE).

Statistical analysis
Log viral titres and RT-qPCR fold changes were analysed by two-tailed Student’s t-test, assuming unequal variance, using Microsoft Excel (Microsoft Office 2013, Version 15.0.5119.1000). Values were compared between wildtype cell lines and each knockout cell line, for each time point. Where both knockout cell lines were significantly different to wildtype (p < 0.05), this is indicated in the figure with an asterisk. Graphs were generated in GraphPad Prism 7 (Version 7.03). Full statistics are available as Underlying data.

An earlier version of this article can be found on bioRxiv (doi: https://doi.org/10.1101/611236).

Results
Ift1 inhibits MNV in RAW264.7 cells
We examined the effect of Ifit1 on calicivirus replication, using MNV as a model. Ifit1 knockout RAW264.7 cell lines were generated by CRISPR-Cas9 gene editing and complete knockout was verified by western blotting. Images of all uncropped blots are available as Underlying data. Ifit1 expression was undetectable in two independent Ifit1 knockout clones after 12 hours of treatment with IFNβ, while expression of Ifit2 and Ifit3 was maintained (Figure 1A). Wild-type and Ifit1 knockout RAW264.7 cells were then infected with MNV1 at low or high MOI and samples were harvested by freezing at the indicated time points. Viral titres were determined by TCID₅₀ assay in BV2 cells. Raw viral titres are available as Underlying data.

In Ifit1 knockout cells infected at a high multiplicity of infection, MNV-1 titres were slightly higher than wild-type cells at 6–8 hours post infection (Figure 1B). By 12–14 hours post infection, viral titres from wild-type and knockout cells were similar. At these times, a high degree of cytopathic effect was observed, hence infection did not progress any further. When infected at low multiplicity, the differences between wild-type and Ifit1 knockout cells were more apparent (Figure 1C). Infection of Ifit1 knockout cells resulted in up to 20x higher MNV-1 yields compared to wild-type cells over the course of the infection, suggesting that Ifit1 has antiviral activity during norovirus infection.

Ift1 cannot inhibit VPg-dependent translation
Ifit1 promotes its antiviral activity by binding to the 5' cap of non-self RNA, to occlude translation factor recruitment and prevent viral translation. However, members of the Caliciviridae family possess a viral protein, VPg, covalently linked to the 5’ end of the genome which promotes viral translation, in place of a 5’ cap. Since knockout of Ifit1 promoted MNV replication in vitro, this suggests that Ifit1 may restrict MNV replication directly, by inhibiting viral translation, or indirectly, by creating a cellular environment which is less permissive to infection. To differentiate these possibilities, we first examined whether Ifit1 could inhibit calicivirus translation in vitro. A similar in vitro translation approach was originally used by Guo et al. to describe the activity of IFIT proteins, and since has been successfully used to investigate IFIT1 translation inhibition on human parainfluenza virus and Zika virus model RNAs.

To generate VPg-linked RNA for examination, total RNA was extracted from cells infected with MNV, PSaV or FCV. For PSaV and FCV, translation from VPg-linked RNA prepared in this way predominantly consists of VP1, the major viral capsid protein, which is translated from a highly abundant subgenomic RNA (Figure 2A). VPg-linked RNAs were transcribed in rabbit reticulocyte lysate in the presence or absence of recombinantly expressed and purified murine Ifit1, Ifit2 and Ifit3. β³S-Met-labelled translation products were separated by SDS-PAGE and detected by autoradiography. Full-length in vitro transcribed cap0 RNA was included as a positive control for Ifit1 activity (Figure 2A). As expected, Ifit1 strongly inhibited translation of the artificial cap0 viral RNA, but had no effect on the translation of VPg-linked RNAs (Figure 2B). Addition of Ifit2 and Ifit3 did not enhance translation inhibition on any RNA tested.

Consistently, we observed no evidence of direct Ifit1 binding to VPg-linked RNA when examined in a primer extension inhibition assay, an approach we have used previously to quantify IFIT binding to different RNAs. Ifit1, alone or with Ifit2 and Ifit3, was incubated with viral RNA before reverse transcription from a radiolabelled primer specific for the full-length genomic RNA of each virus. cDNA products were resolved by denaturing polyacrylamide gel electrophoresis. Ifit1 was capable of forming a toeprint 6-7 nt downstream of the full-length cDNA product on artificial cap0 RNAs, consistent with binding to the 5’ end (Figure 2C). However, VPg-linked RNAs derived from infected cells were not bound by Ifit1. Addition of Ifit2 and Ifit3 did not affect Ifit1 binding.

Ift1 knockout cells have defective innate immune sensing
Ifit1 was previously shown to regulate different stages during innate immune signalling, including signalling downstream of MAVS and TLR3. Therefore, we hypothesised that Ifit1 may mediate its antiviral activity during norovirus infection by promoting the innate immune response to infection. We therefore tested our knockout cell lines for their ability to respond to different stimuli. Wild-type and Ifit1 knockout RAW264.7 cells were incubated with LPS or polyI:C, or transfected with polyI:C, to stimulate TLR4, TLR3 or cytoplasmic RNA sensing pathways, respectively. Samples were taken up to 24 hours post infection and RNA or protein was extracted for analysis.

When polyI:C was transfected, to stimulate cytoplasmic RNA sensing, IFNβ expression was strongly upregulated 3 to 9 hours
post transfection, decreasing by 12 to 24 hours (Figure 3A). Expression was 4- to 10-fold higher in wild-type cells during the peak of expression, compared to Ifit1−/− cells. TNFα was induced to a much lesser extent and expression was comparable between all cell lines (Figure 3B). We observed weak induction of both IFNβ and TNFα when polyI:C was added to the cell culture medium, rather than transfected, and expression levels were comparable between all cell lines tested (Figure 3C–D). This indicates that the differential response in Ifit1−/− cells is specific to cytoplasmic, rather than endosomal, RNA sensing. Cells treated with LPS showed little upregulation of IFNβ mRNA expression when analysed by RT-qPCR (Figure 3E). However, TNFα was strongly upregulated 3 to 6 hours post LPS treatment in all cell lines, returning to near baseline expression by 9 hours post treatment (Figure 3F). At 6 hours, TNFα expression was 2- to 3-fold higher in IFIT1−/− cells compared to wild type, consistent with a recent report29. Raw gene expression data are available as Underlying data42.

**Discussion**

Noroviruses replicate in the cytoplasm, where they establish membrane-associated replication complexes in which the viral genome is replicated via a dsRNA intermediate. Cytoplasmic double-stranded RNA sensors, RIG-I and MDA5, are principally responsible for detecting replicating calicivirus RNA, activating the type I IFN response5–7. This rapid and robust antiviral programme is necessary for viral clearance4. Here, we demonstrated that the antiviral protein Ifit1 promotes type I IFN responses in RAW264.7 cells and as such contributes to the host antiviral response to restrict murine norovirus infection.

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**Figure 2. Calicivirus translation is resistant to Ifit1 inhibition.** (A) Schematic representations of *in vitro* transcribed cap0 genomic RNA or VPg-linked genomic and subgenomic RNAs, purified from infected cells, used for *in vitro* translation and toeprint assays. (B) *In vitro* translation of cap0 or VPg-linked RNA from murine norovirus (MNV), porcine sapovirus (PSaV) and feline calicivirus (FCV). VP1, the dominant protein product produced from the VPg-linked subgenomic RNA, is indicated. (C). Toeprint analysis of MNV, PSaV and FCV VPg-linked and cap0 RNA. Ifit1 binding is indicated by a cDNA product 6-7 nt shorter than the full-length signal (FL), indicated by black arrowheads. Red arrowheads indicate a 1-2 nt shorter full-length signal on VPg-linked RNAs.
We observed that Ifit1−/− RAW264.7 cells were more susceptible to MNV infection. In most cells, Ifit1 is not expressed to detectable levels under basal conditions, but expression is induced within a few hours of IFN treatment or viral infection. As such, we noticed a more pronounced difference between wild-type and Ifit1-deficient cells following a low multiplicity infection, since type I IFN from infected cells will induce naïve cells to establish an antiviral state, including the upregulation of Ifit1 expression.

IFIT proteins have been implicated in regulating different stages of the antiviral and inflammatory responses (reviewed by Mears and Sweeney). In humans, IFIT1 was shown to promote type I IFN expression during alphavirus infection. Consistently, a recent study in human and murine macrophages has shown that IFIT1 stimulates type I IFN expression, but represses the inflammatory gene programme, in the acute response following a number of different stimuli. The authors suggest that a small population of nuclear IFIT1 can modulate the activity of transcription regulatory complex Sin3A-HDAC2, which is responsible for downregulating both type I IFN and inflammatory gene expression.

Another study has suggested that cytoplasmic IFIT1 downregulates IFN expression by disrupting the MAVS-TBK1-STING signalling axis. Together, these studies present a model by which a low level of nuclear IFIT1 promotes type I IFN responses by modulating transcripational activity. Later in infection, when IFIT1 is highly expressed in the cytoplasm, IFIT1 prevents induction of type I IFN by interfering with MAVS signalling. Consistent with this hypothesis, we observed strong IFNβ expression 3 to 9 hours post stimulation in wild-type cells, which sharply decreased from 9 to 24 hours. In Ifit1−/− cells, IFNβ expression was induced to a lesser extent, but remained constant up to 24 hours post stimulation, indicating that Ifit1 may be necessary both to switch on and switch off IFN induction at different stages of the immune response.

In caliciviruses, VPg acts as a substitute for the mRNA 5′ cap, by interacting directly with components of the eIF4F complex, to promote ribosome recruitment via eIF3. Additionally, the VPg of MNV and Norwalk virus, the prototypic strain of human norovirus, may also interact with eIF3 to promote efficient translation initiation. IFIT proteins have been reported to interact with eIF3 and inhibit translation initiation on certain mRNA transcripts. Human IFIT1 binds to the e subunit of eIF3 and can inhibit translation from the hepatitis C virus internal ribosome entry site (IRES). Murine Ifit1 and Ifit2 have both been shown to bind to different domains of the eIF3c subunit, causing translation inhibition on luciferase reporter mRNA at micromolar concentrations. However, while Ifit3 was also reported to bind to eIF3c, it has no impact on translation.

Figure 3. Ifit1 promotes type I IFN expression following cytoplasmic RNA sensing. (A–F) Wild-type (WT) and Ifit1 knockout (KO) RAW264.7 cells were stimulated with (A,B) 2 μg transfected polyI:C (polyI:C), or (C,D) 1 μg/mL polyI:C or (E,F) 10 ng/mL lipopolysaccharide (LPS) in the cell culture medium. RNA was extracted and analysed by RT-qPCR for IFNβ (A,C,E) and TNFα (B,D,F) mRNA, expressed as fold induction over untreated cells, normalised against GAPDH (2−ΔΔCq). Graphs show the mean and the standard error of three biological replicates. Fold induction was compared between WT and KO cells for each time point by two-tailed Student’s t-test. Asterisks indicate that a statistically significant difference (p < 0.05) was observed for both KO cell lines.
We have demonstrated that S' VPg renders calivirus genomic RNA resistant to Ifit1-mediated translation inhibition. We saw no effect on translation of either capped or VPg-linked RNA when Ifit2 and Ifit3 were added to \textit{in vitro} translation lysates, indicating that neither of these proteins can inhibit translation, despite their potential to interact with eIF3. Therefore, it remains to be determined how IFIT-eIF3 interactions can inhibit translation initiation on some transcripts but not on others.

In summary, despite calivirus RNA being refractory to translation inhibition by Ifit proteins, we have shown that Ifit1 knockout cells support a higher degree of MNV infection compared to wild-type cells. We observed that Ifit1 promoted type I IFN expression downstream of cytoplasmic dsRNA sensing, suggesting it may play a role in potentiating the host antiviral state. This work contributes to a growing body of evidence that IFIT proteins can modulate innate immune signalling, complementing their role in translation inhibition.

Data availability

Underlying data

Figshare: Original gels used for making figures. https://doi.org/10.6084/m9.figshare.7998521.v1.

Figshare: Raw and processed data used to generate viral titre and host gene expression figures. https://doi.org/10.6084/m9.figshare.7998563.v1.

This project contains the following underlying data:

- FIG1BC_TCID50_RAW_MNV.xlsx (containing raw data on viral titre).
- FIG3_qPCR_RAW_LPSpolyC.xlsx (containing raw qPCR data for gene expression).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Grant information

This research was funded by a Wellcome Trust/Royal Society Sir Henry Dale Fellowship to T.S,(202471) and a Wellcome Trust Senior Fellowship to I.G. (207498). H.M. is supported by a Department of Pathology PhD studentship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Reviewer Report 29 July 2019

https://doi.org/10.21956/wellcomeopenres.16613.r35948

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Mears et al. describe an analysis of the role of canonical ISG IFIT1 in regulation of murine norovirus infection in RAW264.7 cells. Enhanced MNV-1 viral titers were observed in 2 distinct cell lines lacking Ifit1, and the authors convincingly demonstrate that inhibition of viral replication by IFIT1 is independent of regulation of VPg-dependent translation. The authors suggest an alternate mechanism of regulation via promotion of type I IFN expression by IFIT1, though this is not explored in depth. Overall, this is a well-written manuscript, the data is robust, and this is an important contribution to the understanding of ISG-mediated control of norovirus infection.

Comments:
1. It would be appropriate to reference the B cell model for human norovirus cultivation in addition to HIEs in the introduction.

2. In Figure 1A, IFIT1 is convincingly absent in KO1/2 lines. However, IFIT2 and IFIT3 levels also appear to be somewhat decreased. Can the authors please comment, especially as this may be related to the putative mechanism of suppression of IFN signaling in IFIT1 KO cells?

3. Could the authors please comment in figure legends on the number of experiments performed, and number of blots for which figures are representative? Are replicates within a single experiment or across multiple experiments?

4. The statement in the first paragraph of the discussion, "Ifit1 promotes type I IFN responses in RAW264.7 cells and as such contributes to the host antiviral response to restrict murine norovirus infection", is somewhat overstated, as the authors do not provide any direct evidence that IFN levels are differentially regulated by MNV in the presence or absence of Ifit1. The polyIC data is suggestive, but transfection of MNV RNA or analysis of IFN levels after infection at a variety of MOI would be helpful. If the authors can provide this data, this would be welcome addition to the study, or the conclusion should be softened.
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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Norovirus, interferons, innate immunity.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 22 July 2019
https://doi.org/10.21956/wellcomeopenres.16613.r35949

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Donna MacDuff
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The manuscript from Mears et al., examines the role of murine Iifit1 as an antiviral factor for RNA viruses that utilize a VPg protein on the 5’ end of their RNAs in place of a cap structure. By generating two Iifit1 KO RAW264.7 (murine macrophage) cell lines, the authors show that Iifit1 suppresses murine norovirus (MNV) replication. Iifit1 has been shown previously to block the translation of capped mRNAs by
interacting with the cellular translation machinery. However, using an in vitro translation assay, the authors demonstrate that IFIT1, as well as IFIT2 and IFIT3 do not inhibit translation of VPg-associated MNV, feline calicivirus (FCV) or porcine sapovirus (PSaV) RNAs. Cap0-associated RNAs are included as an important positive control for these assays. Evidence is also presented that, while IFIT1 can bind to Cap0-associated viral RNAs, IFIT1, IFIT2 or IFIT3 cannot bind to VPg-bound viral RNAs. Previous studies have shown that IFIT1 can regulate interferon (IFN) induction downstream of TLR3, TLR4 and MAVS. The authors confirm that IFIT1-deficiency impairs IFN induction after activation of intracellular RNA sensors by polyI:C transfection. However, they are unable to detect differences in IFN induction after stimulation of TLR3 or TLR4 with polyI:C or LPS treatment of WT and IFIT1 KO RAW cells. Overall, the experiments are well-controlled and the data are mostly convincing with multiple viruses tested. While the demonstration that IFIT1 controls MNV replication but cannot inhibit translation of VPg-RNAs is novel, the modulation of IFN induction by IFIT1 has been demonstrated by several other studies, and no further mechanistic insight is provided. The connection between IFIT1-mediated IFN induction after RNA sensing and regulation of MNV replication is inferred, but not demonstrated in this study.

Major comments:

1. The authors conclude that IFIT1 modulation of the interferon response contributes to the restriction of MNV replication. However, no direct evidence is provided either that IFN induction after MNV infection is controlled by IFIT1, or that a defective IFN response is responsible for enhanced MNV replication in IFIT1 KO cells. Does IFNb treatment of cells restore control of MNV replication in IFIT1 KO cells? Since this study focuses of the anti-viral role of IFIT1 during calicivirus infection, it would be beneficial to measure IFN induction after MNV infection, particularly since the authors have shown previously that MNV-1 expresses an ORF that antagonizes the interferon response. Alternatively, the authors could revise this conclusion.

2. The authors should clarify the nature of the WT RAW cells. Are they a polyclonal population, or a single clone derived at the same time as the two KO clones? Individual clonal cell populations can behave very differently. Does complementation of the KO cells with IFIT1 restore control of MNV replication or interferon induction?

3. In Figure 1 B and C, the authors demonstrate that MNV replicates to higher titers within 6-8 hours post-infection. However, viral titers at the time of infection are not shown. Is viral binding and entry equal in WT and KO cells?

4. For Figure 3, the doses of polyI:C and LPS used do not appear to be well optimized. 2 μg polyI:C is a large amount to transfect into cells (is this per ml?). A lower dose (0.2 μg) may allow larger differences in IFN induction to be observed. The IFN response to polyI:C and LPS treatment appears to be too minimal to detect differences in induction between the WT and KO cells and to draw conclusions from the data. Larger doses may be required.

5. The authors state that IFIT1 is not detectably-expressed in basal conditions and is induced within a few hours. The authors should consider how the timing of IFIT1 induction, differences in IFN induction (in Figure 3A or during MNV infection, which may take longer), and differences in MNV replication (Figure 1B and C) fit into their model.
5. The description of the experiments in Figure 3 in the text state that protein was extracted for analysis, but the data are not presented.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Innate immune signaling, interferon regulation, inflammation, murine norovirus biology, in vitro and in vivo models of viral infection.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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Yogesh Karpe
Nanobioscience Group, Agharkar Research Institute, Pune, India

Authors Mears et al. in their manuscript have investigated the antiviral role of IFIT1/ISG56 in response to calicivirus infection. Authors have shown that Ifit1 knockdown promoted MNV replication in a macrophage cell line. Finally, the authors demonstrated that Ifit1 knockdown cells have impaired cytoplasmic dsRNA sensing and weak type I interferon response. Overall this is a well-written manuscript containing results which merit indexing.

Specific comments:
1. Comments should be made on the relevance of RAW264.7 cells for MNV infection studies. Why authors’ selected this particular cells for their studies?

2. In this work, authors have purified recombinant Ifit1, Ifit2, and Ifit3. These recombinant proteins were used for in-vitro translation and primer extension inhibition assays. So please indicate the purity of the recombinant proteins used for the assay. Also please include the gel images of purified recombinant proteins in the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular Virology, Replication, and pathogenesis of Hepatitis E virus.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.