A human and rhesus macaque interferon-stimulated gene screen reveals ARHGEF3/XPLN as an antiviral gene against hepatitis C virus and other flaviviruses

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Abstract

Natural hepatitis C virus (HCV) infection is restricted to humans whereas in other primates such as rhesus macaques, the virus is non-permissive. To identify human and rhesus macaque genes that differ or share the ability to inhibit HCV replication, we conducted a medium-throughput screen of lentivirus-expressed host genes that disrupt replication of HCV subgenomic replicon RNA expressing secreted Gaussia luciferase. A combined total of >800 interferon-stimulated genes (ISGs) were screened. Our findings confirmed established anti-HCV ISGs, such as IRF1, PKR and DDX60. Novel species-specific inhibitors were also identified and independently validated. Using a cell-based system that recapitulates productive HCV infection, we identified the ‘Rho Guanine Nucleotide Exchange Factor 3’ gene (ARHGEF3) from both species as a restriction factor for full-length virus replication. Mechanistically, ARHGEF3-mediated inhibition was ablated by mutating a critical GEF active site residue and deleting the N-terminal portion of the protein. Additionally, replication of two mosquito-borne flaviviruses, yellow fever virus (YFV) and Zika virus (ZIKV), were reduced in ARHGEF3-expressing cell lines compared to controls. In conclusion, we ascribe novel antiviral activity to the cellular gene ARHGEF3 that inhibits replication of HCV and other important human viral pathogens belonging to the Flaviviridae, and is conserved between humans and rhesus macaques.

Introduction

Hepatitis C virus (HCV) is the prototypic member of the genus Hepacivirus - in the family Flaviviridae, alongside yellow fever virus (YFV) and Zika virus (ZIKV) - that now include viruses identified in diverse mammals, such as rodents, bats and horses¹. HCV and other hepaciviruses can efficiently establish long-term chronic infection in the liver². In the case of HCV infection in humans, chronic inflammation, cirrhosis and
liver failure are typical sequelae that arise over a period of decades\(^3\). However, natural
HCV infection is restricted to humans, although chimpanzees can recapitulate aspects
of HCV pathogenesis and immunity following experimental infection\(^4\). Other non-
human primates such as rhesus macaques are not permissive for HCV infection\(^5,6\).
Understanding the factors underlying the species tropism of HCV is of major
importance in order to aid development of better animal models for infection, which
could be used to understand pathogenesis or determine vaccine efficacy\(^7\).

Host cells have evolved numerous defence mechanisms to block viral infection
and limit harmful consequences, while differences in the innate immune systems of
species can underly differential species tropism of viruses\(^8\). In vertebrates, a multitude
of antiviral genes are induced through activation of pathogen recognition receptors
(PRRs) and synthesis of three families (types I, II and III) of cytokines called interferons
(IFNs)\(^9\). Autocrine and paracrine signalling via IFN activation of cell surface receptor
complexes induces the expression of hundreds of genes, so-called ‘IFN-stimulated
genes’ (ISGs), which may have direct or indirect antiviral activity, regulate IFN
signalling or control inflammation\(^10\). Given the impact that IFNs and ISGs have on virus
biology, viruses encode numerous mechanisms to evade or inhibit this response either
at the point of initial detection of infection, blocking of signalling or evasion of specific
antiviral ISGs\(^9\).

HCV infection induces IFN production and signalling \textit{in vivo} during acute and
chronic phases of infection, which can be examined in \textit{in vitro} model cell culture
systems\(^11\)–\(^14\). Furthermore, until recently, recombinant type I IFN – which
demonstrates antiviral activity \textit{in vitro}\(^15\) - was used to treat chronic HCV infection\(^16\).
Given the obvious biomedical interest in understanding how IFN controls HCV
infection, numerous reports have identified a plethora of anti-HCV ISGs over the last
two decades (reviewed in \(^17\)), such as PKR\(^18,19\), MxA\(^19\), IRF9\(^19\), IFI6/IFI6-16\(^20\)–\(^22\),
IFI27\(^19\), 2′-5′-OAS, IRF1\(^23\), IFITM1\(^24,25\) and IFITM3\(^26\), MDA5\(^23\), RIGE\(^23\), OASL\(^23\),
MAP3K14\(^23\), IFI44L\(^23\), NT5C3\(^23\), DDIT4\(^23\), SSBP3\(^23\), ISG56/IFIT1\(^24\) and IFIT3\(^27\),
PLSCR1\(^27\), TRIM14\(^27\), NOS2\(^27\), RSAD2/VIPERIN\(^18,28\), ISG20\(^18\), GBP1\(^29\), CH25H\(^30\),
RTP4\(^31\), ISG15\(^32\), C19orf66\(^33\), and DDX60L\(^34\). These studies have utilised various
approaches, such as HCV pseudoparticle\(^25\), subgenomic replicon\(^34\) and infectious
virus\(^23\) methods to recapitulate the various stages of the virus life cycle. Moreover,
there has been deployment of an array of systems for identifying ISGs with anti-viral
activity, including loss- or gain-of-function approaches\(^20,23\). To date, one of the most
comprehensive and fruitful strategies employed used an arrayed lentiviral system that
expressed ~400 individual human ISGs against infectious HCV, which uncovered a
number of effectors with broad anti-viral activity as well as specific anti-HCV
specificity\(^23\). Whether other anti-HCV ISGs exist in humans or indeed non-permissive
primate species like rhesus macaques against infectious or subgenomic replicon HCV
systems is unknown.

In this study we expanded the previous screens of human ISGs with potential
anti-HCV activity to include a large set of rhesus macaque ISGs\(^35\), to determine
whether additional anti-HCV IFN-mediated effectors could be found with species
specificity. From the potential ISGs that restricted HCV replication, we also examined
their activity against related flaviviruses YFV and ZIKV, identifying ARHGEF3/XPLN
as an anti-flaviviral factor conserved between humans and rhesus macaques.
Results

Screening of human and rhesus macaque ISGs against HCV sub-genomic replicon replication

To determine whether ISGs from rhesus macaques could block HCV replication, and in an attempt to identify additional anti-HCV human ISGs, we established an ISG screening assay to systematically probe the anti-HCV activity of ISGs in the context of transient HCV RNA replication by sub-genomic replicons (SGR). The sensitivity of the assay was enhanced by deploying a system that used transiently-transfected HCV genotype (gt)2a JFH1-based replicon RNA expressing the secreted reporter protein, *Gaussia* luciferase (GLuc), in “wild-type” Huh7 cells. We used libraries of human and rhesus macaque ISGs encoded by lentiviral vectors, which co-express the ISG alongside the puromycin resistance gene and red fluorescent protein (RFP). Huh7 cells in 96 well plates were transduced with the lentivirus libraries and subsequently transfected with HCV SGR RNA. Cells were selected for puromycin resistance and extracellular RLU was measured at 72 hours post (hp) electroporation (Fig 1A). The IRF1 ISG positive control demonstrated robust inhibition of reporter luciferase activity comparable to values obtained from mock transduced cells (sFig1).

Applying the above approach, a medium-throughput screen was performed with the human and rhesus macaque ISG libraries containing ~870 ISG ORFs against the luciferase-expressing transient HCV SGR. Initial analysis using an arbitrary cut-off for hits as defined by >10-fold reduction in reporter activity as compared to controls, revealed 54 inhibitory human ORFs (Fig 1B). This group included known anti-HCV ISGs such as IRF1 (as described above), RIG-I, RSAD2/ VIPERIN and OASL. Using the same criteria, the screen identified 46 inhibitory rhesus macaque ORFs (Fig 1B), including known anti-HCV ISGs (and their rhesus macaque orthologues where applicable) such as IRF7, PKR, and DDX60L. Together, our human and rhesus macaque screens therefore identified 100 candidate antiviral effectors, the majority of which were potential novel ISG inhibitors of HCV RNA replication, such as human ARHGEF3 and TRIM34, or rhesus macaque PHF11 and BTN3A1 (Fig 1B). There were also ISG ORFs that enhanced HCV reporter expression (Fig 1B), including both human and rhesus macaque orthologues for two genes, CYTH1, and HPSE.

Before further validation of these hits, we refined the ISGs revealed by the screen. Firstly, APOBEC genes (e.g. APOBEC3G) were omitted for further study due to their known ability to restrict lentiviral transduction and thus likely to identify false positives. Additionally, the putative rhesus macaque ISG, ‘HLA-X’, was excluded as it was not possible to verify that this allele was authentic due to possible generation of chimeric amplicons via PCR, which would make downstream validation challenging. Reductions in HCV reporter activity could be caused by off-target effects such as activation of a global antiviral state via the induction of the IFN pathway. Therefore, to identify putative HCV restriction factors, we filtered potential hits by removing those that activate the IFN-beta promoter and interferon stimulated response element (ISRE) (>2-fold) using findings from a previously derived dataset. Together, this additional filtering resulted in a final selection of 62 ISGs (32 human and 30 macaque), which we proceeded to validate.
Validation of ISG inhibitors of HCV SGR replication

All potential inhibitory hits (alongside CYTH1 and HPSE that enhanced replication) from the primary screen were tested in an independent secondary validation screen since it is possible that the initial screen could identify false positives other than those that we had excluded. Importantly, this secondary screen was conducted using independently generated lentivirus stocks following plasmid confirmation of gene identity. The same experimental procedure as described above was employed except data were obtained in triplicate. In these experiments, we took a ~2-fold inhibition of the mean values in RLU compared to the ‘no ISG control’ of empty/EGFP-expressing lentivirus transductions to select positive hits (Fig 2A and B). Of the ISGs assessed, about 50% were validated as inhibitory in the secondary screen. In parallel, the transduction efficiency of the lentivirus stocks was also determined by measurement of the number RFP-positive cells using non-puromycin-selected samples by flow cytometry. While 8 ISGs met the inhibition criteria they exhibited poor transduction efficiency and were excluded as likely false positives (e.g. human SAMD9L, NOS2A & PARP10, and rhesus macaque MLKL, C5ORF39 and BST2/TETHERIN (sFig 2A and B). These genes were presumed to be either cytotoxic or possess anti-lentiviral vector activity.

Excluding known inhibitory human ISGs (RNASEL, DDX58/RIGI, IRF1, DDX60, OASL and ADAR as well as rhesus macaque RNASEL, IFNB1, IRF1 and DDX60L), we identified the following ISGs as novel inhibitors of HCV SGR replication: human ARHGAP17, ARHGEF3, MICB, CDKN1A, SRBII, FAM46C, TRIM34 and SLC15A3, and rhesus macaque NCOA7, COMMD3, SRBII, CASP1, PHF11, PTAR1 and BTN3A1. Human SLC15A3 was taken forward as, although it did not reach 2-fold inhibition, it had a strong effect in our experiments compared to the well-established restriction factor RSAD2/VIPERIN. Furthermore, we detected inhibition by two isoforms of the rhesus macaque orthologue to human NCOA7, PTAR1 and BTN3A1; only the longest isoform of each was examined further. One ISG, SRBII, was inhibitory in both human and rhesus macaque screens.

The capacity to restrict HCV replication by ISGs from the primary and secondary screens was further evaluated by monitoring HCV RNA replication following electroporation of SGR RNA into Huh7 cell lines that had constitutive expression of ISGs (Fig 2C-F). In these experiments we used an arbitrary cut-off of 2-fold reduction in viral RNA to be considered inhibitory. RLU reporter measurements were conducted on the WT SGR (Fig 2C) and the replication-defective ‘GND’ mutant (Fig 2D) at 72h after electroporation to ascertain any role of ISGs in disrupting primary translation of SGR RNA. We were able to establish stable Huh7 cell lines expressing ISGs for all genes except human MICB, which exhibited toxicity during long-term expression in cell culture and so was not explored further. Stable expression of most ISGs (n=17) reduced expression of the GLuc reporter following electroporation compared to controls (EGFP or empty cells) except SRBII-Mm (Fig 2C and D). Interestingly, the ISG that previously enhanced reporter expression, CYTH1, did not display the same phenotype in this assay, suggesting that the gene may affect transfection of SGR RNA. From analysis of reporter expression from the GND replication-defective construct, some ISGs (CDKN1A, FAM46C, RNASEL, SRBII-HS, PHF11, CASP1) reduced translation (Fig 2D). The ratio of WT:GND reporter expression was calculated as a measure of the ability of each ISG to inhibit HCV RNA replication compared to translation of input SGR RNA (Fig 2E). By this measure, all ISGs apart from CDKN1A inhibited HCV RNA replication. Furthermore, the relative accumulation of HCV SGR RNA in electroporated Huh7-ISG cells was measured to identify those ISGs that could
promote off-target inhibition by altering reporter expression/secreation rather than specifically interfering with HCV RNA replication. Most ISGs inhibited HCV RNA accumulation as compared to control lines that did not express any ISG, mirroring their inhibition of reporter activity except for PTAR1, NCOA7 and SRBII-Mm (Fig 2F).

Indeed, NCOA7 and SRBII-Mm enhanced HCV RNA accumulation. This increase in HCV RNA accumulation by SRBII-Mm contrasts with the inhibitory effect of SRBII-Hs on viral RNA replication, which may be worthy of future study. Based on these findings, CDKN1A, PTAR1, NCOA7 and SRBII-Mm were excluded from further analysis. Thus, the remaining novel ISGs that interfered with HCV RNA replication were human ARHGAP17, ARHGEF3, FAM46C, SLC15A3, SRBII-Hs, TRIM34, and rhesus macaque BTN3A1, CASP1, COMMD3 and PHF11.

Assessment of inhibitor activity against replication of infectious virus

As replicon-based assays do not represent the complete HCV life cycle, we screened the final selected 10 Huh7-ISGs as stable cell lines using HCVcc Jc1 full-length virus and measured viral RNA accumulation at 72 hours post infection (hpi) (Fig 3). For these assays, we included IRF1 and RSAD2/VIPERIN as additional controls, since these ISGs inhibit RNA replication in both the HCV SGR (Fig 2) and infectious systems. In this experiment, IRF1 and RSAD2/VIPERIN overexpression reduced HCV RNA accumulation by >100 and ~10-fold respectively (Fig 3). Consistent with previous experiments, in this assay, we also used 2-fold cut off as a criterion for an ISG to have inhibitory activity. Only ARHGEF3 achieved this threshold, although other ISGs such as FAM46C and TRIM34 did have an effect albeit to a less dramatic extent. Interestingly, in contrast to replicon-based assays, ARHGAP17 now enhanced HCV RNA accumulation by ~2.5-fold. ISGs exhibiting contradictory effects in the different challenge models (SGR transient versus full-length virus) likely reflects intrinsic differences in respective model systems (e.g. gene content, delivery system etc).

ARHGEF3 inhibits HCV replication

As ARHGEF3 (also known as XPLN) gave the most robust inhibition of HCV replication, including in the infectious virus system, and its role in HCV infection has not been previously examined, we chose to study it in greater detail. Although only human ARHGEF3 was identified in our initial screen, the rhesus macaque ISG library also contained ARHGEF3, although it did not give any apparent antiviral effect in the primary screen. We then examined the capacity of ARHGEF3 to inhibit RNA replication in both the SGR and infectious HCV systems using Huh7 cells that stably expressed the rhesus macaque orthologue. In both systems, the human and rhesus macaque orthologues inhibited RNA replication to similar levels, demonstrating conservation of anti-HCV (SGR and HCVcc) activity for ARHGEF3 in both species (Fig 4A and B).

We next examined the role and potential mechanism of ARHGEF3-mediated inhibition of HCV RNA replication. Hence, three ARHGEF3 mutants from the human orthologue were generated with an N-terminal MYC-tag to facilitate comparative analysis of their abundance. ARHGEF3 has two major established activities, acting as a guanine nucleotide exchange factor (GEF) for various Rho proteins38, and activation of mammalian target of rapamycin complex 2 (mTORC2)39; these activities are encoded by distinct regions of the protein. RhoGEF activity can be blocked by single point mutations in the GEF domain while mTORC2 inhibition is controlled by the N-terminal region of the protein38. To test whether either of these functions contributed to the antiviral activity of ARHGEF3, we made a single RhoGEF knockout point mutant
(W440L hereafter termed 'delGEF'), an N-terminal truncation of ARHGEF3 (termed 'delN') and expressed only the N-terminal fragment of ARHGEF3 (termed 'N'). We generated stable Huh7 cell lines for the WT, mutated and deleted forms of ARHGEF3, monitored protein expression using the Myc tag (sFig 3) and quantified HCV RNA replication by measuring Gaussian luciferase activity using the SGR assay (Fig 4C). The WT version of Myc-tagged ARHGEF3 inhibited HCV RNA replication to a similar degree as the non-tagged WT form of the gene with the HCV SGR system (Fig 4B). By contrast, antiviral activity was ablated for all three variants of ARHGEF3 (Fig 4C). This loss in activity was not due to any major differences in protein levels between the WT and mutants (sFig 3). These data demonstrate that the anti-HCV activity of ARHGEF3 is dependent on functions across the entire gene including both the N-terminal domain, which is responsible for mTORC2 activation, and RhoGEF activity. However, the mTORC2-sufficient N domain failed to demonstrate antiviral activity.

Given their relatedness, we hypothesized that the antiviral activity of ARHGEF3 may extend to other flaviviruses as well as HCV. To test this hypothesis, we challenged stable cell lines expressing ARHGEF3 with yellow fever virus (YFV; derived from vaccine 17D) and Zika virus (ZIKV), two mosquito-borne flaviviruses (Fig 5A and B). The viruses chosen for these experiments were recombinant strains that expressed the nanoluc or HiBiT reporters, similar to the luciferase-expressing HCV systems used herein40,41. Reporter activity following infection with both viruses was decreased in ARHGEF3-expressing Huh7 cells although not to the same extent as the positive control IRF1. YFV replication was inhibited to a greater extent (5-fold) compared to ZIKV (2-fold). These data suggest that ARHGEF3 may exert antiviral effects not only on HCV but other members of the Flaviviridae.

**ARHGEF3 is expressed and modestly up-regulated in the human liver during chronic HCV infection**

Given the observed inhibition of HCV replication by ARHGEF3 following infection of Huh7 human hepatoma cells, we wanted to determine whether the gene was expressed in chronically HCV-infected liver in vivo where it could also contribute to controlling viral replication. By analysing RNAseq data from HCV gt1- and gt3-infected livers42, we compared the levels of ARHGEF3 expression in uninfected and infected liver biopsies along with a number of other anti-HCV ISGs (Fig 6A and B). These data showed that ARHGEF3 transcription was detected in uninfected liver, albeit at a very low level compared to other ISGs such as IFITM3 (Fig 6A). In HCV-infected liver biopsies, ARHGEF3 was modestly upregulated 1.5-2-fold during HCV gt1 and gt3 infection; by comparison, IFI27 and RSAD2/VIPERIN were induced >10-100 fold during HCV infection (Fig 6B). In vitro ISG induction by IFN-alpha and IFN Lambda3 stimulation in Huh7 cells yielded negligible induction of ARHGEF3 compared to MX1. (sFig4). Thus, ARHGEF3 expression could be detected in the liver or liver-derived cells where it may be very modestly induced during infection or IFN stimulation compared with other anti-HCV ISG factors.

**Discussion**

The outcome of acute HCV infection is variable in natural infection of humans and is intimately linked with host immune responses, including the ability to control infection through the IFN system16,43. Similarly, in animal models, the course of infection and clinical pathology also differs from human infection, such as rhesus macaques which are non-permissive to infection in vivo4-6. One hypothesis is that...
species-specific differences in IFN-mediated immunity may contribute to the observed
distinct outcomes. Since the antiviral potency of IFN is mediated through the induction
of a large spectrum of core ISGs\textsuperscript{10,23}, there have been previous screening studies to
identify specific ISGs that interrupt the HCV infectious cycle. The most comprehensive
approach utilized a screening method similar to that adopted here but was not applied
to the rhesus macaque ISG library\textsuperscript{35} and did not utilise HCV SGR systems\textsuperscript{23}. It should
be noted that while rhesus macaques cannot be productively infected \textit{in vivo},
hepatocytes cultured \textit{ex vivo} from the species remain permissive\textsuperscript{44}, although the
relative efficiency of HCV replication between human and rhesus macaque
hepatocytes has not been compared. By way of such an example, we revealed in a
recent report that rhesus macaque and chimpanzee \textit{IFNL4} had greater antiviral activity
than their human orthologue due in-part to an amino acid variant at codon position 154
in the \textit{IFNL4} gene, which may impact outcome of HCV infection\textsuperscript{14}.

Our study has utilised both transient HCV SGR and HCVcc systems,
uncovering \textit{ARHGEF3}/\textit{XPLN} as an antiviral factor conserved between humans and
rhesus macaques. Notably, in our experiments there are differing outcomes between
transient SGR and HCVcc systems. We identified several potential antiviral ISGs (e.g.
\textit{TRIM34}, \textit{FAM46C}) with the SGR approach yet only one hit achieved our arbitrary cut-
off for reducing viral RNA by >2-fold with infectious HCVcc. The SGR and HCVcc
systems are based on the same gt2a viral strain (JFH1/Jc-1\textsuperscript{45,46}) and for certain
experiments, the same method was employed to quantify RNA abundance. However,
there are substantial biological and experimental differences between the approaches
using transient SGR transfection and HCVcc infection. Firstly, SGR RNA is transfected
or electroplated into Huh7 cells unlike authentic infectious virus. Delivery of viral RNA
is therefore distinct, as is the potential to induce an IFN response from introduction of
naked RNA to the cytoplasm as we used Huh7 cells and not Huh7.5 cells, which are
deficient in RIG-I-dependent sensing\textsuperscript{47}. Additionally, by definition, the SGR system
lacks genes (such as HCV-encoded capsid, envelope, p7 and NS2 genes) present in
full-length HCVcc that have been shown to affect sensitivity to certain ISGs\textsuperscript{21}.

Given that our strategy employed an initial screen using transient replication
with the HCV SGR approach followed by validation employing an infectious assay
system, the potential anti-viral host factors would likely target either translation or
replication of HCV RNA. Thus, we would not anticipate identifying host factors that
would only interrupt either virus entry or assembly. Having identified novel ISGs
compared to previous screens using the SGR approach, the only anti-viral factor that
met our strict criteria for reducing replication in an infectious system was
\textit{ARHGEF3}/\textit{XPLN}. Interestingly, the rhesus macaque orthologue showed similar
activity although it was not identified in the initial library screen, which is likely due to
high false negatives in initial assays. Nonetheless, this indicates conservation of
function for both human and rhesus macaque orthologues. Moreover, \textit{ARHGEF3}/\textit{XPLN}
displayed antiviral activity in assays for both ZIKV and YFV
replication. Thus, the gene apparently has broader anti-viral activity against other
positive-sense, single-stranded RNA viruses in the \textit{Flaviviridae} family.

\textit{ARHGEF3}/\textit{XPLN} is a guanine nucleotide exchange factor, which specifically
stimulates and interacts with two Rho GTPases, RhoA and RhoB involved in
cytoskeletal regulation\textsuperscript{48}. In common with other GTPases, ARHGEF3 possesses both
da Dbl homology (DH) domain, responsible for catalytic activity, and a pleckstrin
homology (PH) domain\textsuperscript{38,48}. ARHGEF3 also interacts with and inhibits mTORC2 at
endogenous levels, which regulates cell development\textsuperscript{39}. However, this function of
ARHGEF3 does not require its GEF activity. ARHGEF3 protein is most highly
expressed in the brain and skeletal muscle, and to a lesser extent in heart and kidney
but expression could not be detected in the liver\textsuperscript{48}. More recently, the mouse
orthologue of ARHGEF3 has been implicated in regulating muscle regeneration in a
process that involves autophagy\textsuperscript{49}. Thus, ARHGEF3 influences a number of
physiological processes although detailed insight into all of its possible functions have
not been explored. Additionally, the higher levels of ARHGEF3 expression in several
tissues may partially explain the lack of robust infectivity in non-hepatic cell lines.

Our analysis permitted some mechanistic insight into ARHGEF3/XPLN
inhibition of RNA replication for HCV, YFV and ZIKV \textit{in vitro}. From our experiments
with the different HCV systems available, ARHGEF3 was able to effectively inhibit \textit{de novo}
RNA replication in cells transfected with SGR RNA. However, it could not exert
a similar antiviral effect in cells that constitutively harboured the subgenomic replicon
although the positive control \textit{IRF}1 had muted activity with this approach also (sFig5).
Presumably, persistent HCV RNA replication may adapt the intracellular environment
and regulatory processes such that certain ISGs are less effective. Thus, the factor
may only function as an antiviral upon initial infection when RNA replication is being
established. The ability of the rhesus macaque orthologue to also inhibit replication
indicates that any antiviral activity is conserved in other species. This is not surprising
given that human and rhesus macaque orthologues differ at only 3 amino acid
positions (data not shown). Moreover, ARHGEF3 variants that either introduced a
mutation which blocked GEF activity or removed the region of the protein responsible
for mTORC2 inhibition ablated antiviral activity. Thus, it was not possible to identify the
function in \textit{ARHGEF3} responsible for blocking viral RNA replication. Further studies
would require more detailed analysis of \textit{ARHGEF3} to determine the precise
mechanism for inhibiting viral RNA replication and the cellular processes that limit viral
RNA synthesis.

The IFN system can pose a significant barrier to HCV infection \textit{in vitro} and \textit{in vivo}. One question resulting from our studies is whether this inhibition could be
influenced by \textit{ARHGEF3/XPLN} in the liver in natural infection? Our data shows that it
can be detected in the liver albeit at low levels and we also show that it can be induced,
although not to a substantial extent, by HCV infection \textit{in vivo} and IFN \textit{in vitro} in Huh7
cells. However, compared to other ISGs with potent anti-HCV activity and higher
expression levels, the effect of \textit{ARHGEF3/XPLN} \textit{in vivo} within the liver is likely to be
modest. Nevertheless, we have demonstrated that \textit{ARHGEF3} also exhibits anti-viral
activity against two other flaviviruses, YFV and ZIKV, which have significant extra-
hepatic tropism although YFV can infect hepatocytes \textit{in vivo}. Thus, induction of
\textit{ARHGEF3} may have broader effects on other single-stranded viruses that can infect
tissues where the gene is expressed to high levels. We propose that future studies on
\textit{ARHGEF3}, for example using pre-existing animal models, may focus efforts on its
properties as an anti-viral inhibitor that is stimulated by the IFN response.

\section*{Materials and Methods}

\subsection*{Cells}

The following three continuous human cell lines were used throughout this paper: wild-type
(WT) human hepatoma Huh7 cells, Huh7 cells harbouring a stable HCV SGR based on the HCV gt2a
JFH-1 strain expressing the neomycin resistance gene\textsuperscript{50}, and HEK-293T cells. Huh7 cells were used
for determination of antiviral activity of ISGs, while the HEK-293T cells were used to produce
lentiviruses expressing ISGs. All cell lines were grown in standard high-glucose DMEM 10\% FCS (v/v)
without antibiotics and passaged routinely in cell culture flasks, incubated with 5\% CO\textsubscript{2} at 37°C. Stable
SGR cells were maintained with neomycin/G418 (ThermoFisher Scientific) in every second passage
(1mg/ml). Following transduction with lentiviruses, transduced cells were selected with, and maintained,
in puromycin-containing media (ThermoFisher Scientific) (between 1–3 ug/ml). Cell lines were routinely assessed for mycoplasm contamination by ‘MycoAlert’ luciferase assay (Lanza), but no evidence of infection was identified.

Molecular biology

All plasmid stocks were produced following DNA purification by standard molecular biology procedures. ISG-expressing lentivirus transfer vectors were grown for 24 hours at 30°C before purification in commercial competent bacteria (NEB10beta, New England Biolabs). Human ARHGEF3/XPLN cDNA was amplified from our library vectors and modified by traditional molecular biology approaches. An amino-terminal Myc epitope tag (N-QLISEEDLC) was constructed by standard and overlap high-fidelity PCR (Phusion polymerase, New England Biolabs) as were various truncations and a mutation using primers: MYC(+) 5’SCTCTCTGGCCAGAGGCGATGGTGAGGATCTGGTGCGAAGAGAGTTACCCTTC-3’; Full-length(+) TCTCTGGCCAGAGGCGATGGTGAGGATCTGGTGCGAAGAGAGTTACCCTTC-3’; ARHGEF3(-) 5’TCTCTGGCCAGAGGCGATGGTGAGGATCTGGTGCGAAGAGAGTTACCCTTC-3’; delN(+) TCTCTGGCCAGAGGCGATGGTGAGGATCTGGTGCGAAGAGAGTTACCCTTC-3’; N-only(-) 5’TCTCTGGCCAGAGGCGATGGTGAGGATCTGGTGCGAAGAGAGTTACCCTTC-3’; W440L(+) 5’CAAACACAGCTTTGCTTTGAACTGCTGTTTGGTTGAAAGGATCTGGTGGCGAAGGGATTTCC-3’; W440L(-) 5’ACAGTTAAGCAACTGCTGTTTGTTGAAAGTGTCATTGGCTTG-3’. Amplicons were cloned into a modified “SCRPSY” lentivirus vector containing only two Stil sites flanking the insert. ARHGEF3/XPLN variants were amplified with primers flanked by Stil sites and cloned directly into the SCRPSY lentivirus vector linearised by Stil digestion. Resulting positive clones were verified by nucleotide sequence analysis.

Generation of ISG lentivirus library

An arrayed lentivirus library containing >800 ORFs was produced using standard protocols. Briefly, plasmids (ISG-expressing transfer, heterologous glycoprotein VSV-G and GAG/POL packaging) were co-transfected into HEK-293T cells via lipid-mediated transfection using polyethyleneimine (PEI). Lentivirus-containing supernatant was harvested 2-3 days later, clarified by low-speed centrifugation, filtered using a 0.45um filter and aliquoted into 96 well plates before freezing at -70°C. For validation experiments, lentivirus ISG plasmids were re-generated from banked bacterial stocks, verified by nucleotide sequence analysis, and new lentivirus preparations were produced.

HCV reagents

Two HCV systems were used for the generation of replication-competent HCV; HCV transient SGR containing the GLuc reporter based on the gt2a JFH1 strain\(^{42}\) and the HCVcc gt2a Jc-1 system \(^{45}\). Infectious HCV SGR and HCVcc RNAs were generated using standard protocols\(^{44}\). Briefly, SGR or HCVcc plasmid was linearised with Xba I (New England Biolabs), blunt-ended using Mung bean nuclease (New England Biolabs), column purified and full-length SGR or HCV RNA was transcribed \textit{in vitro} (T7 RiboMAX Express LargeScale RNA Production, Promega) followed by purification on columns and determination of quality and concentration by Nanodrop or agarose gel electrophoresis. HCV SGR RNA was transfected via lipid-mediated means or via electroporation. Lipid-mediated transfection was carried out using either PEI or lipofectamine 2000 at a ratio of 1:1 (1ug RNA: 1ug PEI or 1ul lipofectamine 2000) combined in Optimem 50ul (20 mins PEI, 5 minutes lipofectamine 2000). Huh7 cells in 96 well plates (1x10\(^4\)) were transfected with RNA (200ng/well) when subconfluent in Optimem (Thermo Fisher Scientific). Optimem/ transfection mix was removed at 4 hours for screening. Electroporation was carried out using standard procedures. For generation of infectious HCVcc Jc-1, protocols were carried out in a BSL3 facility and infectious virus was harvested at 3-4 days post electroporation and titrated by TCID\(_{50}\) with detection of infectious foci using an anti-NS5A antibody\(^{51}\). For infection, HCVcc-containing cultures were incubated with Huh7 cells in 12 well plates at pre-defined moi.

YFV and ZIKV reporter assays

We used two previously described recombinant reporter-expressing flaviviruses systems for ZIKV\(^{40}\) and YFV\(^{41}\). Briefly, YFV reporter virus was produced by circular polymerase extension reaction “CPER” in BHK-21 cells, titrated in Huh7 cells by TCID\(_{50}\) and used for infections. Luciferase signal was measured using Nano-Glo HiBiT Lytic Detection System (Promega) in a GloMax microplate reader. ZIKV reporter virus was produced in A549 cells and titrated by TCID\(_{50}\) in Vero cells. The luciferase signal was read using Nano-Glo Luciferase Assay System (Promega) in a GloMax microplate reader.
Huh7 cells expressing ISGs were challenged with the reporter ZIKV for 72 hours and reporter YFV for 48 hours at 37°C, 5% CO2 in humidified conditions.

ISG screening protocol

Huh7 cells were seeded in 96 well plates (1x10^4 cells per well) and transduced using lentivirus (75-90 ul). Two days post transduction, ISG-expressing cell lines were transfected with HCV SGR RNA for 4 hours using the protocol above, followed by media changed and/ or puromycin (3ug/ml) added and incubated for 3 days without changing the media. At 3 days post transfection, media was removed and aliquoted into parallel plate before being read for GLuc activity using kit (Pierce Gaussia Luciferase Glow Assay kit, Thermo Fisher Scientific) using the manufacturer’s instructions and read on a GloMax microplate reader. Relative RLU was calculated based on negative controls and plate average. Hits were defined by at least a 90% decrease in RLU in the screen.

Determination of transduction efficiency

Flow cytometry was used to determine the transduction efficiency and therefore toxicity of ISGs using the SCRPSY lentivirus vector that express the RFP tag alongside ISGs when transduced/expressed in Huh7 cell cultures via quantification of RFP-expressing cells. At 2 days after transduction, cells were trypsinised, fixed in 4% PFA and used for flow cytometry using a Guava EasyCyte flow cytometer (Millipore).

RT-qPCR

RT-qPCR was used to determine the level of HCV RNA and for host gene expression of ISG levels using previously described protocols with the 2-ΔΔCt method. Total RNA was isolated from cell cultures and extracted using a RNeasy kit (Qiagen), and cDNA was synthesized using the Applied Biosystems high-capacity cDNA kit. RT-qPCR was carried out using Taqman protocols for primers and probes against HCV (in-house) as well as human Mx1 (Hs00895608), ARHGEF3 (Hs04241552), and the control GAPDH (Hs402869); assays were performed with TaqMan Fast Universal PCR Master Mix (Applied Biosystems).

Immunoblotting

Immunoblotting was carried out on lysates from transduced cells, typically from a confluent 6 or 12-well plate (0.5-1.0x10^6 cells). Lysates from cell cultures were generated using RIPA buffer extraction using manufacturer’s instructions following a PBS wash. Lysates were clarified by low-speed centrifugation and frozen at -20°C. For running by polyacrylamide gel electrophoresis under reducing conditions (SDS-PAGE), lysates (~10 ul) were boiled along with running buffer containing beta-mercaptoethanol (Pierce Lane Marker Reducing Sample Buffer, Life Technologies). Boiled lysates were then transferred to a hand-cast 10-12% polyacrylamide gel and ran for 2-3 hours at room temperature at 100 volts. Separated polypeptides were transferred to a nitrocellulose membrane using ‘wet transfer’ conditions for 1.5 hours. Membranes were blocked in 50% FCS/blocking solution and probed using primary antibodies (1:1000 dilution of beta tubulin and MYC tag antibodies [Abcam], containing 50% FCS and PBS-T) overnight at 4°C. Membranes were washed in PBS-T. Primary antibodies binding was visualised using corresponding secondary antibodies (Licor) and detected using the Licor imaging technology.

Bioinformatic analysis of ISG expression in HCV-infected human livers

The level of RNA (fragments per kilobase of transcript per million mapped reads; FPKM) encoding putative anti-HCV ISGs was quantified from published RNAseq data produced from liver biopsies of infected (HCV gt1 and gt3 [each n = 5]) and uninfected human livers (n = 4).

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References


**sFigure 1. Optimisation of the ISG screen using HCV-NSR RNA.** WT HCV-NSR RNA was transfected into cells previously transduced to express ISGs, including antiviral human *IRF1* gene. RLU was measured at 72 hpt compared to controls (empty vector, eGFP and mock transduced). All cell cultures had been treated with puromycin and therefore ‘mock’ denotes background RLU values in the assay. Values from triplicate wells were calculated and variation is shown as standard error of the mean.
Figure 1. Screening for ISGs from humans and rhesus macaques that influence HCV- SGR luciferase reporter activity. (A) Schematic depiction of the screening protocol used in the study showing that Huh7 cells were transduced and transfected with luciferase-expressing HCV SGR after 48 hours and the secreted luciferase levels were quantified at 72 hours post-transfection (hpt). (B) Dot plot of RLU detected for ISG-expressing cells transfected with HCV-SGR RNA for human (Hs; green) and rhesus macaque (Mm; red) ISGs, relative to the control ISGs from 72 hpt. The primary screen was performed as single assays. Genes of interest are marked on the figure.
Figure 2. **Secondary screen validation of anti-HCV-SGR ISGs from humans and rhesus macaques detected in the initial screen.** (A and B) RLU detected at 72 hpt in HCV-SGR RNA (wild-type [WT]) transfected cells expressing human (A) or rhesus macaque (B) ISGs. (C and D) Validation of anti-HCV-SGR activity of human and rhesus macaque ISGs against WT (C), or replication-defective HCV-SGR RNA (D) at 72 hpt. The ratio of WT:GND RLU (E) and HCV RNA levels (F) was calculated at 72 hpt. For the data in panel F, WT HCV-SGR RNA was measured by RT-qPCR following electroporation of SGR RNA into stable puromycin-selected ISG-expressing Huh7 cell lines. Negative controls are shown (black) and ISGs that gave a >2-fold reduction in HCV RNA replication are indicated (pink). ISGs that do not achieve this threshold are shown in green. Values from triplicate wells were used and variation is shown as standard error of the mean.
Figure 2. Transduction efficiency of lentiviruses expressing ISGs. In parallel assays from those shown in Figure 2, transduced cells were not selected using puromycin but instead were subjected to flow cytometry analysis to determine the abundance of transduced cells expressing RFP.
Figure 3. **Validation of ISGs with activity against HCVcc.** The effect of ISG expression on replication of full-length Jc1-HCVcc was ascertained following infection of stably transduced ISG-expressing cell lines by quantification of HCV viral RNA abundance by RT-qPCR at 72 hpi (moi = 0.1). Negative controls are shown (black) and ISGs that reached a threshold of >2-fold reduction in viral replication are shown in pink. Values from combined duplicate wells from three independent experiments were used and variation is shown as standard error of the mean.
Figure 4. Validation of the antiviral activity of ARHGEF3/XPLN on HCV RNA replication. (A) Relative HCV RNA abundance from HCVcc infection of Huh7 cells expressing ARHGEF3 from Human (Hs) or rhesus macaque (Mm) as compared to negative control at 72 hpi. (B) Relative RLU (%) following HCV SGR RNA transfection of Huh7 cells expressing ARHGEF3 from Human (Hs) or rhesus macaque (Mm) as compared to negative control at 72 hpi. (C) Effect of ARHGEF3/XPLN-specific mutations (Myc-tagged WT, N-terminus truncation, N-terminus only and the GEF-inactivation mutant W440L) on HCV RNA replication following SGR RNA transfection. RLU activity was measured and compared to controls at 72 hpi.
**Figure 3. Expression of WT and mutant MYC-tagged ARHGEF3/XPLN.**

Representative immunoblots demonstrating production of MYC-tagged ARHGEF3/XPLN variants (A) and beta-tubulin loading control (B) in stably transduced Huh7 cells. Localisation of relevant bands is indicated with an arrow for WT/delGEF, delN, and N in (A), as well as beta-tubulin (in B).
Figure 5. Antiviral activity of ARHGEF3 against flaviviruses. RLU activity (relative to empty control cells) in Huh7 cells stably expressing ARHGEF3/XPLN or IRF1 following infection with recombinant luciferase-expressing YFV (A) and ZIKV (B) as measured at 48 hpi and 72 hpi, respectively. Values from nine replicates for YFV and triplicates for ZIKV were used and variation is shown as standard error of the mean.
Figure 6. Abundance of ARHGEF3/XPLN RNA in vivo in HCV-infected human liver biopsies. RNA levels (FKPM) of known anti-HCV ISGs in liver biopsies from uninfected individuals ($n = 4$) (A). Fold-change in RNA (FPKM) levels of known ISGs in liver biopsies from infected individuals (HCV gt1: pink; gt3: green [each $n = 5$]) compared to uninfected controls (B).
**Figure 4. Expression of ARHGEF3/XPLN in Huh7 cells following IFN stimulation.** Huh7 cells were stimulated with human recombinant IFN-alpha or IFN Lambda3 (1000IU/mL and 100ng/mL, respectively) for 24 hours and the levels of MX1 and ARHGEF3 (IFNa: pink; IFNL3: green) were determined. Changes in expression of MX1 and ARHGEF3 are shown as fold-change compared to mock stimulation. Values from duplicate wells were used and variation is shown as standard error of the mean.
**Figure 5.** Antiviral activity of ISGs against stable HCV-SGR Huh7 cell lines.

Relative RNA abundance (% of empty/EGFP controls) following puromycin selection. Huh7 cells were firstly transduced with lentiviruses expressing each of the ISGs for 48 hours and were then selected with puromycin for a further 48 hours.