TITLE

ISG15/USP18/STAT2 is a molecular hub regulating autocrine IFN I-mediated control of Dengue and Zika virus replication

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SUMMARY

The establishment of a virus infection is the result of the pathogen's ability to replicate in a hostile environment generated by the host's immune system. Here, we found that ISG15 restricts Dengue and Zika viruses' replication through the stabilization of its binding partner USP18. ISG15 expression was necessary to control DV replication driven by both autocrine and paracrine type one interferon (IFN-I) signaling. Moreover, USP18 competes with NS5-mediated STAT2 degradation, a major mechanism for establishment of flavivirus infection. Strikingly, reconstitution of USP18 in ISG15-deficient cells was sufficient to restore the cells' immune response and restrict virus growth,

suggesting that the IFNAR-mediated ISG15 activity is also antiviral. Our results add a novel layer of complexity in the virus/host interaction interface and suggest that NS5 has a narrow window of opportunity to degrade STAT2, therefore suppressing host's IFN-I mediated response and promoting virus replication.

KEYWORDS

Dengue virus, Zika virus, ISG15, USP18, type one interferon, ISGylation, antiviral response, immune evasion, NS5, STAT2

INTRODUCTION

Cells detect infection by recognizing molecular patterns derived from pathogen's constituents (PAMPs) or cell damage (DAMPs). During viral infection, nucleic acid is a major signal that triggers the innate immune response, inducing a type one interferon (IFN-I)-mediated antiviral state (1, 2). IFN-I binds to its cognate receptor and activates the JAK/STAT pathway, leading to expression of hundreds of interferon stimulated genes (ISGs) that make the intracellular environment hostile to viral replication in infected and proximal cells (3). The evolutionary arms race between viruses and its hosts led to evolution of immune evasion mechanisms that are crucial for successful viral replication. Considering IFN-I's importance in viral infection control, many immunomodulatory proteins target this signaling pathway (4).

Several flaviviruses, such as dengue virus (DV), Zika virus (ZIKV) and yellow fever virus (YFV), have emerged and re-emerged over recent years and are the leading cause of human arbovirus infection (5, 6). DV alone infects nearly 400 million people every year (7) with extensive health and economic burden (8). A requirement for effective flavivirus emergence is the ability to counteract the human immune system.

The compact flavivirus genome encodes seven non-structural proteins that are responsible for viral replication and immune evasion. Six of these proteins are not secreted implying that intracellular pathways are central targets for evasion (9–13). For instance, DV non-structural protein 5 (NS5), which is the viral RNA-dependent-RNA-polymerase (RdRp) and a methyltransferase,

mediates STAT2 degradation by facilitating its interaction with UBR4, leading to its ubiquitination and subsequent proteasomal targeting (14). This evasion pathway is functional in humans but not mice due to differences in the amino acid sequence of human and murine STAT2 (15).

ISG15 is an intracellular and secreted ubiquitin-like protein that has three described functions. Extracellular ISG15 acts as a cytokine, leading to the expression of IFN γ and IL-10 in diverse immune cells (16–18). It has been suggested that humans lacking ISG15 have severe mycobacterial disease due to deficiency in IFN γ production by NK cells (19). Moreover, ISG15 mRNA is highly expressed in active tuberculosis and strongly correlates with disease severity (17, 20).

ISG15 is conjugated to other proteins through a three-step ubiquitination-like process (21) in which the main ligase for ISGylation is the HECT domain and RCC1-like domain-containing protein 5 (HERC5) (22, 23). ISGylated proteins are affected in several different ways, such as increased or reduced stability and activity. ISG15 can also be conjugated to viral proteins, impacting their function, and therefore belong in the plethora of ISGs with a direct antiviral function (3, 24).

The third and more recently described role of ISG15 is its IFN-I modulatory function. Non-conjugated ISG15 binds and stabilizes the ISG USP18, a protease that negatively regulates IFN-I signaling and also serves as a deISGylation protein (25–27). Correspondingly, individuals lacking ISG15 are prone to severe interferonopathies due to decreased USP18 function and increased IFN-I signaling. Interestingly, ISG15-deficient patients do not have enhanced susceptibility to viruses suggesting ISG15 is not necessary to control ubiquitous viral infections in vivo (28). In contrast to the indirect role of ISG15 in negative regulation of IFN-I signaling through the USP18 axis in humans (28, 29), murine ISG15 itself blocks replication of human viruses such as Influenza and HSV-1(30). These findings implicate ISG15 as an important molecule inhibiting IFN-I-mediated actions but also suggest that ISG15 may mediate host cell intrinsic mechanisms triggered by viruses. However, how ISG15 bridges these apparently two paradoxical phenomena is unclear.

Specifically, it is possible that ISG15 directly regulates proteins exploited by viruses during early intracellular infection events.

Here, we observed that, unlike several other ISGs, ISG15 is highly expressed in infected cells containing the DV genome. Furthermore, independently of its ISGylation function, ISG15 restricts flavivirus replication primarily in the infected cell by stabilizing USP18, which in turn competes with viral NS5 for binding to STAT2. Our results suggest that flaviviruses exploit an ISG15-mediated IFN-I regulatory mechanism to evade innate immunity and enable replication.

RESULTS

ISG15 is expressed in DV-infected cells

The cell is a fundamental unit for viral infection control and developments in single-cell sequencing technology have enabled examination of host-pathogen interactions in great detail. Recently, Zanini and colleagues generated single-cell RNA sequencing data from two human cell lines (PBMCs and the HuH7 hepatoma cell line) independently infected with DV (31, 32). We re-analyzed available single-cell transcriptomic data dividing cells into three categories: uninfected, infected and bystander. Here we define uninfected cells as those derived from healthy donors, bystander cells as those derived from an infected patient or have been exposed to the virus but did not have the viral RNA detected and infected cells as those in which viral genome was detected. We used t-Distributed Stochastic Neighbor Embedding (tSNE) analysis to visualize cell-to-cell relationships in space of reduced dimensionality. As reported previously (31, 32), global cellular mRNA expression profiling was not sufficient to separate infected or bystander from uninfected cells, suggesting a high variability of gene expression in these samples (Figure 1A and S1A). As IFN-I are key elements in controlling infection, we filtered the results of the differential gene expression analysis using the gene ontology (GO) term for “type one interferon”. The Venn diagram in figure 1B shows that from the 394 differentially expressed (DE)

genes in peripheral blood mononuclear cells (PBMCs) of patients infected with DV or healthy donors, 37 were ISGs (IFN-I GO). ISG15, UBE2L6, HERC5 and USP18, members of the ISGylation pathway, were differentially expressed during DV infection (Figure 1C in bold). Interestingly, in contrast to other single cell experiments using Influenza virus as a model where all the members of the ISGylation pathway seem to be expressed at similar levels in both infected and bystander cells (33), HERC5 was the only member of the ISGylation pathway with a higher expression level in bystander cells (Figure 1C and D). In the data set derived from the HuH7 cell line, ISG15 was the only canonical antiviral protein expressed in DV genome-containing cells (Figure S1C and D). This is in agreement with previous reports that the Huh7 cell line does not produce IFN-I upon viral infection (34–36) and could explain the high number of infected cells (Figure S1A) in comparison with the number of infected PMBCs (Figure 1A). In PBMCs, NK, monocytes and B cells were the infected cells with higher ISG15 expression and similar to the data from Zanini and colleagues (32) B cells and monocytes being proportionately the most infected cells (Figure 1E). These results show that in contrast to most ISGs, ISG15 and other components of the ISGylation pathway are enriched in cells where the DV genome was present.

ISG15 restricts DV and ZIKV replication

The enrichment of ISG15-related genes at a single-cell level led us to investigate how ISG15 might impact flavivirus replication in a human cell. We used an A549 cell line lacking ISG15, previously generated using CRISPR/Cas9 in our lab (17). A549 cells were chosen due to their ability to support flavivirus replication and most importantly to produce and respond to IFN-I (37–40).

ISG15-deficient cells were more susceptible to DV infection as shown by an increased plaque size (Figure 2A and B) and number of infected cells per plaque (Figure 2C). In addition, we determined the kinetics of DV replication and dissemination by using a low multiplicity of infection (MOI), to allow for viral spread through secondary infection events. Percentages of infected cells over time (Figure 2D), relative DV Pre-Membrane RNA quantification in the

169 supernatant (Figure 2E) and infectious particle formation (Figure 2F) were
 170 also increased in the absence of ISG15. DV infection at 4 °C for 2 hours
 171 followed by relative intracellular DV genome quantification indicated no
 172 differences in viral RNA between WT and knockout cells up to 48 hours post-
 173 infection (Figure 2G), indicating that ISG15 plays a role in the DV life cycle at
 174 a stage following viral entry. Importantly, reconstitution of ISG15 expression in
 175 knockout cells led to phenotypic reversion (Figure 2H and I), confirming that
 176 the effects observed in our experiments were caused by depletion of ISG15.
 177 Finally, lack of ISG15 expression led to an increase in plaque size in cells
 178 infected with a phylogenetically related virus, such as ZIKV (Figure 2J and K)
 179 but not with HSV-1 (Figure S2A and B) or VSV (Figure S2C and D). These
 180 results are in line with Speer and colleagues' data where cells isolated from
 181 humans deficient for ISG15 do not have enhanced susceptibility to HSV-1 or
 182 VSV (29). Altogether these results suggest a specific role for ISG15 in the
 183 regulation of flavivirus replication and dissemination.

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185 *ISGylation deficiency does not affect DV spread*

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187 ISG15 is an IFN-I-inducible ubiquitin-like molecule and can be conjugated to
 188 target proteins by HERC5, an ISG15 ligase also induced by IFN (23, 41). Of
 189 note, ISGylation of host or viral proteins was reported to inhibit replication of
 190 several viruses such as influenza (IAV) (42), human cytomegalovirus (HCMV)
 191 (43) and human respiratory syncytial virus (RSV) (44). Moreover, DV proteins
 192 were also shown to be ISGylated (45). To determine whether ISGylation could
 193 be involved in DV restriction, we generated HERC5-deficient A549 cells using
 194 CRISPR/Cas9 (Figure S3A) and accordingly, A549 HERC5 null cells were not
 195 able to perform ISGylation after IFN-I treatment (Figure 3A). In contrast to
 196 ISG15 null cells, we did not observe differences between WT and HERC5-
 197 deficient cells when we evaluated both plaque area (Figure 3B) and number
 198 of infected cells per plaque following infection with DV (Figure 3C). Therefore,
 199 ISGylation is not sufficient to inhibit DV replication.

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204 *ISG15 is necessary for autocrine IFNAR1-mediated control of DV replication*

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206 ISG15 and its binding partner USP18 are crucial for IFN-I pathway down-
207 regulation, which is pivotal for infection control and immune-regulation in
208 humans (28, 29, 46). The A549 ISG15-KO cell line exhibited a lower
209 expression of USP18 and sustained ISG expression, as exemplified by IFIT3,
210 after IFN-I stimulation (Figure S4), reproducing the phenotype observed in
211 cells isolated from humans lacking ISG15 (29).

212 While our single-cell RNA-seq analysis showed that both ISG15 and USP18
213 are upregulated in PMBCs containing DV RNA, there was an asynchrony of
214 ISGs being expressed in infected versus bystander cells, with a greater
215 spectrum present in the latter (Figure 1C). This is expected as flavivirus'
216 control of IFN-I signaling occurs mainly via its intracellular non-structural
217 proteins (47, 48) and few ISGs can be directly induced by IRF3 (49–51).

218 Of interest, STAT2 mRNA is expressed at higher levels in DV RNA-containing
219 cells when compared to bystanders and uninfected cells (Figure 1C). Hence,
220 we hypothesized that ISG15 interference with DV replication is associated
221 with autocrine IFN-I signaling in infected cells. Confirming the results from
222 PMBCs and HuH7 cells, ISG15 mRNA was induced in A549 cells during DV
223 infection (Figure 4A). As expected, this expression was abolished in knockout
224 cells. Therefore, we measured the activation of the IFN-I pathway in WT and
225 ISG15-KO cells infected with DV. Despite ISG15-knockout cells having full
226 machinery to control viral infection as well as hyper-responsiveness to
227 exogenous IFN α (Figure S5, Figure 4E and F) (28, 29), they did not respond
228 properly to DV infection. Infected ISG15-deficient cells had less STAT1
229 phosphorylation and IFIT1 expression when compared to WT cells and as
230 expected had no detectable USP18 (Figure 4B). Infected knockout cells
231 induced IFN β mRNA at higher levels than the WT but had less IFIT1 mRNA
232 (Figures 4C and D) indicating that DV infection impairs the response at both
233 mRNA and protein levels downstream of IFN-I induction.

234 Taken together, our results suggest that ISG15 inhibits DV infection by
235 regulating early events of the IFN-I receptor activation. To test this, we

constructed an ISG15/IFNAR1 double knockout cell line based on the ISG15-KO background (Figure S3B). Cells were infected with DV and plaque area and number of infected cells per plaque was quantified. In parallel, we performed this experiment in IFNAR1 knockout cells, previously generated by our group (52, 53). The same phenotype was observed in all three cell lines, with no additive or synergistic effects in the double knockout (Figures 4G and H), which places intracellular ISG15 downstream of IFNAR1 signaling in the control of DV infection.

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245 *ISG15 counteracts DV IFN-I evasion*

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Flaviviruses are known to counteract IFN-I signaling by inducing the degradation of STAT2, a key protein in the interferon signal transduction pathway (11, 14, 54). As our previous results suggest that ISG15's role during DV infection is downstream of IFNAR engagement (Figure 4), we evaluated DV-mediated STAT2 degradation in the absence of ISG15. In agreement with others (55), STAT2 degradation in the A549 cell line occurs rapidly after infection. Despite showing sustained activation of IFN-I signaling (Figure S4), ISG15-KO cells showed pronounced STAT2 degradation when infected with DV (Figure 5A and E-upper panel). As infected IFN-secreting cells are able to induce an antiviral state in neighboring-bystander cells by inducing the expression of ISGs (56), we then evaluated in which cell population, infected and/or bystander, ISG15 impacted DV infection. DV plaques were co-stained for flavivirus E protein and IFIT3 and confocal microscopy was performed. Both infected and bystander cells were able to respond to infection, producing IFIT3 (Figure 5D, top panel). However, IFIT3 expression in the plaque context was largely impaired and restricted to infected cells in the absence of ISG15 (Figures 5B, C and D). This data suggests that restriction of DV replication and dissemination is achieved by both autocrine and paracrine IFN-I response amplification and is dependent on ISG15 expression. To further investigate this, we sorted DV envelope protein positive and negative cells (Figure S5A and B), therefore enabling us to evaluate the impact of infection in virus-containing and bystander cells, respectively. Strikingly, DV-positive cells had a lower expression of STAT2, IFIT3 and USP18 when compared to bystander

cells; a phenotype that was markedly enhanced in ISG15-deficient cells (Figure 5E, *bottom panel*). While also confirming DV inhibition of IFNAR signaling, our results further suggest that ISG15 function is targeted for successful viral infection.

USP18 expression overcomes ISG15 deficiency

STAT2 degradation mediated by all DV serotypes and ZIKV is largely dependent on NS5 (11, 14), the virus RdRp and methyltransferase. NS5 was shown to bind to the N-terminus of human STAT2 (11, 15). Interestingly, USP18 interacts with STAT2 via its coiled-coil and DNA binding domains. This causes negative regulation of IFNAR signaling by displacing JAK1 from chain 2 of the receptor (25). As shown here (Figures 4B, 5E and S4) and elsewhere (28, 29), the absence of ISG15 results in USP18's destabilization. We therefore examined if NS5 is also present in the STAT2/USP18 complex. In HEK293 cells lacking ISG15 (Figure S3C, D and E) and primed with IFN α for 18 hours, both endogenous STAT2 and overexpressed USP18, immunoprecipitated with ZIKV's NS5 (Figure 6A, lane 2). This interaction was enhanced when a protease-deficient high-expressing USP18 mutant (C64A) was used (Figure 6A, lane 3). We then hypothesized that the interaction of USP18 with STAT2 competes with NS5 binding, which in turn could result in a lower efficiency of virus evasion mechanism. To test this, we performed an endogenous STAT2 pull-down in ISG15-deficient HEK293 cells transfected with NS5, USP18 or a combination of both, followed by treatment with IFN α overnight. In the absence of NS5, USP18 interaction with STAT2 was markedly enhanced (Figure 6B, lanes 2 and 3), suggesting that both proteins indeed compete for the same region of the protein.

Thus, we evaluated whether the presence of USP18 could restore STAT2 expression in ISG15 knockout cells during DV infection. Overexpression of USP18 led to an increase of STAT2 levels similar to ones seen in wild type cells (Figure 6C). Importantly, USP18 reconstitution in ISG15-deficient cells was also able to reduce virus replication (Figure 6D), suggesting that

reestablishment of the IFN-I signaling was sufficient to recover the WT phenotype in ISG15 knockout cells.

Together, the results presented here reveal that human ISG15 restricts DV and ZIKV replication via its ability to stabilize USP18 and regulate the type 1 IFN signaling pathway.

DISCUSSION

A successful infection is dependent on the virus replication machinery and its ability to evade host immunity. One of the first lines of defense a virus has to overcome is a plethora of antiviral genes induced by IFN-I (57).

ISG15 is induced early during infection (33, 58) and has been shown to have a viral restriction role in several infection models. Most of those have been reported to be a consequence of ISGylation (42–44, 59), even though this process was suggested to be both inefficient and unspecific (59). Here we show that during the IFN-I response elicited throughout DV infection, direct viral protein ISGylation is redundant for antiviral immunity; rather, ISG15's ability to stabilize USP18 prevents NS5-mediated STAT2 degradation, thus leading to a more effective interferon response that culminates in DV and ZIKV restriction.

Many of ISG15's functions have been shown to be immunomodulatory. For instance, ISGylation stabilizes IRF3 by occluding its ubiquitylation sites (60), negatively regulates RIG-I (61) and activates PKR (62). Secreted ISG15 functions as a cytokine (16, 63), leading to the production of IFN γ and IL-10 in human cells, crucial to the control of pathogens such as *Mycobacterium tuberculosis* (17, 19). Moreover, free intracellular ISG15 is essential for USP18 stability (Figures, 4B, 5E, S4 and (28, 29) and its absence leads to severe interferonopathy in humans (28). Also there are reports of ISG15 having an antiviral function independent of Ube1L during Chikungunya virus infection in mice (28, 64), where free ISG15 contributes to infection control by blunting potentially pathologic levels of cytokine effectors. Considering this range of functions, it is expected that different pathogens might interact with this pathway in different ways, according to its co-evolutionary history.

Of note, NK, NKT and monocytes were the PBMC populations with higher upregulation of ISG15 mRNA in single-cell gene expression studies. These have been shown to be the major producers and/or targets of free extracellular ISG15 in other contexts (17–19, 63). The influence of extracellular ISG15 during viral infections should be further explored in the future.

Interestingly, humans lacking ISG15 do not have increased susceptibility to common viral infections, such as influenza and HSV-1. The explanation for this, as suggested elsewhere, may lie in the sustained IFN-I response of their cells creating a hostile environment for virus growth (28, 29). Here, we reveal that ISG15 restricts DV and ZIKV replication indirectly by stabilizing USP18 and thereby disrupting NS5-STAT2 interaction: ISG15 promotes competition for a niche exploited by such viruses. We also demonstrate that USP18, STAT2 and NS5 co-immunoprecipitate, suggesting a very narrow window of opportunity that NS5 has to degrade STAT2. As USP18/ ISG15 interaction is reported to down-regulate IFN-I signaling in humans but not mice (29), it is tempting to speculate that NS5 interaction with STAT2, a major flavivirus immune evasion mechanism and also restricted to humans (15), was shaped by the USP18/ISG15 interaction.

Our results suggest an unexpected mechanism by which ISG15 can exert an antiviral function distinct from the debilitating effects of its conjugation to viral proteins. The key role of IFN-I in viral infections might lead to the perception of ISGs having a necessarily direct antiviral function, a paradigm that is recently being reassessed, with a range of ISGs being implicated in infection-independent functions (65, 66). Here we provide mechanistic insight of the arms race between ISG15, USP18 and NS5 which suggests that protein/protein dynamics adjacent to IFNAR are a key determinant for the outcome of flavivirus infection.

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378 **AUTHOR CONTRIBUTIONS**

379 Conceptualization, C.E.E., A.B. and D.S.M.; Methodology, C.E.E., E.L.R. and
380 D.S.M.; Software, E.L.R.; Formal Analysis, C.E.E., E.L.R. and E.G.K.,
381 Investigation, C.E.E., A.A.S., D.O.P., G.M. and JB; Resources, C.E.E., A.A.S.,
382 P.F.S., Z.G., T.J.S., T.F. and T.R.S. Data Curation, E.L.R. and E.G.K., Writing-
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385 Visualization, C.E.E., E.L.R. and D.S.M., Supervision, D.S.M., Project
386 Administration and Funding Acquisition, D.S.M.

387

388 **DECLARATION OF INTERESTS**

389 The authors declare no competing interests.

390

391 **FIGURE LEGENDS**

392 **Figure 1. ISG15 is expressed in DV-infected cells**

393 (A) tSNE was used to visualize the single-cell global transcriptome data. Blue
394 dots represent uninfected cells, derived from healthy donors. Beige dots
395 represent bystander cells and red dots represent infected cells, both derived
396 from patients infected with DV.

397 (B) Differential expression of ISGs in PBMCs of patients infected with DV. The
398 Gene Ontology term “type one interferon” was used to filter the results from
399 the single-cell RNA sequencing.

400 (C) Single cell ISG expression variability in uninfected, bystander and
401 infected. ISGylation related genes are underlined. Blue bar represents
402 uninfected cells, derived from healthy donors. Beige bar represents bystander

403 cells and red bar represents infected cells, both derived from patients infected
404 with DV.

405 (D) Violin plot representing the expression of ISGylation family members in
406 uninfected [U], bystander [B] and infected [I] PMBC.

407 (E) Expression of ISG15 in DV infected PMBCs. Size is proportional to the
408 percentage of infected cells in each cell type. Color intensity represents
409 ISG15 average expression.

410

411 **Figure 2. ISG15 restricts DV and ZIKV replication**

412 (A) A549 WT and ISG15 KO were infected with 20 DV PFUs. At 36 hpi cells
413 were fixed, permeabilized and stained for the flavivirus E protein using 4G2
414 antibody. Displayed images were acquired with a Leica DMI6000 B
415 microscope.

416 (B and C) DV relative plaque area (B) and the number of infected cells per
417 plaque (C) quantified by ImageJ software and analyzed using Welch's t test.
418 Error bars represent mean $\pm$ SD. Results are representative of three
419 independent experiments.

420 (D, E and F) Multiple-step DV growth curve in A549 cells. Shown is the
421 percentage of cells infected as measured by E protein staining (4G2+) (D),
422 extracellular DV mRNA (PrM) relative expression by RT-qPCR (E) and
423 titration by focus forming assay (FFA) (F). Statistical analyses were conducted
424 using unpaired t tests. Error bars represent mean $\pm$ SD. Results are
425 representative of three independent experiments.

426 (G) Changes in genomic DV mRNA relative expression over time following
427 binding of DV to A549 WT and ISG15 KO cells at 4°C and analyzed using
428 unpaired t test. Error bars represent mean $\pm$ SD. Results are representative of
429 two independent experiments.

430 (H and I) Complementation of A549 ISG15 KO cells with ectopically
431 expressed ISG15. Shown is the percentage of cells infected 36 hpi as
432 measured by E protein staining (4G2+) (H) and DV mRNA relative expression
433 by RT-qPCR (I). One-way ANOVA was used to analyze these experiments.
434 EV: empty vector. Error bars represent mean $\pm$ SD. Results are
435 representative of two independent experiments.

(J and I) A549 WT and ISG15 KO were infected with 20 ZIKV PFUs. At 36 hpi cells were fixed, permeabilized and stained for the flavivirus E protein. ZIKV relative plaque area (J) and number of infected cells per plaque (I), quantified by ImageJ software and analyzed using unpaired t test with Welch's correction. Images were acquired with an Olympus IX83 inverted microscope. Error bars represent mean $\pm$ SD. Results are representative of three independent experiments.

Statistical analyses were performed using Prism 8 (GraphPad Software). p values * <0.05 ; ** <0.01 ; *** <0.001 .

Figure 3. ISGylation is not sufficient to restrict DV spread

(A) ISGylation profile of A549 WT and HERC5 KO cells by Western blot. Cells were primed with IFN α 2b (100 IU/ml) for 24 h and cell lysates were analyzed with an ISG15 antibody. (*) indicates antibody unspecific band.

(B and C) A549 cells were infected with 20 DV PFUs. At 36 hpi cells were fixed, permeabilized and stained for the flavivirus E protein. DV relative plaque area (B) and the number of infected cells per plaque (C) quantified by ImageJ software and analyzed by one-way ANOVA. Images were acquired with an Olympus IX83 inverted microscope.

Error bars represent mean $\pm$ SD. Results are representative of three or more independent experiments. Statistical analyses were performed using Prism 8 (GraphPad Software). p values **** <0.0001 .

Figure 4. ISG15 is necessary for autocrine IFNAR1-mediated control of DV replication

(A-D) A549 cells were infected with 20 DV PFU. At 36 hpi, cells were harvested and the indicated mRNA transcripts were quantified by RT-qPCR (A, C and D). Cell lysates were analyzed by immunoblotting (B). Error bars represent mean $\pm$ SD. Results are representative of two independent experiments. Data was analyzed by unpaired t test.

(E and F) A549 ISG15 KO cells were primed with IFN α 2b (100 IU/ml) for 12 h, washed three times with DPBS and allowed to rest 12 h before infection with DV or ZIKV at an MOI of 0.1. Shown is the percentage of cells infected at 36 hpi, as measured by E protein staining (4G2+). Error bars represent mean $\pm$

470 SD. Results are representative of three independent experiments. Data was
471 analyzed using unpaired t test.

472 (G and H) A549 cells were infected with 20 DV PFUs. At 36 hpi cells were
473 fixed, permeabilized and stained for the flavivirus E protein. DV relative
474 plaque area (E) and number of infected cells per plaque (F) quantified by
475 ImageJ software and analyzed by one-way ANOVA. Images were acquired
476 with an Olympus IX83 inverted microscope.

477 Error bars represent mean $\pm$ SD. Results are representative of three
478 independent experiments. Statistical analyses were performed using Prism 8
479 (GraphPad Software). p values * <0.05 ; *** <0.001 .

480

481 **Figure 5. ISG15 counteracts DV IFN-I evasion.**

482 (A) A549 WT and ISG15 KO were infected with 20 DV PFU. Cells were
483 harvested at the indicated time points after infection (hpi) and cell lysates
484 were analyzed by Western blot using STAT2 and Actin antibodies.

485 (B, C and D) A549 WT and ISG15 KO immunofluorescence assay (IFA) 36
486 hpi for cellular IFIT3 and flavivirus E protein expression (D). Percentage of
487 IFIT3 (B) and DV (C) positive cells per plaque were quantified by ImageJ
488 software and analyzed using unpaired t test with Welch's correction when
489 appropriate. Displayed images were acquired with a Leica DMI6000 B
490 microscope.

491 (E) A549 cells were infected with DV at MOI 0.01. At 36 hpi, cells were fixed,
492 permeabilized and stained for flavivirus E protein. Cell lysates were analyzed
493 by Western blot with the indicated antibodies before (upper panel) and after
494 (bottom panel) cells were sorted by fluorescence-activated cell sorting (FACS)
495 based on E protein expression.

496 U: uninfected. B: bystander. I: infected.

497 Error bars represent mean $\pm$ SD. Results are representative of two
498 independent experiments. Statistical analyses were performed using Prism 8
499 (GraphPad Software). p values *** <0.001 .

500

501 **Figure 6. USP18 expression overcomes ISG15 deficiency**

502 (A) Flag-tag immunoprecipitation (IP) assay and Western blot analysis of
503 HEK293 ISG15 KO cells transfected with ZIKV NS5-FLAG, human USP18

WT, human USP18 C64A mutant or the empty vector (pcDNA3.1) plasmids, followed by IFN α 2b (100 IU/ml) priming for 18 h. WCL, whole cell lysate. Results are representative of three independent experiments. (B) STAT2 IP assay and Western blot analysis of HEK293 ISG15 KO cells transfected with the indicated plasmids, followed by IFN α 2b (100 IU/ml) priming for 18 h. Results are representative of three independent experiments. (C and D) Complementation with USP18 in A549 ISG15 KO cells. Cells were stably transfected with human USP18 or the empty vector and infected with DV at an MOI of 0.01. At 36 hpi, cells were harvested and cell lysates were analyzed by Western blot with the corresponding antibodies (C). Percentage of cells infected as measured by E protein staining (D). Error bars represent mean $\pm$ SD. Results are representative of two independent experiments. Statistical analyses were conducted using Mann-Whitney's test in Prism 8 (GraphPad Software. p value **<0.01 (D). EV: empty vector.

519

520 **LEAD CONTACT AND MATERIAL AVAILABILITY**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Daniel Santos Mansur (daniel.mansur@ufsc.br)

524

525 **MATERIAL AND METHODS**

526

527 **Cells and viruses**

Mammalian cell lines were maintained at 37 °C under the conditions of a humidified atmosphere and 5% CO₂. The human alveolar adenocarcinoma-derived A549 cells, human embryonic kidney HEK293 and the African green monkey kidney-derived Vero cells were cultured in Dulbecco's modified Eagle's medium F-12 (DMEM F12) (Gibco) supplemented with 5% fetal bovine serum (FBS) (Gibco) and streptomycin/penicillin (100 U/ml) (Gibco). The *Aedes albopictus* mosquito-derived cell line C6/36 was maintained at 28°C in a BOD in Leibovitz's L-15 medium (L-15) (Gibco) supplemented with 10% FBS (Gibco), 0.26% tryptose phosphate broth (Sigma) and 50 μ g/ml

gentamicin (Sigma). A549 ISG15 KO and IFNAR1-KO have been described elsewhere (17, 52, 53). All cells were negative for mycoplasma. Dengue virus serotype 4 (DENV-4 TVP/360 – GenBank accession number: KU513442) and Zika virus (ZV BR 2015/15261 - GenBank accession number: MF073358) stocks were propagated in C6/36 cells and titrated in Vero cells. Vesicular stomatitis virus-green fluorescent protein (VSVeGFP) (Indiana strain, Marques-JT, Plos Pathogens 2013) and Herpes simplex virus-1-green fluorescent protein (HSV-1eGFP) (SC16) viruses were propagated and titrated in Vero cells. HSV-1eGFP was a kind gift of Professor Stacey Efstathiou.

547

548 **Single-cell RNA sequencing analysis**

549 Processed, publicly available single-cell RNA-seq data are available through
550 the GEO accession numbers GSE116672 and GSE110496. We downloaded
551 processed single-cell data and metadata from the supplementary information
552 from the respective publications (31, 32).

553 Then, we used CellRouter to analyze these datasets. To perform the tSNE
554 analysis using single-cell data generated by Zanini 2018 (32), we set the
555 parameters num.pcs=10, seed=1 and max_iter=1000 in the computeTSNE
556 function. Next, we identified genes preferentially expressed in Uninfected,
557 Bystander and Infected cells using a cutoff for the log2 fold change of 0.25.
558 We used a customized script to obtain all genes containing the keywords
559 “type I interferon” in the Gene Ontology Biological Processes (package
560 versions: org.Hs.eg.db_3.10.0, GO.db_3.10.0). Next, we took the overlap of
561 type I interferon genes with the genes preferentially in each condition reported
562 above. The remaining analyses were focused on these genes.

563 To perform the tSNE analysis using the single-cell data generated by Zanini
564 2018 (31), we set the parameters num.pcs=20, seed=1 and max_iter=1000 in
565 the computeTSNE function. We used a strategy similar to the one described
566 above to identify genes differentially expressed in each condition but used a
567 cutoff of 0.15 for this dataset. The parameter num.pcs was determined using
568 the elbow approach, as described in the CellRouter tutorial at
569 <https://github.com/edroaldo/cellrouter>.

570

CRISPR/Cas9-mediated gene editing

A549 WT and ISG15 KO cells were co-transfected with two Herc5 or Ifnar1-targeting gRNA CRISPR/Cas9-GFP plasmids, respectively. HEK293 WT cells were transfected with three lsg15-targeting gRNA CRISPR/Cas9-GFP plasmids (Table S1) (Horizon Cambridge, UK). After 72 h, cells were sorted by FACS (FACSMelody, BD) and single-cell derived clones were initially screened by PCR genotyping. Additionally, both HERC5 and ISG15/IFNAR1 (dKO) clones were functionally tested by assessing their ISGylation profile and expression of ISGs, respectively, after IFN α priming. Briefly, A549 WT and HERC5 and dKO clones were primed with IFN α 2b (100 IU/ml) (PBL Assay Science) for 24 h and the expression of ISG15-conjugates and IFIT3 was analyzed by Western blot. HEK293 cells were primed with IFN α 2b (1000 IU/ml) for 8 h, total RNA was isolated and lsg15 mRNA expression was assessed by RT-qPCR.

Viral infection

A549 cells were seeded one day prior to infection in appropriate multi-well plates. For plaque assay, a viral inoculum containing 20 plaque forming units (PFU) of the corresponding virus was added to each well, and virus adsorption was performed in DMEM supplemented with penicillin/streptomycin (100 U/ml) for 90 min at 37°C. Cells were washed with PBS to remove un-adsorbed virus, and maintained in DMEM 1.5% carboxymethylcellulose sodium (CMC) (Sigma). Alternatively, cells were infected with DV at the indicated MOI, as described above, and maintained in DMEM supplemented with 1% FBS.

Titration

DV titration was performed by focus forming assay (FFA) in C6/36 cells. Briefly, C6/36 cells were seeded (1×10^5 cells/well in 24 well plate) and after overnight incubation were infected with a 10-fold serial dilution of virus samples (cell culture supernatants) in L-15 with 0.26% tryptose and 25 μ g/ml of gentamicin. After 90 minutes, the inoculum was removed and a CMC overlay media (L-15 media with 5% FCS, 0.26% tryptose, 25 μ g/ml of gentamicin and 1.6% of CMC) was added and the plates were incubated for 7

605 days at 28°C. After incubation, cells were washed, fixed with 3%
606 paraformaldehyde (PFA) (Sigma-Aldrich) and permeabilized with 0.5% triton
607 X100 (Sigma-Aldrich). After washing, cells were immunostained with mouse
608 monoclonal anti flavivirus E protein antibody 4G2 (ATCC® HB-112™, dilution
609 1:100), followed by goat anti-mouse immunoglobulin conjugated to alkaline
610 phosphatase (Promega S3721, dilution 1:7500). Focuses of infection were
611 revealed using NBT/BCIP reagent (Promega), following the manufacturer's
612 instructions and the virus titer calculated as follow: media of focus
613 number/inoculum volume x dilution. The results are expressed as FFU_{C6/36}/ml
614 (Gould et al., 1985).

615 VSVeGFP and HSV-1eGFP titrations were performed by plaque assay.
616 Briefly, Vero cells were seeded in 24-well plates 24 h prior to infection. Cell
617 monolayers were washed with PBS and inoculated with 0.3 ml of serial 10-
618 fold dilutions of the virus in duplicates. After 90 min adsorption at 37°C, each
619 well was re-suspended in DMEM 1.5% CMC. At 48 hpi, cells were fixed with
620 3% PFA (Sigma) for 30 min, washed 3 times with PBS and stained with 1%
621 crystal violet (Sigma) for 30 min at room temperature. Virus yield was
622 calculated and expressed as plaque forming units per ml (PFU/ml).

623

624 **Immunofluorescence**

625 At 24 hpi and 18 hpi, respectively, HSVeGFP and VSVeGFP infected cells
626 were fixed with 3% PFA for 20 min at room temperature, permeabilized with
627 0.5% Triton X100 in PBS for 4 min and stained with DAPI counterstain
628 (Molecular Probes). At 36 hpi, DV and ZIKV infected cells were fixed,
629 permeabilized and stained with mouse monoclonal 4G2 antibody (10 µg/ml
630 dilution 1:100), followed by incubation with Alexa Fluor 488 rabbit anti-mouse
631 IgG (H2+2L, Life Technologies, dilution 1:500), and DAPI counterstain.
632 Images were acquired with an Olympus IX83 inverted microscope. Briefly,
633 virus plaques, determined by eGFP or flavivirus E protein expression, were
634 delimited; images were converted to 16-bit and processed to be analyzed with
635 the ImageJ Software Cell Counter Plugin (W. S. Rasband, ImageJ, US
636 National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>, 1997–
637 2006).

For confocal analysis, A549 cells grown on glass coverslips were mock infected or infected with 10 DV PFU. At 36 hpi, cells were fixed and permeabilized. Following washes with PBS, cells were stained with mouse monoclonal anti-E protein (4G2, dilution 1:100) and rabbit polyclonal anti-IFIT3 (Proteintech, 15201-1-AP, dilution 1:200) for 1 h at room temperature. The cells were washed with PBS and stained with secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H+L, Life Technologies, dilution 1:500) and Alexa 568 goat anti-rabbit IgG (H+L, Life Technologies, dilution 1:500) and DAPI counterstain (Molecular Probes). Cells were washed and coverslips mounted using Prolong antifade reagent (Invitrogen). Z-stack and max intensity projection images were generated with a Leica DMI6000 B confocal microscope and Leica Application Suite X software for image analysis (Leica Microsystems).

651

652 **Flow cytometry and cell sorting**

Cells were fixed with 2% PFA in PBS, washed twice with PBS and permeabilized with 0.5% saponin in 1% BSA in PBS. Anti-flavivirus E protein mAb 4G2 was conjugated to Alexa Fluor 488 5-SDP (Life Technologies) following the manufacturer's instructions. Cells were incubated with FITC-conjugated 4G2 (dilution 1:1000) in permeabilization buffer for 40 minutes at room temperature, washed once and resuspended in FACS buffer. The cell suspensions were analyzed by flow cytometry on a FACSVerse instrument (BD Biosciences) and analyzed using FlowJo V10 software (BD). Cell sorting experiments were performed on a FACSMelody cell sorter (BD Biosciences).

662

663 **RT-qPCR**

vRNA was isolated by using QIAamp viral RNA Mini Kit (Qiagen). Intracellular total RNA was isolated with TRIZOL (Thermo Life) following manufacturer's instructions. A total of 1 µg was reverse transcribed using the High capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCRs (qPCRs) were performed with GoTaq® qPCR Master mix (Promega) using StepOne Plus real time PCR system (Applied Biosystems). VSVeGFP and 18S mRNA were used as control housekeeping genes. Amounts of DV or ISG

mRNA were calculated by using the $\Delta\Delta CT$ method. Primers specific the mRNA analyzed are listed in Table S1.

673

Western blot

Human antibodies used for immunoblot were as follows: mouse mAb to β -actin (Abcam, ab6276, dilution 1:4000), rabbit mAb to IFIT1 (Abcam, ab137632, dilution 1:1000), mouse mAb to pSTAT1(Y701) [M135] (Abcam, ab29045, dilution 1:1000), mouse mAb to STAT1 (Abcam, ab3987, dilution 1:1000), rabbit pAb to IFIT3 (ProteinTech, 15201-1-AP, dilution 1:1000), rabbit mAb to USP18 [D4E7] (Cell Signaling Technologies, 4813, dilution 1:1000), rabbit mAb to STAT2 [D9J7L] (Cell Signaling Technologies, 72604, dilution 1:1000), mouse mAb to ISG15 (R&D System, MAB4845, dilution 1:1000), home-made mouse mAb anti-GFP (67, 68) (dilution 1:1000) and mouse mAb to FLAG tag (Sigma, F3165, dilution 1:500).

Cells were treated as indicated, wash two times with ice-cold PBS and then lysed in RIPA buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA] supplemented with 1x protease inhibitors (Mini Protease Inhibitor Tablets, Roche). Total protein concentration was determined by BSA Protein Assay Kit (Thermo Life). For western blotting, 20 μ g of total protein were prepared in dithiothreitol-containing Laemmli sample buffer, separated and transferred to a nitrocellulose blotting membrane (GE Healthcare Amersham). After transfer, the membrane was blocked with 5% nonfat dry milk in TBS 0.1% Tween 20 (TBST) for 1 h at room temperature. Membrane was incubated with primary Abs diluted in 2% BSA in TBST at 4°C with gentle shaking overnight. Membrane was washed three times with TBST and then incubated with the appropriate secondary HRP-linked antibody for 1 h at room temperature. Membranes were washed and covered with ECL developing solution (PierceTM ECL WB substrate, Thermo Fisher Scientific).

700

Plasmids and transfections

Herc5 and Ifnar1 sgRNA/Cas9/GFP plasmids were provided by Horizon (Cambridge, UK). sgRNA sequences are described in Supplementary Table 1. Expression plasmid for ISG15 was described elsewhere (17). PmaxTM GFP

expression vector was acquired from Lonza (cat numb #D-00061). Expression plasmid for ZIKV NS5 was generated by amplifying the NS5 coding sequence (amino acids 2521-3423 in the polyprotein) from a previously described plasmid-based ZIKV reverse genetic system (69) using primers containing an N-terminal FLAG tag and inserted into pcDNA3.1. Expression plasmids for human USP18 WT and USP18 C64A mutant were kindly provided by Dr Carsten Münk (70). Transfections were performed with FuGene6 (Promega), following the manufacturer's instructions. Stably transfected cells were selected with geneticin (500 µg/ml) (Sigma).

714

715 **Immunoprecipitation**

For Stat2 co-immunoprecipitation assays, cells were lysed in RIPA buffer composed of 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM CaCl₂, 0.1% v/v Triton X-100, 10% v/v glycerol and complete protease inhibitor cocktail (Mini Protease Inhibitor Tablets, Roche). The supernatant was separated by centrifugation at 12.000 g at 4 °C for 10 min and incubated with STAT2 antibody (2 µg) (Santa Cruz Biotechnology, 514193) for 3 h at 4°C with gentle shaking. Complexes were precipitated with protein A/G Plus-agarose (Santa Cruz Biotechnology), washed with TBS and resuspended in SDS sample buffer. Immunoprecipitates were subjected to SDS-PAGE and western blotting, as described above.

FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2-agarose (Sigma), following the manufacturer's instructions.

All assays were performed three times and representative blots are presented.

730

731 **QUANTIFICATION AND STATISTICAL ANALYSIS**

Details concerning the statistical analysis methods are provided in each figure legend. Briefly, all data were analyzed using GraphPad Prism 8 software and were shown as mean and the standard deviation (SD). Statistical significance was determined by Welch's t test or one-way ANOVA for plaque size analyses, unpaired t test for virus multiple-step growth curve, cellular mRNA quantification and percentage of cells infected. Statistical significance is indicated by *, p < 0.05; **, p < 0.01; ***, p < 0.001.

739

740 SUPPLEMENTAL INFORMATION

741

742 Figure S1

743 (A) tSNE was used to visualize the single-cell global transcriptome data. Blue
744 dots represent uninfected cells, derived from healthy donors. Beige dots
745 represent bystander cells and red dots represent infected cells, both derived
746 from patients infected with DV.

747 (B) Differential expression of ISGs in HuH7 cells infected with DV. The Gene
748 Ontology term “type one interferon” was used to filter the results from the
749 single-cell RNA sequencing.

750 (C) Single cell ISG expression variability in uninfected, bystander and
751 infected. ISGylation related genes are underlined. Blue bar represents
752 uninfected cells. Beige bar represents bystander cells and red bar represents
753 infected cells, both derived from cells exposed to DV.

754 (D) Violin plot representing the expression of ISGylation family members in
755 uninfected [U], bystander [B] and infected [I] HuH7 cell line.

756

757

758 Figure S2. ISG15 does not restrict HSV-1 and VSV spread

759 (A and B) A549 cells were infected with 20 HSV-1eGFP PFU. At 24 hpi, cells
760 were fixed and stained with DAPI counterstain. HSV-1eGFP relative plaque
761 area (A) and number of cells per plaque (B). Images were acquired with an
762 Olympus IX83 inverted microscope and quantified by ImageJ software.

763 (C and D) A549 cells were infected with 20 VSVeGFP PFU. At 18 hpi, cells
764 were fixed and stained with DAPI counterstain. VSVeGFP relative plaque
765 area (C) and number of cells per plaque (D). Images were acquired with an
766 Olympus IX83 inverted microscope and quantified by ImageJ software.

767 Error bars represent mean $\pm$ SD. Results are representative of three or more
768 independent experiments. Statistical analyses were conducted using Mann-
769 Whitney’s test in Prism 8 (GraphPad Software).

770

771 Figure S3. Characterization of knockout cell lines

772 (A) Agarose gel electrophoresis of A549 WT and HERC5 KO PCR products
 773 using primers surrounding CRISPR/Cas9 ISG15 sgRNA guides editing region.
 774 PCR product size: WT: 310 bp; HERC5 KO: ~250 bp. (*) indicates PCR
 775 unspecific band. WT: A549 WT cell line; KO: A549 HERC5 KO; B: blank
 776 (B) Clonal isolates of A549 cells were immunoblotted for IFIT3 after 24 h
 777 treatment with IFN α 2b (100 IU/ml) (+, control IFIT3 -proficient cells; 1–9,
 778 different clonal isolates after limiting dilution).
 779 (C) Agarose gel electrophoresis of HEK293 WT and ISG15 KO PCR products
 780 using primers surrounding CRISPR/Cas9 ISG15 sgRNA guides editing region.
 781 PCR product size: WT: 1016bp; ISG15 KO: ~180bp. (*) indicates PCR
 782 unspecific band. WT: HEK293 WT cell line; KO: HEK293 ISG15 KO; B: blank
 783 (D) HEK293 WT and ISG15 KO cells were stimulated with IFN α 2b (1000
 784 IU/ml) for 8 h. Cells were harvested and total RNA was isolated. Isg15 mRNA
 785 was analyzed by RT-qPCR.
 786 (E) HEK293 ISG15KO PCR product was cloned into pGEM vector and
 787 sequenced by Sanger method. Nucleotide sequence was aligned with Isg15
 788 reference sequence retrieved from GenBank (NM_005101) and translated
 789 into primary amino acid sequence. (.) indicates the same sequence; (-) gap;
 790 (*) stop codon.

791

792 **Figure S4. A549 ISG15 KO cell line exhibits sustained ISG expression**

793 (A) A549 cells were primed with IFN α 2b (100 IU/ml) for 12 h, washed three
 794 times with DPBS and allowed to rest. Cells were harvested at the indicated
 795 time point and cell lysates were analyzed by Western blot with the
 796 corresponding antibodies.

797 Results are representative of two independent experiments.

798

799 **Figure S5. Sorting of infected cells**

800 (A and B) A549 cells were infected with DV at MOI 0.01.
 801 Representative FACS profile and DV mRNA qPCR of WT (A) and ISG15-KO
 802 (B) cells sorted by flavivirus E protein expression (4G2, FITC-A axis).
 803 B: bystander. I: infected
 804

805 **REFERENCES**

- 806 1. D. S. Mansur, G. L. Smith, B. J. Ferguson, Intracellular sensing of viral DNA by
807 the innate immune system. *Microbes Infect.* **16**, 1002–1012 (2014).
- 808 2. A. Gebhardt, B. T. Laudenbach, A. Pichlmair, Discrimination of Self and Non-
809 Self Ribonucleic Acids. *J. Interferon Cytokine Res.* **37**, 184–197 (2017).
- 810 3. J. W. Schoggins, Interferon-Stimulated Genes: What Do They All Do? *Annu Rev*
811 *Viro* (2019) <https://doi.org/10.1146/annurev-virology-092818-015756>.
- 812 4. A. García-Sastre, Ten Strategies of Interferon Evasion by Viruses. *Cell Host*
813 *Microbe* **22**, 176–184 (2017).
- 814 5. T. C. Pierson, M. S. Diamond, The emergence of Zika virus and its new clinical
815 syndromes. *Nature* **560**, 573–581 (2018).
- 816 6. A. D. T. Barrett, The reemergence of yellow fever. *Science* **361**, 847–848
817 (2018).
- 818 7. S. Bhatt, *et al.*, The global distribution and burden of dengue. *Nature* **496**, 504–
819 507 (2013).
- 820 8. D. S. Shepard, E. A. Undurraga, Y. A. Halasa, J. D. Stanaway, The global
821 economic burden of dengue: a systematic analysis. *Lancet Infect. Dis.* **16**, 935–
822 941 (2016).
- 823 9. L. Miorin, A. M. Maestre, A. Fernandez-Sesma, A. García-Sastre, Antagonism of
824 type I interferon by flaviviruses. *Biochem. Biophys. Res. Commun.* **492**, 587–596
825 (2017).
- 826 10. A. E. Ngono, S. Shresta, Immune Response to Dengue and Zika. *Annu. Rev.*
827 *Immunol.* **36**, 279–308 (2018).
- 828 11. A. Grant, *et al.*, Zika Virus Targets Human STAT2 to Inhibit Type I Interferon
829 Signaling. *Cell Host Microbe* **19**, 882–890 (2016).
- 830 12. M. Laurent-Rolle, *et al.*, The interferon signaling antagonist function of yellow
831 fever virus NS5 protein is activated by type I interferon. *Cell Host Microbe* **16**,
832 314–327 (2014).
- 833 13. S. Chen, Z. Wu, M. Wang, A. Cheng, Innate Immune Evasion Mediated by
834 Flaviviridae Non-Structural Proteins. *Viruses* **9** (2017).
- 835 14. J. Morrison, *et al.*, Dengue virus co-opts UBR4 to degrade STAT2 and
836 antagonize type I interferon signaling. *PLoS Pathog.* **9**, e1003265 (2013).
- 837 15. J. Ashour, *et al.*, Mouse STAT2 restricts early dengue virus replication. *Cell Host*
838 *Microbe* **8**, 410–421 (2010).
- 839 16. P. F. Dos Santos, D. S. Mansur, Beyond ISGylation: Functions of Free
840 Intracellular and Extracellular ISG15. *J. Interferon Cytokine Res.* **37**, 246–253
841 (2017).
- 842 17. P. F. Dos Santos, *et al.*, ISG15-Induced IL-10 Is a Novel Anti-Inflammatory
843 Myeloid Axis Disrupted during Active Tuberculosis. *J. Immunol.* **200**, 1434–1442
844 (2018).
- 845 18. C. D. Swaim, A. F. Scott, L. A. Canadeo, J. M. Huibregtse, Extracellular ISG15

- 846 Signals Cytokine Secretion through the LFA-1 Integrin Receptor. *Mol. Cell* **68**,
847 581–590.e5 (2017).
- 848 19. D. Bogunovic, *et al.*, Mycobacterial disease and impaired IFN- γ immunity in
849 humans with inherited ISG15 deficiency. *Science* **337**, 1684–1688 (2012).
- 850 20. M. Delgobo, *et al.*, An evolutionary recent IFN-IL-6-CEBP axis is linked to
851 monocyte expansion and tuberculosis severity in humans
852 <https://doi.org/10.1101/514943>.
- 853 21. B. Skaug, Z. J. Chen, Emerging role of ISG15 in antiviral immunity. *Cell* **143**,
854 187–190 (2010).
- 855 22. A. Dastur, S. Beaudenon, M. Kelley, R. M. Krug, J. M. Huibregtse, Herc5, an
856 interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in
857 human cells. *J. Biol. Chem.* **281**, 4334–4338 (2006).
- 858 23. J. J. Y. Wong, Y. F. Pung, N. S.-K. Sze, K.-C. Chin, HERC5 is an IFN-induced
859 HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of
860 protein targets. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 10735–10740 (2006).
- 861 24. Y.-C. Perng, D. J. Lenschow, ISG15 in antiviral immunity and beyond. *Nat. Rev.*
862 *Microbiol.* **16**, 423–439 (2018).
- 863 25. K.-I. Arimoto, *et al.*, STAT2 is an essential adaptor in USP18-mediated
864 suppression of type I interferon signaling. *Nat. Struct. Mol. Biol.* **24**, 279–289
865 (2017).
- 866 26. M. P. Malakhov, O. A. Malakhova, K. I. Kim, K. J. Ritchie, D.-E. Zhang, UBP43
867 (USP18) specifically removes ISG15 from conjugated proteins. *J. Biol. Chem.*
868 **277**, 9976–9981 (2002).
- 869 27. O. Malakhova, M. Malakhov, C. Hetherington, D.-E. Zhang, Lipopolysaccharide
870 activates the expression of ISG15-specific protease UBP43 via interferon
871 regulatory factor 3. *J. Biol. Chem.* **277**, 14703–14711 (2002).
- 872 28. X. Zhang, *et al.*, Human intracellular ISG15 prevents interferon- α/β over-
873 amplification and auto-inflammation. *Nature* **517**, 89–93 (2015).
- 874 29. S. D. Speer, *et al.*, ISG15 deficiency and increased viral resistance in humans
875 but not mice. *Nat. Commun.* **7**, 11496 (2016).
- 876 30. D. J. Lenschow, *et al.*, IFN-stimulated gene 15 functions as a critical antiviral
877 molecule against influenza, herpes, and Sindbis viruses. *Proc. Natl. Acad. Sci.*
878 *U. S. A.* **104**, 1371–1376 (2007).
- 879 31. F. Zanini, S.-Y. Pu, E. Bekerman, S. Einav, S. R. Quake, Single-cell
880 transcriptional dynamics of flavivirus infection. *Elife* **7** (2018).
- 881 32. F. Zanini, *et al.*, Virus-inclusive single-cell RNA sequencing reveals the
882 molecular signature of progression to severe dengue. *Proc. Natl. Acad. Sci. U.*
883 *S. A.* **115**, E12363–E12369 (2018).
- 884 33. I. Ramos, *et al.*, Innate immune response to influenza virus at single-cell
885 resolution in human epithelial cells revealed paracrine induction of interferon
886 lambda 1. *J. Virol.* (2019) <https://doi.org/10.1128/JVI.00559-19>.

- 887 34. P. Keskinen, *et al.*, Impaired antiviral response in human hepatoma cells.
888 *Virology* **263**, 364–375 (1999).
- 889 35. B. Schmid, *et al.*, Live Cell Analysis and Mathematical Modeling Identify
890 Determinants of Attenuation of Dengue Virus 2'-O-Methylation Mutant. *PLoS*
891 *Pathog.* **11**, e1005345 (2015).
- 892 36. H. Roth, *et al.*, Flavivirus Infection Uncouples Translation Suppression from
893 Cellular Stress Responses. *MBio* **8** (2017).
- 894 37. K. Wang, *et al.*, Interferon-stimulated TRIM69 interrupts dengue virus replication
895 by ubiquitinating viral nonstructural protein 3. *PLoS Pathog.* **14**, e1007287
896 (2018).
- 897 38. J. N. Whelan, Y. Li, R. H. Silverman, S. R. Weiss, Zika Virus Production Is
898 Resistant to RNase L Antiviral Activity. *J. Virol.* **93** (2019).
- 899 39. R. C. Gullberg, *et al.*, Stearoyl-CoA desaturase 1 differentiates early and
900 advanced dengue virus infections and determines virus particle infectivity. *PLoS*
901 *Pathog.* **14**, e1007261 (2018).
- 902 40. J. Zhang, *et al.*, Flaviviruses Exploit the Lipid Droplet Protein AUP1 to Trigger
903 Lipophagy and Drive Virus Production. *Cell Host Microbe* **23**, 819–831.e5
904 (2018).
- 905 41. C. Zhao, C. Denison, J. M. Huibregtse, S. Gygi, R. M. Krug, Human ISG15
906 conjugation targets both IFN-induced and constitutively expressed proteins
907 functioning in diverse cellular pathways. *Proc. Natl. Acad. Sci. U. S. A.* **102**,
908 10200–10205 (2005).
- 909 42. C. Zhao, T.-Y. Hsiang, R.-L. Kuo, R. M. Krug, ISG15 conjugation system targets
910 the viral NS1 protein in influenza A virus-infected cells. *Proc. Natl. Acad. Sci. U.*
911 *S. A.* **107**, 2253–2258 (2010).
- 912 43. Y. J. Kim, *et al.*, Consecutive Inhibition of ISG15 Expression and ISGylation by
913 Cytomegalovirus Regulators. *PLoS Pathog.* **12**, e1005850 (2016).
- 914 44. R. González-Sanz, *et al.*, ISG15 Is Upregulated in Respiratory Syncytial Virus
915 Infection and Reduces Virus Growth through Protein ISGylation. *J. Virol.* **90**,
916 3428–3438 (2016).
- 917 45. T. Hishiki, *et al.*, Interferon-mediated ISG15 conjugation restricts dengue virus 2
918 replication. *Biochem. Biophys. Res. Commun.* **448**, 95–100 (2014).
- 919 46. V. François-Newton, *et al.*, USP18-based negative feedback control is induced
920 by type I and type III interferons and specifically inactivates interferon α
921 response. *PLoS One* **6**, e22200 (2011).
- 922 47. N. A. Dalrymple, V. Cimica, E. R. Mackow, Dengue Virus NS Proteins Inhibit
923 RIG-I/MAVS Signaling by Blocking TBK1/IRF3 Phosphorylation: Dengue Virus
924 Serotype 1 NS4A Is a Unique Interferon-Regulating Virulence Determinant.
925 *MBio* **6**, e00553–15 (2015).
- 926 48. J. L. Muñoz-Jordán, *et al.*, Inhibition of alpha/beta interferon signaling by the
927 NS4B protein of flaviviruses. *J. Virol.* **79**, 8004–8013 (2005).
- 928 49. C. Daly, N. C. Reich, Double-stranded RNA activates novel factors that bind to

- 929 the interferon-stimulated response element. *Mol. Cell. Biol.* **13**, 3756–3764
930 (1993).
- 931 50. C. Daly, N. C. Reich, Characterization of specific DNA-binding factors activated
932 by double-stranded RNA as positive regulators of interferon alpha/beta-
933 stimulated genes. *J. Biol. Chem.* **270**, 23739–23746 (1995).
- 934 51. B. K. Weaver, K. P. Kumar, N. C. Reich, Interferon regulatory factor 3 and
935 CREB-binding protein/p300 are subunits of double-stranded RNA-activated
936 transcription factor DRAF1. *Mol. Cell. Biol.* **18**, 1359–1368 (1998).
- 937 52. E. S. Guimarães, *et al.*, Cyclic Dinucleotides Trigger STING-Dependent
938 Unfolded Protein Response That Favors Bacterial Replication. *J. Immunol.* **202**,
939 2671–2681 (2019).
- 940 53. K. H. Antunes, *et al.*, Microbiota-derived acetate protects against respiratory
941 syncytial virus infection through a GPR43-type 1 interferon response. *Nat.*
942 *Commun.* **10**, 3273 (2019).
- 943 54. J. Ashour, M. Laurent-Rolle, P.-Y. Shi, A. García-Sastre, NS5 of dengue virus
944 mediates STAT2 binding and degradation. *J. Virol.* **83**, 5408–5418 (2009).
- 945 55. M. Jones, *et al.*, Dengue virus inhibits alpha interferon signaling by reducing
946 STAT2 expression. *J. Virol.* **79**, 5414–5420 (2005).
- 947 56. U. Rand, *et al.*, Multi-layered stochasticity and paracrine signal propagation
948 shape the type-I interferon response. *Mol. Syst. Biol.* **8**, 584 (2012).
- 949 57. W. M. Schneider, M. D. Chevillotte, C. M. Rice, Interferon-stimulated genes: a
950 complex web of host defenses. *Annu. Rev. Immunol.* **32**, 513–545 (2014).
- 951 58. K. R. Loeb, A. L. Haas, The interferon-inducible 15-kDa ubiquitin homolog
952 conjugates to intracellular proteins. *J. Biol. Chem.* **267**, 7806–7813 (1992).
- 953 59. L. A. Durfee, N. Lyon, K. Seo, J. M. Huibregtse, The ISG15 conjugation system
954 broadly targets newly synthesized proteins: implications for the antiviral function
955 of ISG15. *Mol. Cell* **38**, 722–732 (2010).
- 956 60. H.-X. Shi, *et al.*, Positive regulation of interferon regulatory factor 3 activation by
957 Herc5 via ISG15 modification. *Mol. Cell. Biol.* **30**, 2424–2436 (2010).
- 958 61. M.-J. Kim, S.-Y. Hwang, T. Imaizumi, J.-Y. Yoo, Negative feedback regulation of
959 RIG-I-mediated antiviral signaling by interferon-induced ISG15 conjugation. *J.*
960 *Virol.* **82**, 1474–1483 (2008).
- 961 62. F. Okumura, *et al.*, Activation of double-stranded RNA-activated protein kinase
962 (PKR) by interferon-stimulated gene 15 (ISG15) modification down-regulates
963 protein translation. *J. Biol. Chem.* **288**, 2839–2847 (2013).
- 964 63. J. D’Cunha, E. Knight Jr, A. L. Haas, R. L. Truitt, E. C. Borden,
965 Immunoregulatory properties of ISG15, an interferon-induced cytokine. *Proc.*
966 *Natl. Acad. Sci. U. S. A.* **93**, 211–215 (1996).
- 967 64. S. W. Werneke, *et al.*, ISG15 is critical in the control of Chikungunya virus
968 infection independent of UbE1L mediated conjugation. *PLoS Pathog.* **7**,
969 e1002322 (2011).

- 970 65. F. Vuillier, Z. Li, P.-H. Commere, L. T. Dynesen, S. Pellegrini, USP18 and ISG15
971 coordinately impact on SKP2 and cell cycle progression. *Sci. Rep.* **9**, 4066
972 (2019).
- 973 66. S. Dabo, *et al.*, Inhibition of the inflammatory response to stress by targeting
974 interaction between PKR and its cellular activator PACT. *Sci. Rep.* **7**, 16129
975 (2017).
- 976 67. C. Zanluca, G. A. C. A. Mazzarotto, J. Bordignon, C. N. Duarte Dos Santos,
977 Development, characterization and application of monoclonal antibodies against
978 Brazilian Dengue virus isolates. *PLoS One* **9**, e110620 (2014).
- 979 68. G. F. Silveira, *et al.*, Human T Lymphocytes Are Permissive for Dengue Virus
980 Replication. *J. Virol.* **92** (2018).
- 981 69. M. Mutso, *et al.*, Reverse genetic system, genetically stable reporter viruses and
982 packaged subgenomic replicon based on a Brazilian Zika virus isolate. *J. Gen.*
983 *Virol.* **98**, 2712–2724 (2017).
- 984 70. E. Osei Kuffour, *et al.*, USP18 (UBP43) Abrogates p21-Mediated Inhibition of
985 HIV-1. *J. Virol.* **92** (2018).
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Figure 1-Espada *et al.*

A

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Figure 2-Espada et al.

Figure 3-Espada *et al.*

Figure 4-Espada et al.

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Figure 5-Espada *et al.*

Figure 6-Espada et al.

A

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