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Mouse STAT2 Restricts Early Dengue Virus Replication

Joseph Ashour,[1] Juliet Morrison,[1] Maudry Laurent-Rolle,[1] Alan Belicha-Villanueva,[1] Courtney Ray Plumlee,[5] Dabeiba Bernal-Rubio,[1] Katherine L. Williams,[4] Eva Harris,[4] Ana Fernandez-Sesma,[1,2,3] Christian Schindler,[5] and Adolfo García-Sastre[1,2,3,*]
[1]Department of Microbiology
[2]Division of Infectious Diseases, Department of Medicine
[3]Global Health and Emerging Pathogens Institute
Mount Sinai School of Medicine, New York, NY 10029, USA
[4]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, Berkeley, CA 94720, USA
[5]Department of Microbiology and Immunology, Columbia University, New York, NY 10032, USA
*Correspondence: adolfo.garcia-sastre@mssm.edu
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SUMMARY

Dengue virus encodes several interferon antagonists. Among these the NS5 protein binds STAT2, a necessary component of the type I interferon signaling pathway, and targets it for degradation. We now demonstrate that the ability of dengue NS5 to associate with and degrade STAT2 is species specific. Thus, NS5 is able to bind and degrade human STAT2, but not mouse STAT2. This difference was exploited to demonstrate, absent manipulation of the viral genome, that NS5-mediated IFN antagonism is essential for efficient virus replication. Moreover, we demonstrate that differences in NS5 mediated binding and degradation between human and mouse STAT2 maps to a region within the STAT2 coiled-coil domain. By using *STAT2*$^{-/-}$ mice, we also demonstrate that mouse STAT2 restricts early dengue virus replication in vivo. These results suggest that overcoming this restriction through transgenic mouse technology may help in the development of a long-sought immune-competent mouse model of dengue virus infection.

INTRODUCTION

Despite its global health impact, there is currently no vaccine or effective antiviral therapeutic available for dengue virus (DENV) (Sampath and Padmanabhan, 2009; Whitehead et al., 2007). One of the primary obstacles to developing such a tool is the lack of robust animal models in which efficacy of a given vaccine or drug can be tested prior to its administration in humans (Chaturvedi et al., 2005). Difficulties in developing mouse models for DENV infection result mostly from the animal's high resistance to viral infection, manifested by a transient low viremia even after high-dose challenges (reviewed in Yauch and Shresta, 2008). Several studies have elucidated the critical role of type I interferon (IFN) in mediating this resistance. Specifically, mice deficient in type I IFNα/β receptor (IFNAR) or in signal transducer and activator of transcription 1 (STAT1) expression are compromised in their ability to clear DENV at early time points. By way of comparison, *IFNGR1*$^{-/-}$ mice, which are IFNα/β signaling competent but lack the type II IFNγ receptor (IFNGR), remain nonviremic upon DENV challenge. However, enhanced morbidity and mortality can be achieved by infecting mice that are deficient for both IFNAR and IFNGR (AG129 mice), indicating a greater role for the type II IFN pathway at later stages postinfection (Shresta et al., 2004b; Shresta et al., 2005). Though valuable insight has been obtained from these mouse strains, their immune deficiencies limit the scope of questions that can be addressed, including questions on the efficacy of vaccines and therapeutics.

The STAT protein family consists of seven members. Of the seven, only STAT1 and STAT2 are required for the type I IFN pathway as they (in association with IRF9) make up the transcription factor complex interferon-stimulated gene factor 3 (ISGF3) (Fu et al., 1990; Kessler et al., 1988). STAT domain organization is conserved and includes an N-terminal domain, a coiled-coil domain, a DNA-binding domain, an SH2 domain, and a transactivation domain. With the exception of STAT2, the protein sequence of the STATs is highly conserved between mouse and human (Park et al., 1999; Paulson et al., 1999). STAT2 is a frequent target for degradation by viral proteins including the V protein of hPIV2 and the NS1 protein of RSV (Andrejeva et al., 2002; Lo et al., 2005; Parisien et al., 2001).

DENV encodes several proteins which block the IFN production or IFN signaling capabilities of the infected cell. These include the NS2b-3 protease which limits IFN production (Rodriguez-Madoz et al., 2010), and the NS2a, NS4a, and NS4b proteins which can independently inhibit the IFN signaling pathway to some extent (Munoz-Jordan et al., 2003). In addition, the NS5 protein was demonstrated to bind to STAT2 and target it for degradation through as-yet-unidentified mechanisms (Ashour et al., 2009; Mazzon et al., 2009). This loss of STAT2 protein recapitulates what is observed during dengue infection (Ashour et al., 2009; Jones et al., 2005). Curiously, the degradation activity but not the binding function of NS5 requires that NS5 be expressed within the context of a polyprotein which undergoes proteolytic processing for its maturation. This manner

of expression mirrors closely how NS5 is translated and matured during infection, though it remains unclear why it results in a gain of function for this viral protein.

In this study we show that DENV NS5 is able to bind and degrade human STAT2 (hSTAT2) in both human and mouse cells, but is unable to bind or degrade mouse STAT2 (mSTAT2) in either cell type. Furthermore, we show that DENV is sensitive to the action of IFN in mouse cells, but not in human cells when treatment occurs after infection, and that this phenotype correlates with the species origin of STAT2. Finally, we map the ability of NS5 to bind and degrade STAT2 to a region within the hSTAT2 coiled-coil domain. Taken together, we conclude that mSTAT2 is a restriction factor for DENV and that mutating this protein through transgenic technology may result in an improved mouse model for DENV infection.

RESULTS

NS5 of DENV Mediates Human STAT2 but Not Mouse STAT2 Degradation

As mice are resistant to DENV infection, and this resistance is dependent on a functioning IFN system, we were interested in determining whether DENV could degrade mouse STAT2 (mSTAT2). To address this question, we generated clonal *STAT2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and U6A cells (human cells deficient in STAT2 expression derived from the nondeficient 2FTGH cell line) to stably express FLAG-tagged versions of either hSTAT2 or mSTAT2. These cell lines expressed similar levels of FLAG-tagged STAT2 protein and recovered their ability to respond to IFN treatment (relative to their parental cells) as measured by a bioassay (Park et al., 2003b) utilizing the IFN-sensitive Newcastle disease virus expressing GFP (NDV-GFP), (Figure 1A, see Figures S1A and S1B available online).

We next infected these cell lines with DENV and compared the levels of FLAG-tagged STAT2 protein 24 hpi. As shown in both Figure 1B and Figure 1D, DENV infection results in a loss of hSTAT2-FLAG expression, but not mSTAT2-FLAG, in both human and mouse cells, respectively. To rule out that this was not a result of clonal selection, we utilized an NS5 transfection-based assay to demonstrate STAT2 degradation. We have shown previously that transfection of NS5 in a form that undergoes proteolytic processing is capable of decreasing levels of overexpressed hSTAT2 in a manner sensitive to proteasome inhibition, suggesting this is an active degradation process (Ashour et al., 2009). For these experiments, we utilized an NS5 fused downstream of the E protein and processed by either the viral protease (E-NS5-HA) or host deubiquitinating proteins (E-Ub-NS5-HA). As demonstrated in Figure 1C, E-NS5-HA was able to mediate loss of hSTAT2-FLAG but not mSTAT2-FLAG in transfected U6A cells.

To determine if NS5 alone was able to degrade hSTAT2 without additional human host factors, we transfected BHK21 cells (hamster derived) with E-Ub-NS5-HA and either hSTAT2-FLAG or mSTAT2-FLAG. As a negative control for this experiment, we replaced full-length NS5 with NS5 containing an N-terminal truncation of 277 residues that results in a loss of hSTAT2-FLAG binding (E-Ub-NS5-HA 278-900) (Ashour et al., 2009). Akin to the previous results, Figure 1E demonstrates that E-Ub-NS5-HA expression results in loss of hSTAT2-FLAG expression but not mSTAT2-FLAG expression. Similar results were obtained when we cotransfected E-NS5-HA and NS2b-3-HA with either hSTAT2-FLAG or mSTAT2-FLAG in *STAT2*$^{-/-}$ MEFs (Figure S1C). Finally, we infected human U2A cells (IRF9 deficient) and U3A cells (STAT1 deficient) to determine whether additional factors of the ISGF3 complex were required for DENV-mediated hSTAT2 degradation, as is the case with some paramyxoviruses that degrade STAT proteins (Horvath, 2004). Figure 1F demonstrates that hSTAT2 degradation occurs in the absence of either of these proteins, indicating they are not essential for this process. Thus the species specificity responsible for STAT2 degradation observed occurs at the level of STAT2 and is not due to additional host factors.

NS5 Associates with hSTAT2 but Not mSTAT2

As NS5 associates with hSTAT2 and this association is most likely required for hSTAT2 degradation during infection, we performed immune-precipitation experiments to determine whether NS5-HA was able to associate with mSTAT2-FLAG. STAT1-FLAG was used as a negative control for these experiments, as it has been demonstrated to not associate with NS5. Immune precipitation against the HA tag of full-length NS5 pulled down hSTAT2-FLAG but not mSTAT2-FLAG (Figure 2A). Of note, levels of hSTAT2-FLAG in the whole-cell extract (WCE) of samples cotransfected with E-Ub-NS5-HA 1–900 are lower as compared to samples cotransfected with E-Ub-NS5-HA 278–900, most likely the result of active degradation (Figure 2A, panel 3). We then performed the reciprocal immune precipitation within the context of viral infection. As seen in Figure 2B, NS5 coprecipitated only in the hSTAT2-FLAG-transfected samples. Of note, levels of NS5 in the WCE of the hSTAT2-FLAG sample are significantly lower than NS5 levels in the remaining three WCE lanes (panel three). We speculate that this could be due to the fact that high levels of hSTAT2-FLAG in the cell most likely titrate NS5 away from the replication complex, resulting in lower levels of DENV infection.

Additional evidence of the species-specific difference in association between NS5 and STAT2 was observed through analysis of NS5 localization in the presence of overexpressed hSTAT2 or mSTAT2 in BHK21 cells. In the presence of endogenous levels of hSTAT2, DENV NS5 is predominantly nuclear when overexpressed and during infection (Brooks et al., 2002; Forwood et al., 1999; Kapoor et al., 1995). Given the nuclear localization of NS5 and the predominantly cytoplasmic localization of unstimulated STAT2, we hypothesized that high levels of STAT2 (capable of associating with NS5) may interfere with NS5 nuclear localization. Figure 2C demonstrates that whereas NS5-GFP and NS5-HA localize to the nucleus when coexpressed with mSTAT2-FLAG, NS5-GFP and NS5-HA coexpressed with hSTAT2-FLAG localize to the cytoplasm. Thus, through the use of two independent assays, we conclude that NS5 association with STAT2 is a species-specific event.

Finally, we examined the ability of NS5 derived from DENV1 (Western Pacific strain) to associate with hSTAT2-FLAG and mSTAT2-FLAG. Similar to results obtained with DENV2 NS5 (16681), DENV1 NS5 coprecipitated hSTAT2-FLAG but not mSTAT2-FLAG (Figure 2D). Thus, NS5 species specificity can be extended to at least one other serotype and is not a feature unique to DENV2 16681.

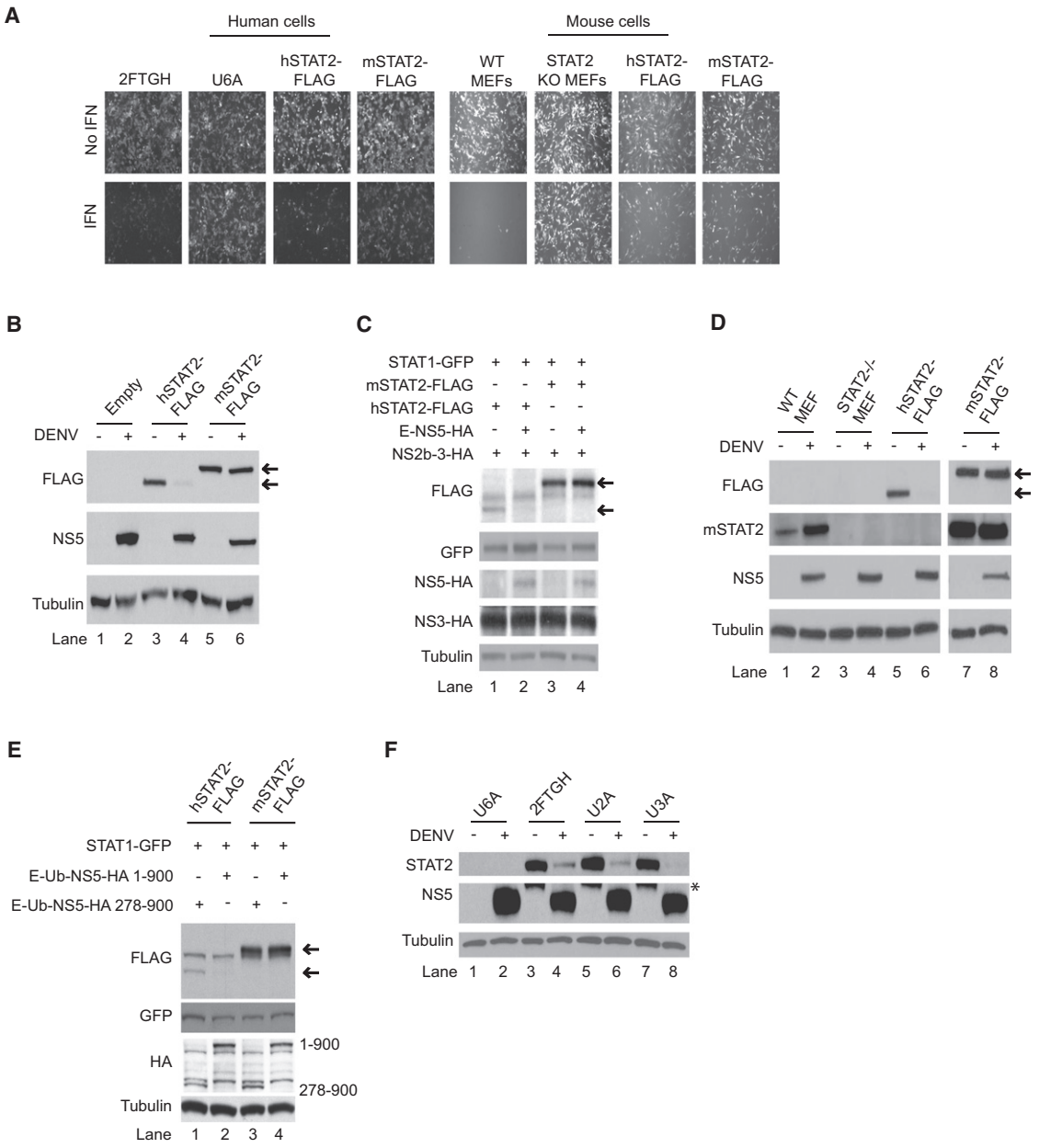


Figure 1. NS5-Mediated STAT2 Degradation Is Species Specific and Does Not Require Additional Species-Specific Host Factors

(A) Cells were treated with 100 μ/mL type I IFN; 24 hpt, cells were challenged with NDV-GFP. Virus replication levels were recorded 12 hpi by live microscopy.

(B) Human cells were infected with DENV at an moi of 10; 24 hpi, cells were lysed and levels of protein analyzed by SDS-PAGE and immune blotting.

(C) U6A cells were cotransfected with the given constructs; 24 hpt, cells were lysed and levels of protein analyzed by SDS-PAGE and immune blotting.

(D) Mouse cells were infected and processed as in (B).

(E) Cells were cotransfected with the given constructs; 24 hpt, cells were lysed and levels of protein analyzed by SDS-PAGE and immune blotting.

(F) Cells were infected with DENV at an moi of 10; 24 hpi, cells were lysed and analyzed by SDS-PAGE and immune blotting. Asterisk denotes residual signal from immune blot against STAT2. All western blots and microscopy data are representative of experiments performed several times. Upper arrows indicate FLAG-tagged mSTAT2 expected migration. Lower arrows indicate FLAG-tagged hSTAT2 expected migration. See also Figure S1.

NS5 Is Unable to Block IFN Signaling in the Presence of mSTAT2-FLAG

To determine if mSTAT2 expression would restore IFN signaling in NS5-expressing cells, we performed IFN signaling reporter assays in 293T cells. Increasing amounts of FLAG-tagged STAT1, IRF9, and either hSTAT2 (hISGF3) or mSTAT2 (mISGF3) were cotransfected with E-Ub-NS5-HA and the IFN-driven chloramphenicol-acetyltransferase (CAT) reporter plasmid ISRE-54-CAT-GFP. As a negative control, we used E-Ub-NS5-HA 278–900. In addition, we included a truncated form of NS5

(E-Ub-NS5-HA 10–900) that is deficient in degrading hSTAT2 but retains hSTAT2 binding and IFN antagonism activity (Ashour et al., 2009).

ISGF3 dose-dependent increases in GFP (Figure 3A) and CAT (Figure 3B) activity were observed in both hISGF3- and mISGF3-transfected cells in E-Ub-NS5-HA 278–900-containing samples. E-Ub-NS5-HA 1–900 containing samples showed significant inhibition of hISGF3-FLAG activity when hISGF3-FLAG plasmids were cotransfected relative to the E-Ub-NS5-HA 278–900 negative control. E-Ub-NS5-HA 10–900 also significantly inhibited hISGF3-FLAG activity at the 0.2 ng, 2.0 ng, and 20 ng amounts, although to a lesser extent than full-length NS5, indicating that binding to STAT2 inhibits ISGF3, possibly through inhibition of STAT2 phosphorylation (Mazzon et al., 2009), but that degradation further enhances this inhibition. In contrast to the hISGF3-FLAG results, cells transfected with mISGF3-FLAG showed intact signaling activity at all three doses in E-Ub-NS5-HA 10–900-containing samples. Of note, E-Ub-NS5-HA 1–900 cotransfected with the lowest amount of mISGF3 showed some inhibition of reporter activity. This may be due to a present but low affinity of NS5 for mSTAT2, or it may represent the loss of transcriptional activity contributed by the endogenous hSTAT2 (which would be targeted for degradation in the presence of full-length NS5). FLAG-tagged STAT1, STAT2, and IRF9 protein levels were undetectable at the 0.2 and 2.0 ng levels across all samples (Figure 3C, top panels). Notably, opposing effects on NS5 expression level were observed in samples dependent on the ability of NS5 to associate with the cotransfected STAT2. This may reflect differences in the stability of STAT2 associated versus nonassociated NS5 and/or NS5 stability during IFN signaling activation and will be followed up on in future studies. Nevertheless, we conclude that NS5-mediated signaling antagonism is compromised in cells expressing mSTAT2. Furthermore, the fact that mSTAT2 expression was sufficient to block NS5-mediated inhibition in these experiments suggests that STAT2 is the primary target of the IFN antagonist activity of NS5.

mSTAT2 Expression Inhibits Virus Production in an IFN-Dependent Manner

As NS5 antagonist function is abrogated in cells expressing mSTAT2, we next determined whether mSTAT2 expression would have a deleterious effect on DENV production. For this purpose, we measured virus titers in the supernatant of IFN-treated hSTAT2 or mSTAT2 stable expressing cells. These experiments were performed in both a human background (using 2FTGH cells and the STAT2-deficient U6A derivatives) and a mouse background (using WT MEFs and the STAT2 KO derivatives). Infected cells (moi of 0.1) were treated 12 hpi with universal IFN and supernatant was collected 36 hpi. We chose these conditions to reflect mainly the antiviral effects of IFN in cells already infected with DENV. The use of universal IFN allowed us to treat both mouse and human cells with the same IFN. IFN treatment of 2FTGH cells resulted in less than 10% inhibition in virus titer as compared to the untreated 2FTGH cells (Figure 4A, see also Figure S2 for the virus titers obtained in these experiments). A similar low level of IFN-mediated inhibition was observed in the hSTAT2-FLAG stable expressing U6A cells. This is in contrast to cells expressing mSTAT2-FLAG which show an 85% decrease in virus titer after IFN treatment (Figure 4A). We repeated these experiments using WT MEFs, STAT2 KO MEFs, or STAT2 KO MEFs stably expressing hSTAT2-FLAG or mSTAT2-FLAG. Whereas WT MEF cells and mSTAT2-FLAG-expressing cells demonstrated 95% and 67% inhibition of virus titer after IFN treatment, respectively, inhibition was not observed in the STAT KO cells and hSTAT2-FLAG-expressing cells (Figure 4B). Taken together, these results demonstrate that mSTAT2 but not hSTAT2 inhibits virus replication in an IFN-dependent manner. Furthermore, these results suggest that STAT2 degradation is an important step in the virus life cycle and is necessary for optimal virus infection in the presence of the IFN response.

mSTAT2 Is Required for Early Control of DENV Replication In Vivo and in Mouse Macrophages

The importance of both the type 1 IFN receptor and STAT1 in controlling DENV infection in mice has been shown previously (Shresta et al., 2004b, 2005). In order to determine whether mSTAT2 was also required, we monitored replication of a mouse-adapted DENV, D2S10 (Shresta et al., 2006), in infected WT and *STAT2*$^{-/-}$ mice. Infections were performed by intravenous (Figures 5A and 5B) or intracranial (Figure 5C) injection. In the case of intravenous injection, viral RNA was detectable in the lymph node (Figure 5A) and spleen (Figure 5B) of WT mice at 8 hr postinfection, but was undetectable by 18 hr postinfection. This in contrast to results obtained in the *STAT2*$^{-/-}$ mice, where we could detect viral RNA in both organs up to 32 hr postinfection. Notably, at 60 hr postinfection, no virus was detected in the spleen or lymph node of the WT or *STAT2*$^{-/-}$ mice. This suggests that while STAT2 is important for control of virus replication early in infection, it is not required at later time points for clearance of dengue virus from these tissues. Similar results were seen when we infected mice intracranially. Levels of virus in WT mice were low at 24 hr postinfection and were undetectable by 48 hr postinfection. However, we were still able to detect DENV in the *STAT2*$^{-/-}$ mouse at up to 72 hr postinfection (Figure 5C). In addition, no death was observed when we monitored survival past 7 days of one STAT2-deficient mouse infected through the intravenous route and one STAT2-deficient mouse infected intracranially (data not shown). Finally, we wanted to know whether macrophages, a primary in vivo target cell for DENV (Jessie et al., 2004), also required mSTAT2 for controlling DENV replication. To test this, we infected primary mouse monocyte-derived macrophages from the bone marrow of WT (WT mBMDMs) or *STAT2*$^{-/-}$ (KO mBMDMs) mice. No virus could be detected in the WT mBMDM samples (Figure 5D, squares). This is in contrast to DENV in the *STAT2*$^{-/-}$ mBMDMs, which reached detectable levels of replication at 24 and 48 hr postinfection (Figure 5D, triangles). These results indicate that mSTAT2 is necessary for early control of DENV replication in vivo and also in macrophages.

NS5 Interacts with the Coiled-Coil Domain of hSTAT2

To determine which specific region(s) of hSTAT2 is targeted by NS5, we first used a functional assay based on an ISRE-CAT-GFP reporter system that is activated by a construct encoding hSTAT2-FLAG sequence fused downstream of IRF9 (Kraus et al., 2003). As can be seen in Figure 6A, in contrast to the

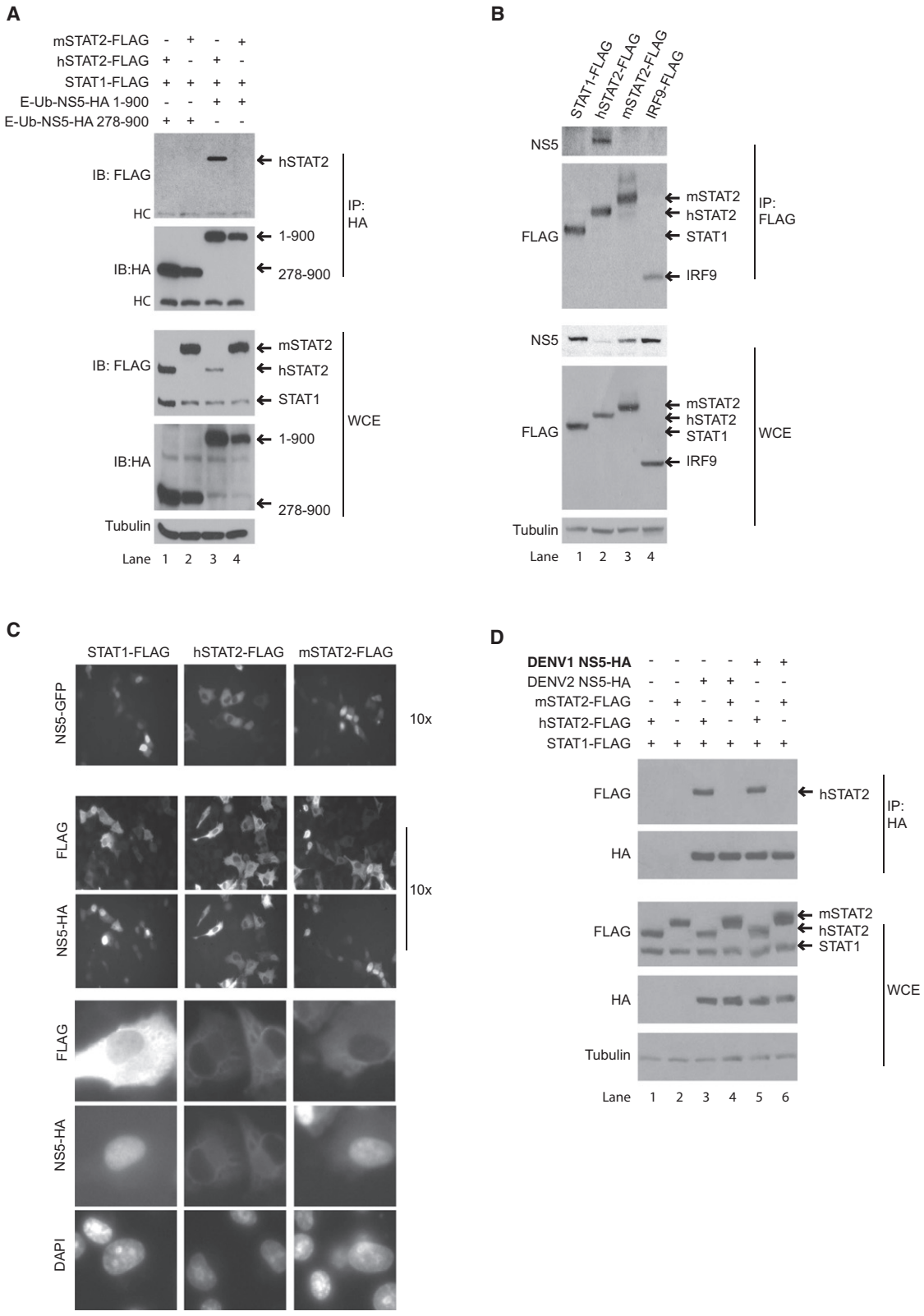
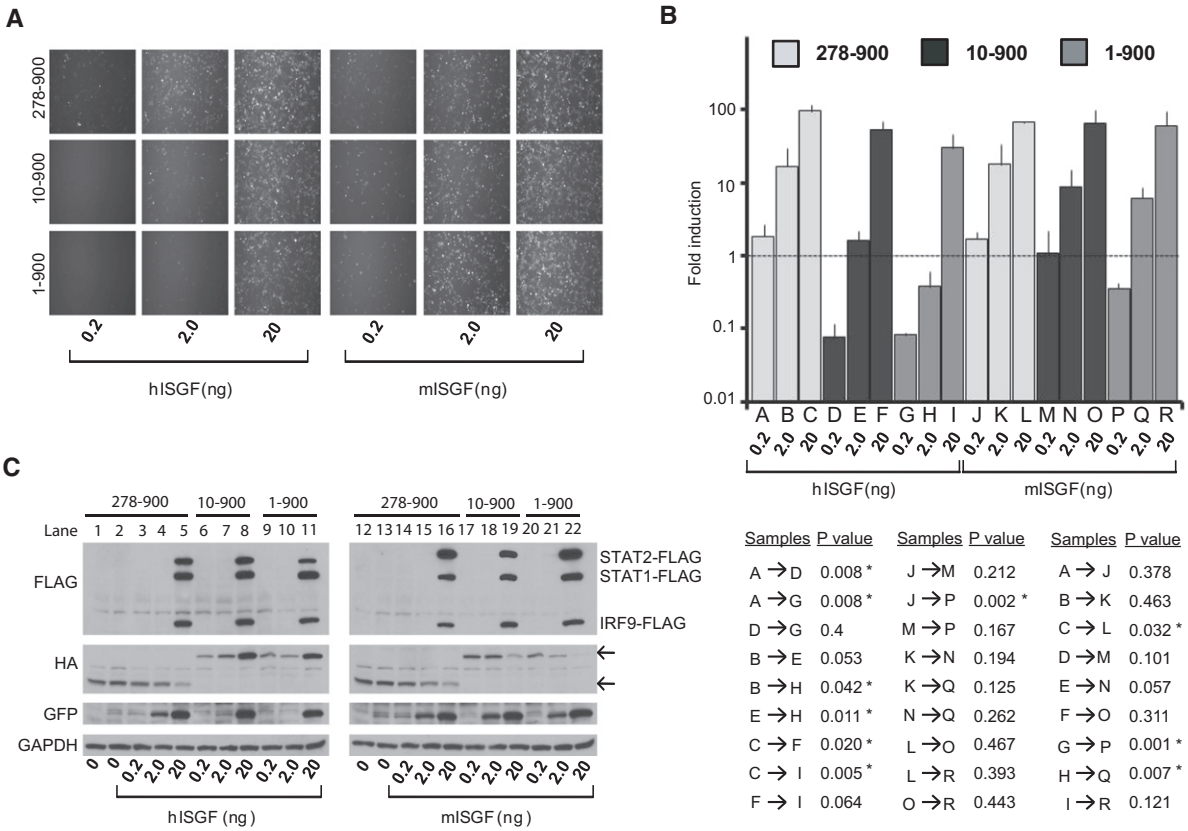


Figure 2. NS5 Associates with hSTAT2 but Not mSTAT2

(A) BHK21 cells were transfected with plasmids encoding for FLAG-tagged STAT1 and STAT2, and HA-tagged NS5 proteins. Cells were lysed 24 hpt, and immune precipitation was performed against the HA epitope.



Samples	P value	Samples	P value	Samples	P value
A → D	0.008 *	J → M	0.212	A → J	0.378
A → G	0.008 *	J → P	0.002 *	B → K	0.463
D → G	0.4	M → P	0.167	C → L	0.032 *
B → E	0.053	K → N	0.194	D → M	0.101
B → H	0.042 *	K → Q	0.125	E → N	0.057
E → H	0.011 *	N → Q	0.262	F → O	0.311
C → F	0.020 *	L → O	0.467	G → P	0.001 *
C → I	0.005 *	L → R	0.393	H → Q	0.007 *
F → I	0.064	O → R	0.443	I → R	0.121

Figure 3. NS5 Inhibits hSTAT2-Mediated Signaling but Not mSTAT2-Mediated Signaling

(A) Cells were cotransfected with increasing amounts of hISGF3-FLAG (hSTAT2-FLAG, STAT1-FLAG, and IRF9-FLAG) or mISGF3-FLAG (mSTAT2-FLAG, STAT1-FLAG, and IRF9-FLAG) plus ISRE-CAT-GFP, pCAGGS-Firefly luciferase, and either E-Ub-NS5-HA 278-900, E-Ub-NS5-HA 10-900, or E-Ub-NS5-HA 1-900; 24 hpt, cells were treated with 100 μ/mL type I IFN. Reporter activity (measured by GFP signal) was visualized 48 hpt by live microscopy. Results are representative of two independent experiments.

(B) Cells were transfected and IFN treated the same as in (A); 48 hpt, cells were lysed and CAT activity was measured. Fold induction of the ISGF3-FLAG-transfected cells is calculated relative to the fold induction observed with ISRE-54-GFP-CAT-transfected IFN-treated cells versus untreated cells, both in the absence of overexpressed ISGF3 components. This value is set as 1 and symbolized by the dashed horizontal line. Data were obtained from one experiment performed in triplicate using independent sources for plasmids and cells. Bars indicate standard deviation of the samples. P values of the statistical differences calculated between each sample (using unpaired, one-tailed, Student's t test), are provided in the bottom panel. Those differences that were found to be significant (p value $\leq$ 0.05) between two samples are signified by an asterisk.

(C) Western blot analysis of one representative experiment. Lanes 1 and 12 represent the control samples that do not overexpress ISGF3 components or receive IFN treatment. Lanes 2 and 13 represent the control samples that do not overexpress ISGF3 components and receive IFN treatment. Bands corresponding to the predicted molecular weights of NS5 1–900 and 10–900 are indicated by the upper arrow in the second panel. Bands corresponding to the predicted molecular weight of NS5 278–900 are indicated by the lower arrow of the second panel. Antibodies against FLAG, HA, GFP, and GAPDH were used.

negative control Core-HA, NS5-HA is capable of preventing the transcriptional activity of IRF9-hSTAT2-FLAG 1–851 (full-length hSTAT2) or 100–851 (hSTAT2 lacking the first 99 residues). However, this antagonist function is lost when we transfected IRF9 fused to hSTAT2 residues 572–851 or 747–851. This indicates that NS5 requires residues somewhere between hSTAT2 region 100 and 572 for its antagonism. It is unlikely that the loss of antagonism is due to defective folding of hSTAT2 resulting from large truncations, as the fusion protein is still capable of activating transcription and western blot controls show comparable levels of IRF9-hSTAT2 expression in all transfections (Figure 6B).

(B) 293T cells were transfected with plasmids encoding for FLAG-tagged STAT1, STAT2, and IRF9 proteins; 24 hpt, cells were infected with DENV at an moi of 10; 24 hpi, cells were lysed and immune precipitation was performed against the FLAG epitope.

(C) Plasmids encoding for STAT1, hSTAT2, mSTAT2, and NS5 proteins were transfected into BHK21 cells; 24 hpt, cells were live imaged for GFP fluorescence (top panels) and subsequently fixed for immune staining using antibody against HA (secondary FITC fluorophore) and FLAG (secondary Texas Red fluorophore) epitopes as well as DAPI stain to visualize DNA. Panels 2 and 3 are 10× images of the cells after fixing and staining for NS5 and the STAT protein. The lower three panels are from the same experiment (though not necessarily from the same field as panels 2 and 3) and have been enlarged for easy visualization of the cytoplasmic and nuclear compartments.

(D) Same as (A). All western blots and microscopy data are representative of experiments performed several times.

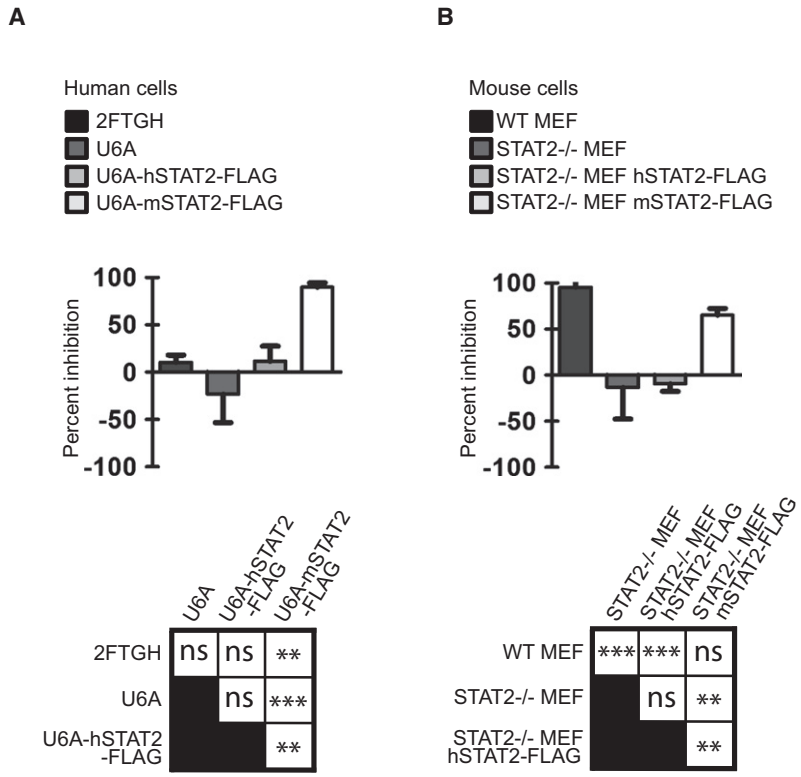


Figure 4. DENV Is Sensitive to mSTAT2 Expression in an IFN-Dependent Manner

Human cells (A) or mouse cells (B) were infected with DENV at an moi of 0.1; 12 hpi, cells were treated with 100 μ/mL of type I IFN; 36 hpi, supernatant was collected and levels of virus were measured by titration in Vero cells. Percent inhibition is measured relative to the levels achieved in the same cell line not treated with IFN (see the Experimental Procedures). No virus was detected at the 0 hour time point. Data was obtained from three independent experiments and bars indicate standard deviation of the samples. ANOVA results (Bonferroni multiple comparison test) are presented on the lower panels. Ns, no significance. ***, extremely significant (p value < 0.001). **, very significant (p value 0.001–0.01). See also Figure S2.

We next generated chimeric STAT2 proteins using hSTAT2 and mSTAT2 sequence. Portions of the N terminus of mSTAT2-FLAG were replaced with the homologous region of hSTAT2 (h/mSTAT2). As expected, immune precipitation against the FLAG epitope of mSTAT2 failed to pull down NS5-HA (Figure 7A, lane 3). IP of h/mSTAT2 containing the first 181 residues of hSTAT2-FLAG (h/mSTAT2-FLAG 1–181) did not pull down NS5, indicating that the first 181 residues of hSTAT2 were not sufficient for interaction (Figure 7A, lane 4). However, h/mSTAT2-FLAG expressing the first 301 residues of hSTAT2 coprecipitated NS5 (Figure 7A, lane 5). Thus, species-specific sequence between residues 181 and 301 is required for interaction with NS5.

To determine whether the h/mSTAT2-FLAG constructs that could bind to NS5 resulted in them becoming susceptible to NS5-mediated degradation, cells were cotransfected with the h/mSTAT2 construct and either E-Ub-NS5-1–900 or E-Ub-NS5-278–900. As expected, mSTAT2-FLAG in this assay showed no detectable changes in protein level upon cotransfection with E-Ub-NS5-1–900-HA as compared to the negative control (Figure 7B, lane 4 versus lane 3). Similar results were obtained with h/mSTAT2-FLAG 1–181 (Figure 7B, lane 6 versus lane 5). However, cotransfection of h/mSTAT2-FLAG 1–301 with E-Ub-NS5-HA 1–900 resulted in a significant decrease in h/mSTAT2 (Figure 7B, lane 8 versus lane 7). Therefore, the ability of STAT2 to bind NS5 correlates with the susceptibility to NS5-mediated degradation. We constructed additional h/mSTAT2 chimeras and placed them into the degradation assay. We first generated m/h/mSTAT2-FLAG in which the internal mouse STAT2 residues 181–301 were replaced with the analogous human sequence. As can be seen in Figure 7C, this construct was susceptible to NS5-mediated degradation. Finally, HA-tagged chimeras in which only the first 200, 210, and 250 residues of mSTAT2 were replaced with the analogous hSTAT2 sequence were all susceptible to degradation (Figure 7C, lanes 3–8). These data indicate that mSTAT2 and hSTAT2 amino acid differences between region 181 and 200 play a role in NS5-mediated association and degradation of STAT2.

We next generated a panel of chimeras where the N-terminal portion of STAT1 was replaced using increasing N-terminal sequence of hSTAT2 (chSTAT2/1). ChSTAT2/1-FLAG 1–124, which expresses the first 124 residues of hSTAT2 and the remaining 125–751 residues derived from STAT1, failed to be pulled down by NS5-HA (Figure 7D, lane 2). Increasing the amount of N-terminal hSTAT2 sequence so that we express the first 239 or 316 residues resulted in the ability to coprecipitate the NS5-HA protein (Figure 7D). Thus, the mapped region in the h/mSTAT2 chimeras required for binding/degradation (181–200) is consistent with the binding region mapped using the STAT2/1 chimeras (124–239). This falls within the coiled-coil region of hSTAT2, which encompasses residues 138–230 (Martinez-Moczygemba et al., 1997). We next placed these chSTAT2/1 chimeras into the STAT2 degradation assay (Figure 7E). ChSTAT2/1-FLAG 1–124 levels were not decreased in the presence of NS5. STAT2/1-FLAG chimeras 1–239 and 1–316, which bind NS5, also failed to be degraded. This suggests that additional hSTAT2 sequence downstream of the first 316 residues is important for STAT2 degradation. These sequences are shared with mSTAT2, as similar h/mSTAT2 chimeras are degraded (Figure 7B). Overall, our results indicate that the N-terminal region of hSTAT2 is needed for interaction with NS5, but that additional STAT2 specific sequences are required for degradation.

DISCUSSION

In this manuscript, we have demonstrated that DENV NS5-mediated antagonism of the IFN pathway is species specific and is nonfunctional in the presence of mSTAT2, suggesting that species specificity occurs at the level of hSTAT2 and is

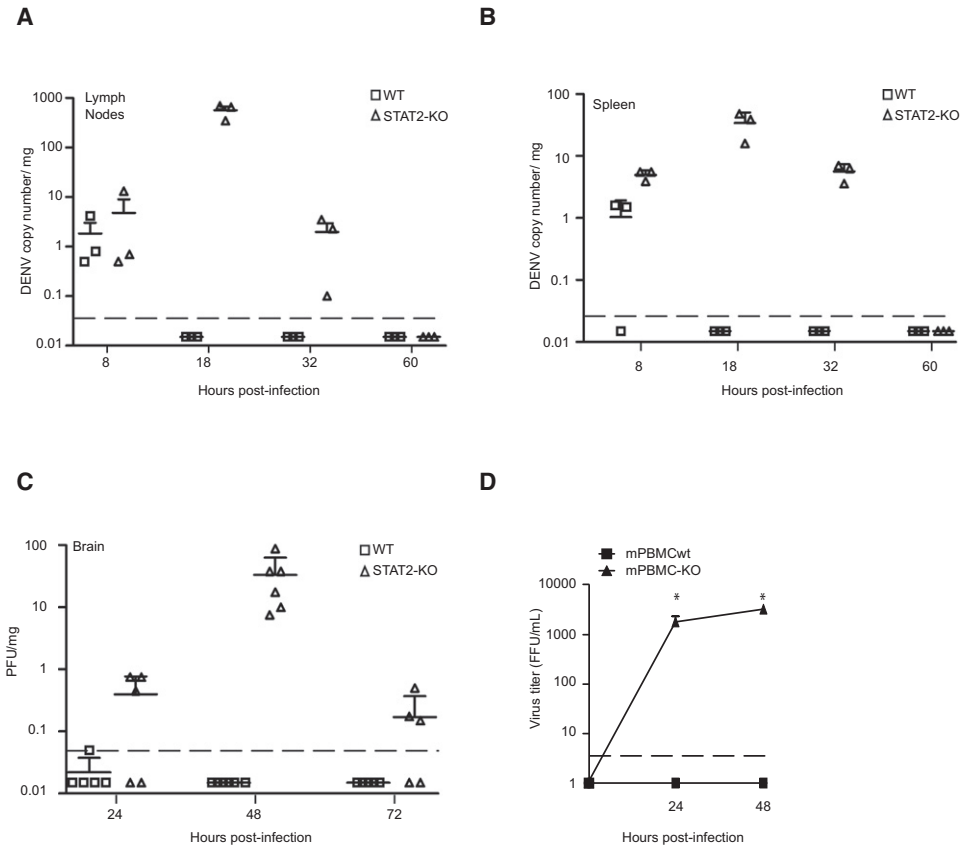


Figure 5. mSTAT2 Is Required for Control of DENV Production In Vivo and in Mouse Monocyte-Derived Macrophages

(A and B) WT and STAT2$^{-/-}$ mice were infected intravenously with DENV2 DS210. Lymph nodes (A) or spleen samples (B) were collected and viral load determined by qPCR for NS5. Results are from a single experiment (n = 3 for each time point) and error bars indicate standard deviation. The p value for the effect of genotype was 0.0001. Limit of detection in this assay was 0.03 copies/mg represented by the dashed line.

(C) WT and *STAT2*$^{-/-}$ mice were infected intracranially with DENV2 DS210. Brain tissue was collected and viral load determined by plaque assay in BHK21 cells. The limit of detection was 0.05 PFU/mg. Results are from a single experiment (n = 5 for 24 hpi and 72 hpi, n = 6 for 48 hpi) and error bars indicate standard deviation. The p value for the effect of genotype was 0.0001.

(D) Mouse bone marrow monocyte-derived macrophages (mBMDMs) from WT and *STAT2*$^{-/-}$ mice were infected with DENV2 16681. Supernatant was removed and analyzed for virus titer. Limit of detection in this assay is 4 fluorescing foci units/mL (FFU/mL) represented by the dashed line. No virus was detected at the 0 hour time point. Data were obtained from one infection performed in triplicate, and bars indicate standard deviation of the samples. Statistically significant difference ($p \leq 0.05$) was determined using Student's t test (unpaired, one-tailed). ND, not detected.

independent of additional species-specific host factors. While additional host factors are most certainly required, their identities remain unknown, though we believe they will most likely include components of the ubiquitin/proteasome degradation pathway. DENV strain variation has been observed with respect to manipulation of the IFN pathway (Umareddy et al., 2008). Importantly, we have shown that the species-specific nature of NS5 association with STAT2 is not unique to DENV2 (strain 16681) and can be extended to DENV1. Given the relatively high percentage NS5 sequence identity when comparing DENV2 to the other three serotypes (77% for DENV1, 77% for DENV3, and 72% for DENV4) (Khan et al., 2006), we surmise that our results could extend to DENV3 and DENV4. We note that DENV encodes multiple IFN antagonists including NS2a, NS4a, NS4b, and proteolitically active NS2b-3 (Leitmeyer et al., 1999; Munoz-Jordan et al., 2003, 2005; Rodriguez-Madoz et al., 2010), and while they were not a focus for this study, their antagonist function may influence species tropism as well.

In addition, we note that DENV is not the only virus whose replication is restricted by species-dependent differences of STAT2 or the IFN pathway. PIV5, a member of the Paramyxoviridae family, utilizes its V protein to bind hSTAT2 and the E3 ligase DDB1 (Precious et al., 2005; Ulane and Horvath, 2002). Consistent with this, hSTAT2 transgenic knockin mice showed higher levels of PIV5 replication in vivo (Kraus et al., 2008). In related fashion, NDV replication in human cells is facilitated through swapping the V protein with the influenza NS1 protein, an IFN antagonist that functions in human cells (Park et al., 2003a). Thus, the ability to antagonize the IFN pathway can be a limiting factor in determining species host range.

The results we have obtained using WT and *STAT2*$^{-/-}$ mice demonstrate that mSTAT2 is important for efficient control of virus replication in vivo and in primary macrophages. It is therefore likely that a STAT2-deficient cellular environment in vivo can be achieved, permitting detectable early levels of DENV replication, after DENV infection in an *mSTAT2*$^{-/-}$ mouse expressing

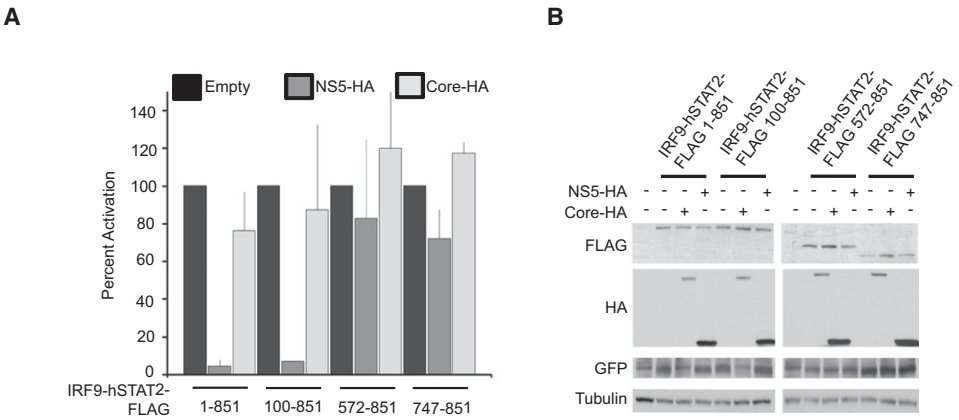


Figure 6. Residues Found within Region 100–572 of hSTAT2 Are Required for NS5-Mediated Inhibition of STAT2-Dependent Transcription

(A) Cells were cotransfected with plasmids encoding an ISRE-54-CAT-GFP reporter; pCAGGS-Firefly luciferase; the IRF9-hSTAT2-FLAG construct stated; and either empty, NS5-HA, or Core-HA protein; 24 hpt, cells were lysed and analyzed for CAT activity. Percent activation is calculated relative to samples transfected with ISRE-54-CAT-GFP, pCAGGS-Firefly luciferase, and empty plasmid with no IRF9-hSTAT2-FLAG construct. Data were obtained from three independent experiments, and error bars indicate standard deviation.

(B) Western blot of one representative experiment from the reporter assay. Lysates were analyzed by SDS-PAGE and immune blot analysis.

endogenous levels of hSTAT2. Thus, although the substitution of mSTAT2 by hSTAT2 certainly will not overcome all murine restrictions of DENV replication, especially at late times of infection, it may be a first step toward the development of an immune-competent mouse model, though it should be noted that the remaining DENV restriction at late times in *STAT2*$^{-/-}$ mice might be mediated by type II IFN, as this antiviral factor does not require STAT2 for signaling, and it is known to contribute to decreased disease in DENV-infected mice (Shresta et al., 2004a, 2004b, 2005).

Finally, through the generation of STAT chimeras, we identified two separate regions within STAT2 that contain sequences required for NS5 association and NS5-mediated degradation. Based on our results, we hypothesize that NS5 binds to the N-terminal region of hSTAT2 and that an as-yet-unidentified third protein containing E3 ligase activity is recruited to the complex through a motif located at the N terminus of NS5. This protein would then interact with the C-terminal half of STAT2, resulting in STAT2 ubiquitin conjugation and degradation. Further investigation of the cellular proteins required for STAT2 degradation in the presence of DENV is necessary to test this hypothesis.

Our understanding of DENV pathogenesis has benefitted greatly through the use of animal models. Specifically, through utilization of several genetically engineered mouse strains, the critical roles of the type I and type II IFN pathways in murine resistance to DENV infection have been elucidated (Shresta et al., 2004b); we note that while both STAT1 and STAT2 activity have been implicated in type III IFN signaling, it remains to be seen whether the activation of this pathway influences DENV replication (Dumoutier et al., 2003; Kotenko et al., 2003). Nevertheless, while the type I and type II IFN-deficient mice have proven invaluable with respect to our understanding of some aspects of dengue pathogenesis, their immune deficiencies limit the scope of questions that can be addressed, particularly those relevant to the development of vaccines and therapeutics. The use of humanized SCID or NOD/SCID mouse strains has overcome this barrier to some degree, though this approach requires a degree of skill and time (An et al., 1999; Bente et al., 2005; Blaney et al., 2002; Kuruvilla et al., 2007; Lin et al., 1998; Wu et al., 1995). Therefore, the development of an immune-competent mouse strain that is susceptible to DENV infection is desirable. Given the results obtained in our study, we speculate that initial resistance to DENV in mice results from an inability of NS5 to associate with and target STAT2 for degradation. Therefore, development of a transgenic mouse expressing a functional chimeric STAT2 capable of being degraded by an NS5-dependent mechanism could result in an immune-competent mouse susceptible to early DENV infection.

EXPERIMENTAL PROCEDURES

Cells and Viruses

All cell lines used in this work were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. All clonal cell lines were generated by cotransfection of the STAT2 encoding pCAGGS plasmid and pCDNA-zeo and subsequently selecting for zeocin resistance. Recombinant NDV expressing GFP was grown in 10-day-old embryonated chicken eggs. High-titer stocks of dengue virus (DENV2 16681) were obtained by passage in C6/36 cells. DENV1 D2S10 was kindly provided by Eva Harris. All transfections were performed using Lipofectamine 2000 (Invitrogen). For derivation of BMDMs from wild-type and STAT2 knockout mice (Park et al., 2000), cells were isolated from the bone marrow and cultured for 5 days in RPMI 1640, 10% FCS, pencillin/streptomycin, and 20% L929 conditioned media.

Plasmids and Antibodies

All DENV protein-encoding plasmids were generated in the pCAGGS (chicken β-actin promoter) background. Primer sequences used in the generation of these constructs are available upon request. Plasmids encoding human STAT1 and STAT2 were a kind gift from Dr. Megan Shaw. Plasmid encoding for human IRF9 was kindly provided by Estanislao Nistal-Villan. ISRE-54-CAT-GFP reporter, pCAGGS-Firefly, and luciferase plasmids were kind gifts from Dr. Luis Martinez-Sobrido. pCDNA-zeo was a kind gift from Dr. Ben tenOever. Mouse STAT2 cDNA was kindly provided by Dr. David Levy. Antibodies utilized for this manuscript include those raised against HA (Sigma), FLAG (Sigma), STAT1 (BD), STAT2 (Santa Cruz SC476), mSTAT2 (Santa

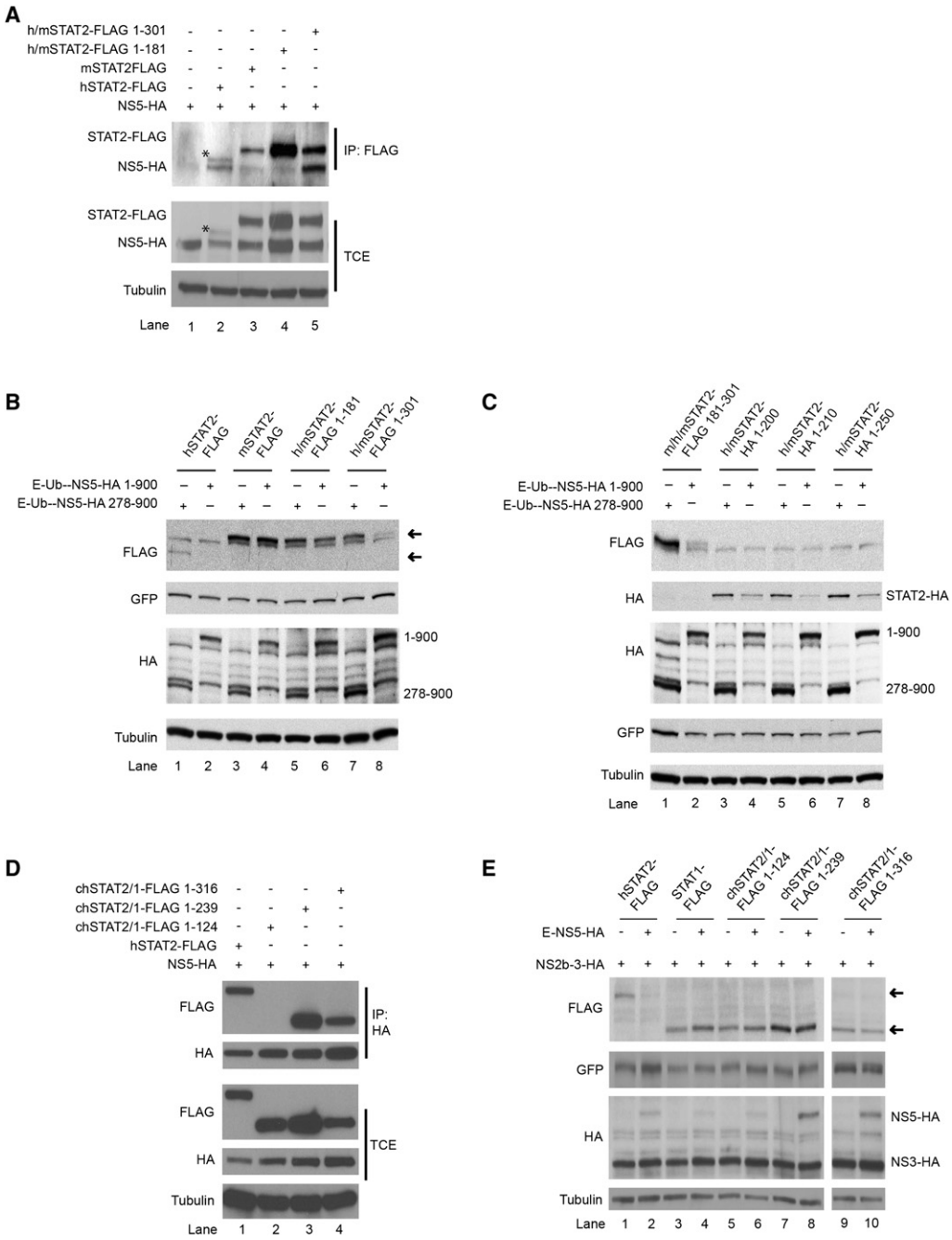


Figure 7. NS5 Binding Domain Maps to the N Terminus of hSTAT2

(A) Immune precipitation of FLAG-tagged STAT protein. U6A cells were cotransfected with NS5-HA and the FLAG-tagged STAT2 constructs stated; 24 hpt, cells were lysed and immune precipitation performed against the FLAG epitope. Asterisks denote full-length hSTAT2-FLAG, which has a faster mobility in SDS-PAGE compared to the h/mSTAT2-FLAG chimeras.

(B) BHK21 cells were transfected with the stated FLAG-tagged STAT2, STAT1-GFP, and NS5-HA-tagged constructs; 24 hpt, cells were lysed and protein levels analyzed by SDS-PAGE and immune blotting using antibodies against FLAG, GFP, HA, and tubulin. Upper arrow indicates expected mobility of mSTAT2-FLAG and chimeras, and the lower arrow indicates expected mobility of hSTAT2-FLAG.

(C) BHK21 cells were transfected with the stated FLAG-tagged STAT2 or HA-tagged STAT2, STAT1-GFP, and NS5-HA-tagged constructs; 24 hpt, cells were lysed and protein levels analyzed by SDS-PAGE and immune blotting. Panel 2 and panel 3 are two different exposure times of the same HA blot.

(D) BHK21 cells were cotransfected with NS5-HA and FLAG-containing STAT constructs; 24 hpt, cells were lysed and immune precipitation performed against the HA epitope.

(E) U6A cells were transfected with the FLAG-tagged STAT2, STAT1-GFP, and HA-tagged constructs; 24 hpt, cells were lysed and protein levels analyzed by SDS-PAGE and immune blotting. Upper arrow indicates expected mobility of hSTAT2-FLAG and lower arrows indicates expected mobility STAT1-FLAG and chimeras. All western blots are representative of experiments performed several times.

Cruz SC839), GFP (Sigma), GAPDH (Research Diagnostics Incorporated), and tubulin (Sigma). Rabbit antibody raised against DENV2 NS5 was generated by serial injection of bacterially expressed and purified DEN2 NS5 protein.

In Vivo DENV Infection and Quantification from Organs

For intravenous delivery of DENV, 24 hr prior to infection, 12 WT C57BL/6 and 12 *STAT2*$^{-/-}$ C57BL/6 mice were injected intraperitoneally with 200 μl anti-DENV1 serum that has previously been shown to enhance DENV2 infection (Balsitis et al., 2010). The mice were then infected with 10^6 pfu of a mouse-adapted DENV2 strain, D2S10, via tail vein injection. Organs were harvested at 8, 18, 32, and 60 hr. DENV2 copy number was measured by the PCR Core Facility of Mount Sinai School of Medicine using a SYBR Green qPCR assay and primers against DENV NS5 and three housekeeping genes (*rps11*, *β-actin*, and *α-tubulin*). For intracranial delivery of DENV, 16 WT C57BL/6 and 16 *STAT2*$^{-/-}$ C57BL/6 mice were injected intracranially with 10^5 pfu of D2S10. Brains were harvested at 24, 48, and 72 hr postinfection. The viral loads in the cleared homogenates were measured by plaque assay on BHK21 cells. Statistical analysis was performed to analyze the effect of genotype using two-way ANOVA (Bonferroni post-test). Values were log10 transformed to approximate Gaussian distribution. Values below the limit of detection were substituted for the limit of detection value (for example, a value of 0.025 in Figure 5A would be replaced with a value of 0.03), thus giving a more conservative calculation of p value.

Virus Quantification from Cell Supernatant

DENV titer present in infected cell supernatant was calculated by overlaying 250 μl of 1 ml total volume serial dilutions of the supernatant onto Vero cells and incubating for 2 hr at room temperature. Supernatant was then removed and replaced with fresh media containing 1% agar. The infection was then continued for 72 hr at 37°C. Infected cells were then fixed and stained for DNA (using DAPI) and for NS5 protein using anti-NS5 antibody and a secondary anti-rabbit FITC fluorophore. The number of positive foci was counted by eye under a fluorescent microscope. Infectious particles are expressed as fluorescing foci forming units/mL (FFU/mL). The limit of detection value reflects the sensitivity obtained when titrating virus stocks of known quantity alongside the infected cell supernatants (i.e., 1 FFU is observed with 250 μl of virus stock diluted to 4 FFU/mL). To calculate percentage of inhibition of dengue virus replication in IFN-treated cells, the FFU/ml obtained in IFN-treated samples was divided by the FFU/ml obtained in IFN untreated samples, and this number was subtracted from 1 and expressed as percent.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.chom.2010.10.007.

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