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The Interferon Signaling Antagonist Function of Yellow Fever Virus NS5 Protein Is Activated by Type I Interferon

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SUMMARY

To successfully establish infection, flaviviruses have to overcome the antiviral state induced by type I interferon (IFN-I). The nonstructural NS5 proteins of several flaviviruses antagonize IFN-I signaling. Here we show that yellow fever virus (YFV) inhibits IFN-I signaling through a unique mechanism that involves binding of YFV NS5 to the IFN-activated transcription factor STAT2 only in cells that have been stimulated with IFN-I. This NS5-STAT2 interaction requires IFN-I-induced tyrosine phosphorylation of STAT1 and the K63-linked polyubiquitination at a lysine in the N-terminal region of YFV NS5. We identified TRIM23 as the E3 ligase that interacts with and polyubiquitinates YFV NS5 to promote its binding to STAT2 and trigger IFN-I signaling inhibition. Our results demonstrate the importance of YFV NS5 in overcoming the antiviral action of IFN-I and offer a unique example of a viral protein that is activated by the same host pathway that it inhibits.

INTRODUCTION

There is an urgent need for therapeutics against flaviviruses. There were 5,674 cases of West Nile virus (WNV) infection in the United States in 2012 resulting in 286 deaths (Hadler et al., 2014), yet this number pales in comparison to the more than 200,000 cases of hemorrhagic disease and death caused annually by dengue virus (DENV). Even in the case of yellow fever virus (YFV), for which there is a highly efficacious vaccine, there are 200,000 reported cases annually with at least 30,000 deaths. Despite the success of the live-attenuated YFV-17D vaccine, occurrences of vaccine-associated viscerotropic and neurotropic disease have brought its safety into question (Barrett et al., 2007; Biscayart et al., 2014; Breugelmans et al., 2013; Monath et al., 2005).

The flavivirus genome is a single-stranded, positive-sense RNA that encodes a polyprotein that is cleaved co- and post-translationally by viral and host proteases generating three structural proteins, C (core), prM-M (membrane protein and its precursor), and E (envelope) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form the virion particle, whereas the NS proteins have roles in viral RNA replication, polyprotein processing, and host immune evasion (Aguirre et al., 2012; Chambers et al., 1990a, 1990b; Diamond, 2009; Rice et al., 1985). NS5 is the largest and most conserved of the flavivirus proteins. Its C terminus encodes the viral RNA-dependent RNA polymerase (Lindenbach and Rice, 2003), whereas its N terminus contains an S-adenosyl-methyltransferase (SAM) domain and is involved in methylation of the 5′ RNA cap structure of the viral RNA (Issur et al., 2009; Zhou et al., 2007a). The middle portion contains at least two nuclear localization sequences whose roles in viral replication are still uncharacterized (Brooks et al., 2002).

Type I interferon (IFN-I or IFN-α/β) induces an antiviral state in cells to curb viral replication and dissemination. Binding of IFN-I to its receptor activates JAK1 and TYK2, which phosphorylate and activate STAT1 and STAT2, resulting in the formation of IFN-stimulated gene factor 3 (ISGF3), a transcription factor complex comprised of IRF9 and phosphorylated STAT1 and STAT2. ISGF3 translocates to the nucleus where it binds to IFN-I-stimulated response elements (ISREs) and promotes the transcription of IFN-stimulated genes (ISGs), which encode proteins with antiviral activities (Der et al., 1998; Horvath et al., 1996; Schoggins et al., 2011).

Several flaviviruses including DENV, WNV, Japanese encephalitis virus (JEV), Langat virus (LGTV), and tick-borne encephalitis virus (TBEV) encode IFN-I antagonists (Ashour et al., 2009; Best et al., 2005; Jones et al., 2005; Laurent-Rolle et al., 2010; Lin et al., 2006; Werme et al., 2008; Mazzon et al., 2009; Morrison et al., 2013). Our present work describes a mechanism of virus-mediated IFN-I signaling inhibition that is unique among all viruses described thus far. We show that IFN-I induces binding of YFV NS5 to STAT2, preventing its transcriptional activity by (1) increasing K63-linked polyubiquitination at residue K6 of YFV NS5, thereby promoting the capacity of YFV NS5 to inhibit IFN-I

signaling via STAT2 binding; and (2) promoting STAT1 tyrosine (Tyr) phosphorylation, which is required for NS5/STAT2 binding.

RESULTS

YFV Antagonizes IFN-I-Stimulated Gene Expression

YFV and DENV share a similar host tropism, are spread by the same vector species, and can both cause hemorrhagic disease. Because DENV was known to antagonize IFN-I signaling (Ashour et al., 2009; Jones et al., 2005), we examined whether YFV (YFV-17D) would also have this ability. YFV infection caused a 5-fold reduction in IFN-I-mediated ISRE promoter activation (Figure 1A), but did not reduce IFN-II (IFN-γ)-stimulated GAS activity (Figure 1B). In addition, IFN-I but not IFN-II signaling was reduced in YFV-infected Vero cells as demonstrated with an NDV-GFP bioassay (Park et al., 2003; Figure 1C).

To identify the step of the IFN-I signaling pathway that is targeted by YFV, the expression, phosphorylation, and nuclear translocation of STAT1 and STAT2 were examined in YFV-infected cells. Immunofluorescence analysis (Figures 1D and 1E) and western blots (Figure 1F) revealed that STAT1 and STAT2 levels were not reduced by YFV infection. If anything, STAT2 levels were increased (Figure 1F). Furthermore, IFN-I-induced phosphorylation and nuclear translocation of the STATs were unaffected (Figures 1D–1F), suggesting that YFV interference is downstream of tyrosine phosphorylation and nuclear translocation of the STATs.

We next examined whether YFV inhibits IFN-I signaling at the level of DNA binding of ISGF3. For this purpose, electrophoretic mobility shift assays were carried out with lysates from IFN-I-treated YFV-infected Vero cells. ISGF3 failed to bind to the ISRE derived from the Oas1b promoter in extracts from IFN-I-treated, YFV-infected, or DENV-2-infected Vero cells (Figure 1G). Antibodies against IRF7 did not disrupt DNA-protein complex formation, whereas antibodies against STAT2 did, confirming the identity of the complex as ISGF3 (Figure 1G).

YFV NS5 Inhibits IFN-I Signaling

Because the NS5 proteins of LGTV, JEV, DENV, and WNV had previously been identified as IFN antagonists (Ashour et al., 2009; Best et al., 2005; Laurent-Rolle et al., 2010; Lin et al., 2006; Mazzon et al., 2009; Morrison et al., 2013), we reasoned that YFV NS5 might also be an IFN-I signaling antagonist. Expression of YFV NS5 reduced IFN-I mediated ISRE promoter activation comparable to the positive controls, Nipah virus (NiV) V, DENV-2 NS5, and WNV NS5 (Figure 2A). Expression of DENV-2 Core did not inhibit CAT activity (Figure 2A).

The NS5 proteins of the virulent YFV-Asibi strain and the YFV-17D vaccine strain differ by three amino acids (aa; dos Santos et al., 1995; Hahn et al., 1987). We examined the ability of both YFV NS5 proteins to inhibit IFN-I signaling by investigating their impact on ISG54 promoter activation after stimulation with exogenous IFN-β. Both proteins behaved similarly in the ISG54 reporter assay (Figure 2B).

YFV NS5 Interacts with STAT2 in the Presence of IFN-I and IFN-III

YFV's antagonism of IFN-I but not IFN-II signaling (Figure 1) suggested that the virus was targeting a factor such as TYK2, STAT2, or IRF9 that is specific to the IFN-I pathway. Because DENV-2 NS5 targets STAT2, and DENV and YFV NS5 are ~60% identical at the aa level, we examined whether YFV NS5, like DENV-2 NS5, would interact with STAT2 (Ashour et al., 2009). Immunoprecipitation experiments in 293T cells expressing YFV-17D NS5, YFV-Asibi NS5, empty plasmid, or DENV-2 NS5 revealed STAT2 coprecipitation with DENV-2 NS5 but not YFV NS5 (Figure 2C). The DENV NS5 expression construct used in this study does not enable proteolytic processing of its N terminus; therefore, only STAT2 binding, and not degradation, is observed (Ashour et al., 2009). When immunoprecipitation experiments were performed in cells stimulated with IFN-I, both YFV-17D NS5 and YFV-Asibi NS5 associated with STAT2 (Figure 2C). Similar results were observed in cells stimulated with IFN-III (IFN-λ), but not IFN-II (Figure 2D). Like IFN-I, IFN-III signals through JAK1/TYK2 and ISGF3, leading to an IFN-I-like transcriptional profile (Ank et al., 2006; Zhou et al., 2007b). Because both the 17D and Asibi NS5 proteins behaved similarly in the reporter and binding assays (Figures 2B and 2C), we used YFV-Asibi NS5 for the remainder of the study.

IFN-I-Induced Modification of STAT2 Is Not Required for STAT2 Interaction with YFV NS5

YFV NS5 localizes to both the cytoplasm and nucleus but is predominantly nuclear (Figure 1F and Figure S1A available online). Because YFV NS5 binds STAT2 in cells treated with IFN-I, we examined whether IFN-I-mediated nuclear translocation of STAT2 was required for YFV NS5 to interact with STAT2. Cell fractionation assays revealed that YFV NS5 bound STAT2 in the cytoplasm and the nucleus of IFN-I-treated cells (Figure S1A). Thus IFN-I-induced nuclear translocation of STAT2 is not required for binding to YFV NS5.

We examined whether IFN-I-mediated modification of STAT2 was required for YFV NS5 to interact with STAT2. Coimmunoprecipitation assays were performed with lysates from U6A cells, which lack endogenous STAT2 (Leung et al., 1995), transfected with YFV NS5 along with wild-type STAT2 or a STAT2 mutant that was incapable of IFN-induced tyrosine phosphorylation (STAT2 Y690F; Leung et al., 1995; Qureshi et al., 1996). YFV NS5 associated with both wild-type STAT2 and mutant STAT2 in IFN-I-treated cells, indicating that IFN-I-mediated STAT2 tyrosine phosphorylation is not required for this interaction (Figure S1B). We also confirmed that STAT1 phosphorylation remained intact in U6A cells upon IFN stimulation (Figure S1C). Confocal immunofluorescence showed YFV NS5 colocalization with wild-type and mutant STAT2 only after IFN-I treatment (Figure 3A).

We next determined if IFN-I was inducing modifications of YFV NS5 and/or cellular proteins other than STAT2 to promote YFV NS5-STAT2 interaction. U6A cells were transfected with a construct expressing HA-tagged YFV NS5. After 24 hr, the cells were either mock stimulated or treated with IFN-I. U6A cell lysates were subjected to immunoprecipitation using anti-HA beads to precipitate NS5. The beads were washed then incubated with lysates from mock-treated or IFN-I-treated 2fTGH cells, the STAT2-proficient U6A parental line. YFV NS5 interacted with STAT2 only when YFV NS5 had been precipitated from IFN-I-treated U6A cells, regardless of whether 2fTGH cells were IFN-I-treated or mock-treated (Figure 3B). In contrast,

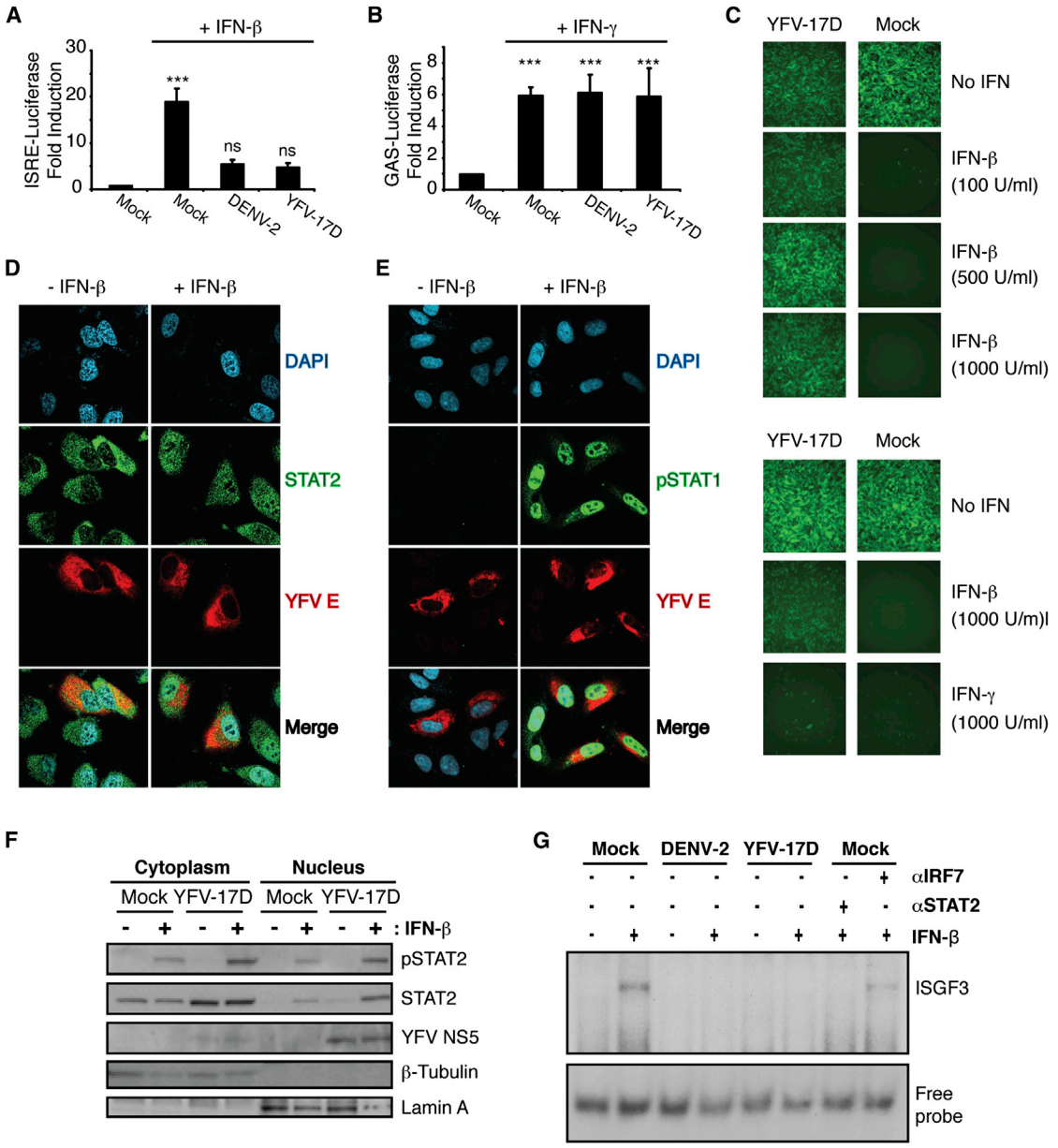

Figure 1. YFV Inhibits IFN-I but Not IFN-II Signaling

(A and B) 293T cells were transfected with (A) an IFN-I-inducible firefly-luciferase (f-luc) reporter plasmid (ISRE-luciferase) or (B) an IFN-II-inducible f-luc reporter (GAS-luciferase) along with a constitutively expressing *Renilla* luciferase (R-Luc) gene plasmid. Cells were infected with DENV-2 or YFV-17D viruses (moi = 5) for 24 hr and treated with IFN-β or IFN-γ (1,000 U/ml) for 16 hr prior to assaying for luciferase activity. Fold induction of f-luc was normalized to R-luc. The bars represent the mean fold induction of three independent experiments compared to untreated, mock-infected controls.

(C) Vero cells were mock infected or infected with YFV-17D virus (moi = 5). At 24 hr postinfection (hpi), cells were mock treated or treated with the indicated amounts of IFN-β or IFN-γ for 16 hr then infected with NDV-GFP. NDV-GFP replication was monitored by fluorescence microscopy at 14 hpi.

(D and E) Confocal microscopy images showing subcellular localization of the indicated protein in mock-infected or YFV-17D-infected (moi = 1) Vero cells before or after treatment with IFN-β for 30 min.

(F) Intracellular fractionation of mock infected or YFV-17D-infected HeLa cells (moi = 10). The proteins in the cytoplasmic and nuclear fractions were detected by western blotting with the indicated antibodies.

(G) Vero cells were mock-infected or infected with DENV-2 or YFV-17D viruses for 24 hr (moi = 10), then either mock-stimulated or stimulated with IFN-β for 8 hr. Cell extracts were analyzed by electrophoretic mobility shift assays with ISRE elements derived from the Oas1b gene. Statistical analyses were conducted using the unpaired t test in Prism 4 for Macintosh (GraphPad Software). ns, no significance, where $p > 0.05$; ***$p < 0.0001$.

DENV-2 NS5 bound in all cases to STAT2. The difference in binding efficiencies of YFV NS5 and DENV NS5 to STAT2 is likely due to (1) the complexes are made in different lysates decreasing complex formation; (2) DENV NS5 may interact directly with STAT2, whereas YFV NS5 interaction may be indirect; and (3) YFV NS5 may require both STAT1/2 to be complexed before

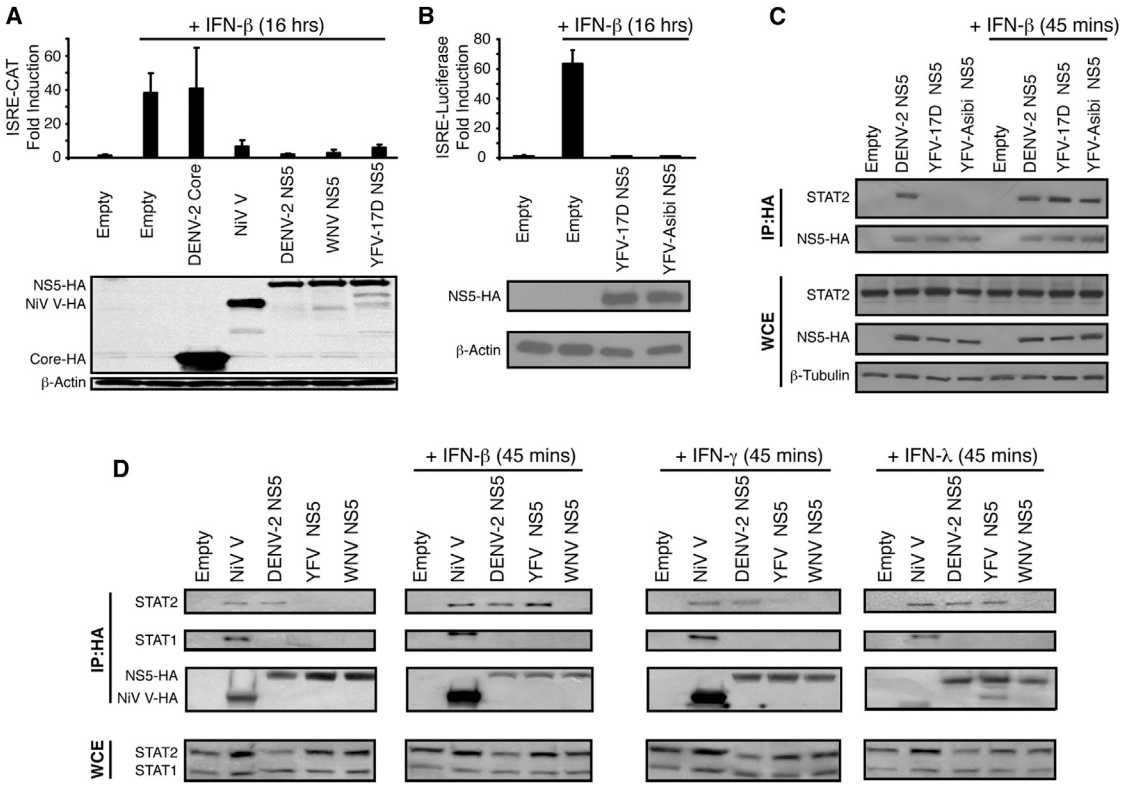

Figure 2. YFV NS5 Inhibits IFN-I Signaling and Interacts with STAT2 in IFN-I- and IFN-III-Treated Cells

(A) 293T cells were cotransfected with a plasmid encoding an ISRE-54-CAT-GFP reporter and with a constitutively expressing firefly-luciferase (f-luc) plasmid plus an empty vector or viral protein encoding plasmids. At 24 hr posttransfection, cells were treated with IFN-β for 16 hr prior to assaying for CAT activity. Induction of CAT activity was normalized to f-luc activity. Lower: Western blot showing the relative expression of each transfected viral protein.

(B) 293T cells were cotransfected with an IFN-I-inducible f-luc reporter plasmid (pISRE-luc) along with a constitutively expressing *Renilla* luciferase (R-luc) gene plasmid and empty vector or YFV-17D and YFV-Asibi NS5 expression plasmids. At 24 hr posttransfection, cells were treated with IFN-β for 16 hr prior to assaying for luciferase activities. Fold induction of f-luc was normalized to R-luc. The bars represent the mean fold induction of three independent experiments compared to untreated empty vector controls. Lower: Western blot (WB) showing the relative expression of each transfected viral protein.

(C) 293T cells were transfected with the indicated NS5 expression plasmids. At 24 hr, cells were mock stimulated or stimulated with IFN-β for 45 min. Cells were lysed and immunoprecipitation was performed followed by WB with the indicated antibodies.

(D) Same experiment as in (C) with cells mock-stimulated or stimulated with 1,000 U/ml of IFN-β, IFN-γ, or IFN-λ for 45 min prior to lysis. WCE, whole cell extract; CAT, chloramphenicol acetyl transferase.

its interaction with STAT2 whereas DENV NS5 does not. Thus, these data suggest that IFN-I signaling modifies YFV NS5 and/or a cellular interactor promoting NS5 binding to STAT2.

The N Terminus of YFV NS5 Is Required for IFN-I Signaling Antagonism

To map the domain of YFV NS5 required for IFN-I signaling inhibition, N-terminally truncated NS5 mutants were tested for STAT2 binding. Removal of the first ten aa resulted in a loss of YFV NS5-STAT2 association (Figure S2). Chimeric proteins were constructed to ensure that mutant YFV NS5 was expressed in the context of a full-length NS5 molecule then tested for their ability to bind STAT2 and inhibit IFN-I signaling. Substitution of the first ten aa of YFV NS5 with the first ten or 11 residues of WNV, DENV-2, and Modoc virus (MODV; Figure 4A) revealed that only WNV-YFV NS5 associated with STAT2 and inhibited IFN-I signaling (Figures 4B and 4C). Next we examined whether the first ten aa of YFV NS5 were sufficient for binding STAT2 by substituting the first ten aa of WNV NS5 for those of YFV NS5 (YFV-WNV NS5; Figure S3). YFV-WNV NS5 did not bind STAT2, indicating that the first ten aa of YFV NS5 were necessary but insufficient for this association.

A Lysine Residue within the First Ten Amino acids of YFV NS5 Is Required for Its IFN-I Antagonist Function

A comparison of the first ten aa of the flaviviral NS5 proteins revealed a lysine (K) at position 6 of YFV NS5 and a K at position 4 (K4) of WNV NS5 that could account for the YFV NS5/STAT2 interaction. Importantly, the ten aa of DENV and MODV did not have K residues. Mutation of K6 to an arginine, YFV NS5 K6R (Figure 4A), abolished the ability of YFV NS5 to associate with STAT2 after IFN-I stimulation (Figure 4D) and inhibit the ISRE promoter (Figure 4E). Substitution of the proline at position 7 of MODV-YFV NS5 with a K (MODV-YFV NS5 P7K; Figure 4A) rescued MODV-YFV NS5's capacity to bind STAT2 and inhibit ISRE promoter activity (Figures 4D and 4E).

The dissimilarity of the N-termini of YFV and MODV and the ability of MODV-YFV NS5 P7K to bind STAT2 suggested that

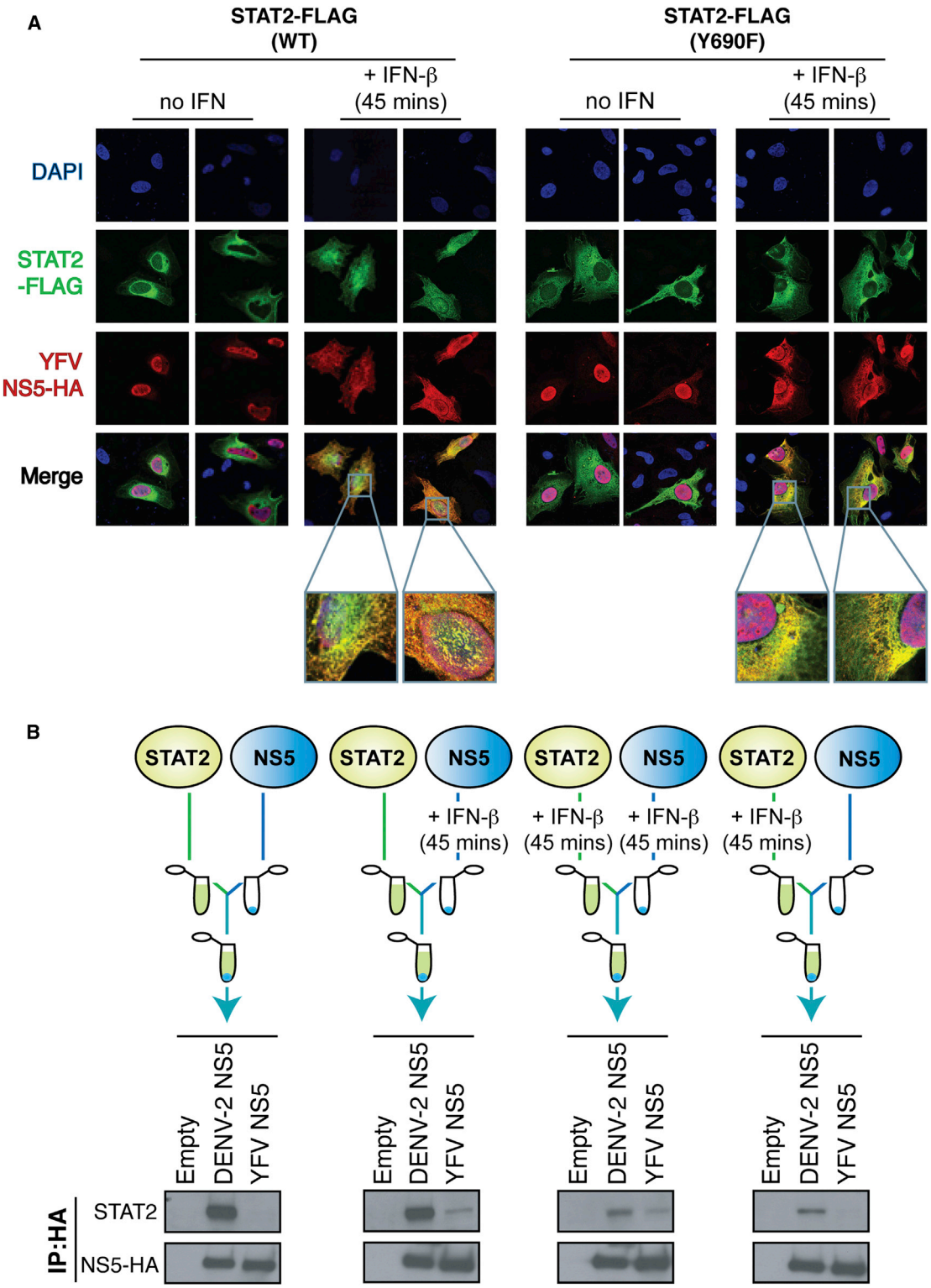

Figure 3. Neither IFN-I-Mediated Phosphorylation nor Nuclear Translocation of STAT2 Are Required for STAT2 Interaction with YFV NS5

(A) Confocal microscopy images showing subcellular localization of the indicated proteins in Vero cells before or after treatment with IFN-β for 45 min.

(B) U6A cells were transfected with the indicated plasmids. At 24 hr, cells were mock stimulated or stimulated with IFN-β for 45 min. Cells were lysed and immunoprecipitation was performed against the HA epitope for 2 hr using anti-HA beads. The beads were then washed three times with lysis buffer. 2fTGH cells were mock stimulated or stimulated with IFN-β, then lysed and added to the NS5-bound anti-HA beads for 2 additional hours. The protein was eluted from washed beads then used for western blot.

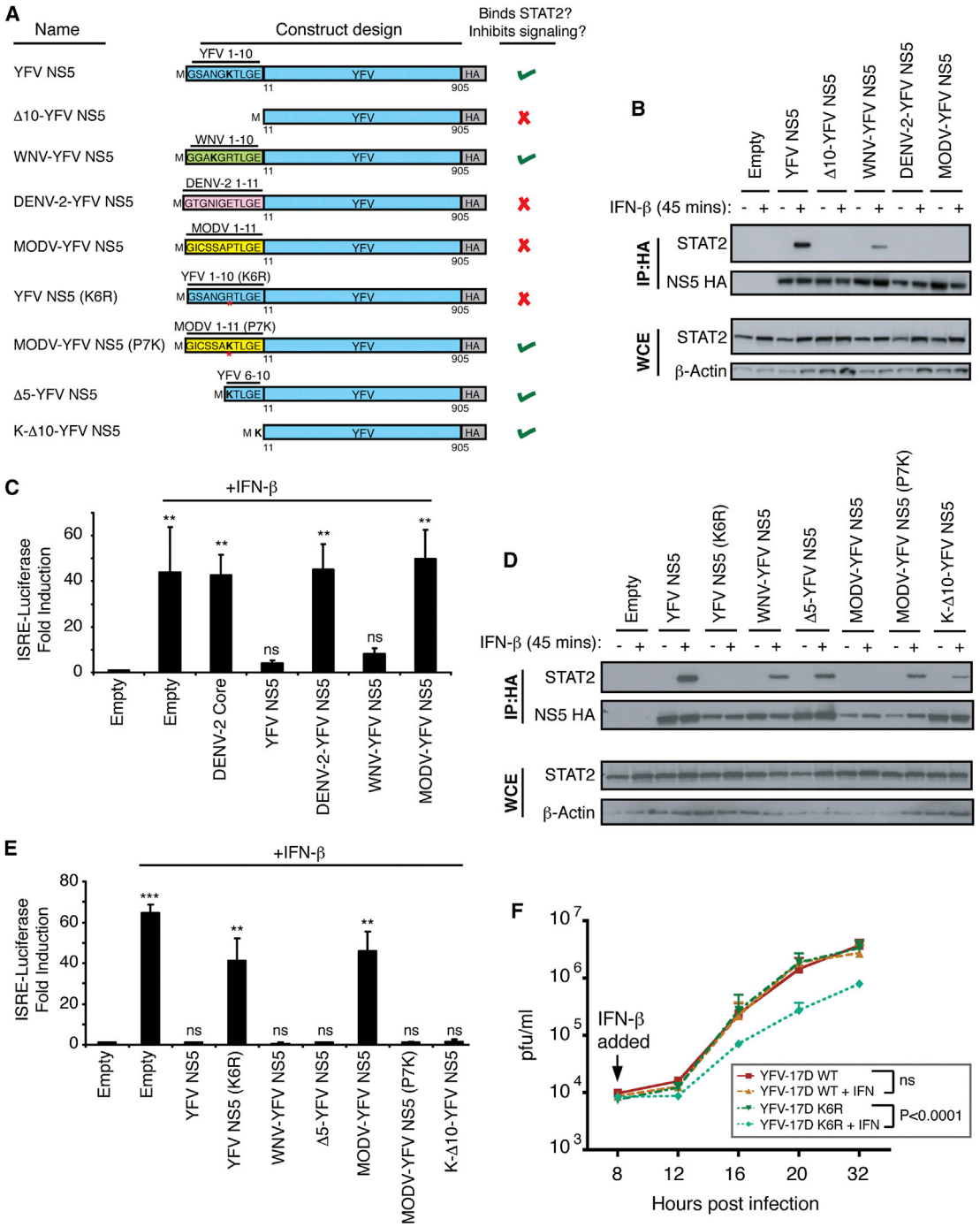

Figure 4. A Single Lysine Residue at Position 6 of YFV NS5 Is Critical for IFN-I Antagonist Function

(A) Diagram of the YFV NS5 expression constructs used in Figure 4.

(B and D) 293T cells were transfected with the indicated plasmids. At 24 hr posttransfection, cells were mock stimulated or stimulated with IFN-β for 45 min. Cells were lysed and immunoprecipitation was performed followed by western blot analysis with the indicated antibodies.

(C and E) 293T cells were cotransfected with an IFN-β-inducible firefly-luciferase (f-luc) reporter plasmid (pISRE-luc) along with a constitutively expressing R-luc plasmid plus empty vector or plasmids encoding the indicated viral proteins. At 24 hr, cells were treated with IFN-β for 16 hr prior to assaying for luciferase activities. Fold induction of f-luc activity was normalized to R-luc activity. The bars represent the mean fold induction of three independent experiments compared to the untreated, empty vector controls.

(F) Vero cells were infected with either YFV-17D WT or YFV-17D K6R at an moi of 10. At 8 hpi, the cells were either mock-treated or treated with 100 U/ml IFN-β. Virus was harvested at the indicated time points and viral titers were quantified by plaque assay on BHK-21 cells. Each point on the graph represents the mean of four independent experiments. Statistical analyses were conducted using the unpaired t test in Prism 4 (GraphPad Software). ns, no significance, where $p > 0.05$; ***$p < 0.0001$; **$p < 0.001$.

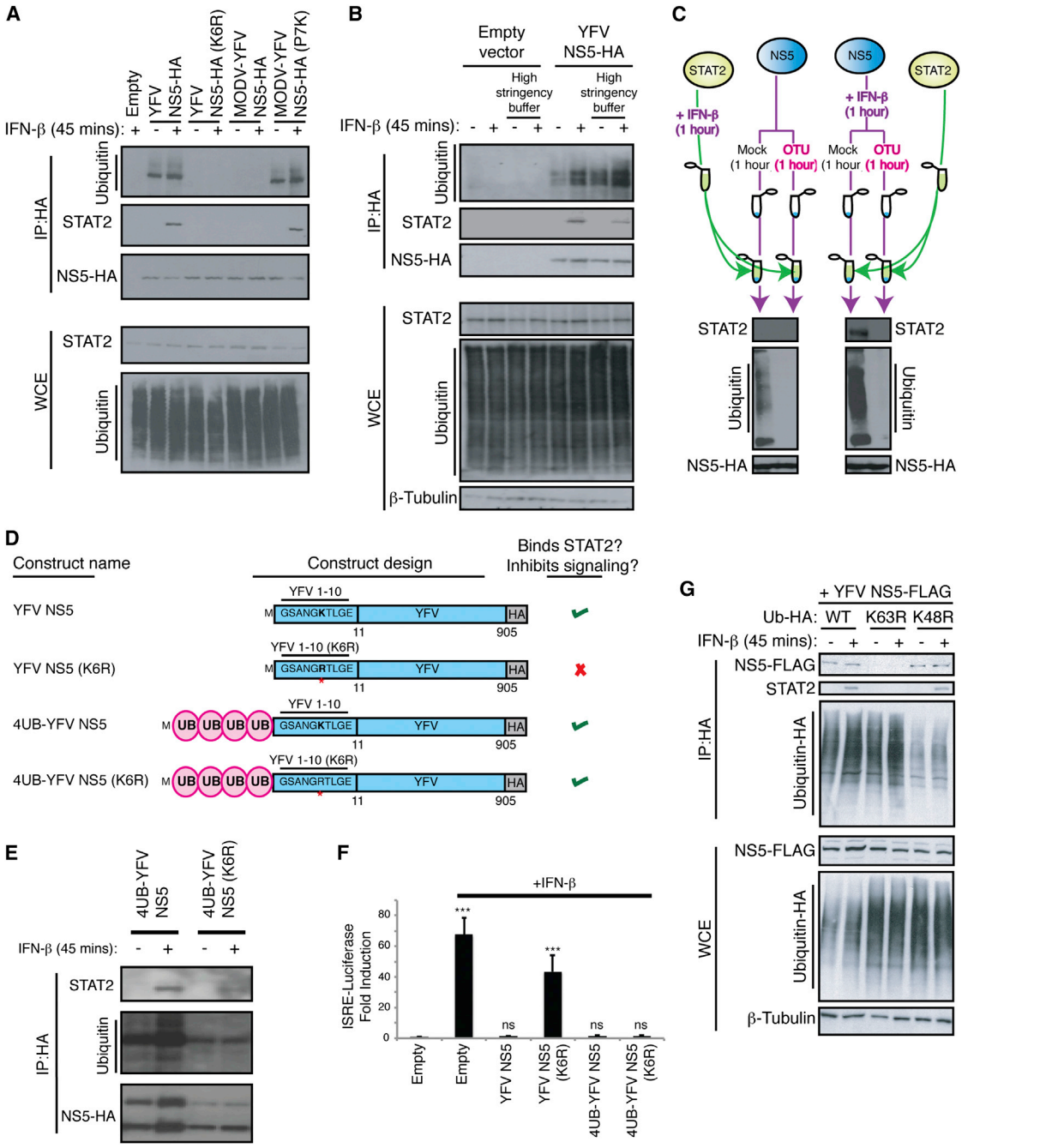

Figure 5. K63-Linked Ubiquitination Is Required for Binding of YFV NS5 to STAT2

(A) 293T cells were transfected with various NS5 mutants. At 24 hr posttransfection, cells were mock stimulated or stimulated with IFN-β for 45 min. Cells were lysed and immunoprecipitation was performed followed by western blot analysis (WB) with the indicated antibodies.

(B) Same as (A), but with more stringent conditions.

(C) U6A cells were transfected with HA-tagged YFV NS5. At 24 hr, cells were mock stimulated or stimulated with IFN-β. Cells were lysed and immunoprecipitation was performed against the HA epitope for 2 hr using HA beads. Beads were washed three times with lysis buffer followed by incubation with reaction buffer or OTU. 2fTGH cells were mock stimulated or stimulated with 1,000 U/ml IFN- β. Cells were lysed and were added to the HA beads for 2 hr. Beads were then washed followed by WB with the indicated antibodies.

(D) Diagram of the YFV NS5 constructs used in (E) and (F).

(E) Same experiment as in (A) with the indicated constructs.

(F) 293T cells were cotransfected with an IFN-α/β-inducible firefly-luciferase (f-luc) reporter plasmid (pISRE-luc) along with a constitutively expressing Renilla luciferase (R-luc) gene plasmid plus empty vector or plasmids encoding the indicated viral proteins. At 24 hr, cells were treated with IFN-β for 16 hr prior to

(legend continued on next page)

the only residue of importance in the first ten aa of YFV NS5 was lysine. We generated YFV NS5 constructs missing the first five aa (Δ5-YFV NS5) or the first ten aa except K (K-Δ10-YFV NS5; Figure 4A). Coimmunoprecipitation assays (Figure 4D) and reporter assays (Figure 4E) showed that these proteins were able to bind STAT2 and inhibit IFN signaling, emphasizing the requirement of the lysine residue in the N-terminal region of NS5. An alignment of the YFV genome sequences in the virus pathogen resource revealed that K6 of YFV NS5 is conserved across strains (Figure S4A).

Introduction of the K6R mutation into YFV-17D (YFV-17D K6R) did not affect viral replication as compared to wild-type virus (YFV-17D WT; Figure 4F). However, IFN-I treatment at 8 hr postinfection, decreased YFV-17D K6R titers by one log relative to YFV-17D WT (Figure 4F). In addition, the upregulation of ISGs in YFV-17D K6R-infected cells was comparable to that in IFN-treated mock-infected cells (Figures S4B and S4C). These results indicate that K6 of YFV NS5 is critical for NS5-STAT2 interaction and inhibition of IFN-I-mediated antiviral activity.

IFN-I Enhances K63-Linked Polyubiquitination of YFV NS5 and Promotes YFV NS5-STAT2 Interaction

Lysine residues are critical for the posttranslational modification of proteins through conjugation to acetyl (acetylation), ubiquitin (ubiquitination), small ubiquitin-like modifier (SUMO; SUMOylation), Nedd8 (NEDDylation), and interferon-stimulated gene-15 (ISG15; ISGylation; Chen and Gerlier, 2006; Hemelaar et al., 2004). Thus, we investigated whether the K6 residue of YFV NS5 was posttranslationally modified and if that modification was required for STAT2 binding. The 293T cells were cotransfected with empty vector, MODV-YFV NS5, YFV NS5, TRIM25, or TRIM19 along with UB, ISG15, or SUMO (Figure S5). TRIM25 serves as a positive control for ubiquitination and ISGylation (Gack et al., 2007), whereas TRIM19 serves as a positive control for SUMOylation (El Bougrini et al., 2011). Coimmunoprecipitation assays demonstrated that YFV NS5 was possibly ubiquitinated, but not ISGylated or SUMOylated (Figure S5). An increase in ubiquitination of YFV NS5 was also observed upon the addition of IFN (compare lanes 3 and 7 of Figure S5A). Immunoprecipitation experiments further demonstrated that a lysine in the first ten aa of the protein was required for the interaction of YFV NS5 and STAT2 as well as the association of YFV NS5 with ubiquitin (Figure 5A).

Our results suggested direct ubiquitination of YFV NS5 K6, although K6 could also be required for NS5 to associate with unanchored polyubiquitin chains or an ubiquitinated protein. When immunoprecipitated YFV NS5 was washed in a high salt buffer, to disrupt NS5/STAT2 interaction, a reduction in STAT2 binding was evident but there was no change in ubiquitin binding to NS5 (Figure 5B). The strength of the association between ubiquitin and NS5 suggested covalent linkage of ubiquitin to NS5. We next assessed the effect of a deubiquitinating enzyme, OTU domain of Crimean-Congo hemorrhagic fever virus L protein, L(1-169; Frias-Staheli et al., 2007) on the NS5-STAT2 interaction. Incubation of NS5 with OTU decreased the NS5-STAT2 interaction (Figure 5C), confirming that ubiquitination of NS5 at K6 is required to mediate the interaction with STAT2.

Given these results, we reasoned that covalently attaching an ubiquitin chain to the N terminus of YFV NS5 K6R might allow this protein to behave like wild-type NS5. We constructed clones with a linear four-ubiquitin chain fused to the N terminus of wild-type or the K6R mutant. Because cellular hydrolases normally remove ubiquitin chains by cleaving the LRGG sequence of the ubiquitin C terminus, we designed ubiquitin fusions lacking the GG portion of this sequence between ubiquitins and between ubiquitin and NS5 (Figure 5D). Coimmunoprecipitation assays (Figure 5E) and reporter gene assays (Figure 5F) showed that the YFV NS5 K6R regained the ability to bind STAT2 and inhibit IFN-I signaling when fused to linear ubiquitin. However, binding to STAT2 was still dependent on IFN-I treatment of cells. This may indicate that in addition to promoting YFV NS5 ubiquitination, IFN-I signaling is required for another modification that allows YFV NS5 to bind to STAT2. On the other hand, it might also be possible that YFV NS5 K6R coupled to linear ubiquitin is not competent for binding to STAT2, and that IFN-I treatment results in the formation of competent polyubiquitin chains that conjugate to the fused linear tetraubiquitin at the N terminus of YFV NS5 K6R. In any case, the functional replacement of K6 in YFV NS5 by linear ubiquitin clearly indicates that ubiquitination mediates the role of K6 in promoting STAT2 binding.

Most polyubiquitinated proteins are modified through K48 or K63 ubiquitin linkage. We examined whether the NS5-STAT2 interaction was mediated by K63- or K48-linked polyubiquitin. Upon IFN-I treatment, wild-type and K48R but not K63R ubiquitin coimmunoprecipitated with both NS5 and STAT2 (Figure 5G), indicating that K63-linked ubiquitination of YFV NS5 is necessary for the interaction.

TRIM23 Interacts with and Promotes YFV NS5 Ubiquitination

Because TRIM E3 ligases are important modulators of IFN signaling pathways, (McNab et al., 2011; Uchil et al., 2013; Versteeg et al., 2013), and IFN-I treatment increased ubiquitination of YFV NS5, we screened a panel of TRIM proteins from the different TRIM family subgroups (groups I–IX) and examined their ability to increase ubiquitination of YFV NS5. Of the TRIMs examined, expression of TRIM23 was observed to increase ubiquitination of NS5 in IFN-I-stimulated cells (data not shown). We examined the cellular distribution of TRIM23 with wild-type or K6R NS5 using confocal immunofluorescence. Overexpressed TRIM23 localized in punctate perinuclear patterns, while overexpression of NS5 resulted in a diffuse pattern predominantly in the nucleus. However, coexpression of TRIM23 and YFV NS5 resulted in relocalization of NS5 to the punctate dot-like structures

assaying for luciferase activities. Fold induction of f-luc activity was normalized to R-luc activity. The bars represent the mean fold induction of three independent experiments compared to the untreated empty vector controls.

(G) 293T cells were transfected with FLAG-tagged wild-type YFV NS5 and various HA-tagged ubiquitin mutants. At 24 hr, cells were mock stimulated or stimulated with IFN-β for 45 min. Cells were lysed and immunoprecipitation was performed against the HA epitope followed by WB with the indicated antibodies.

Statistical analyses were conducted using the unpaired t test in Prism 4 (GraphPad Software). ns, no significance, where $p > 0.05$; ***$p < 0.0001$.

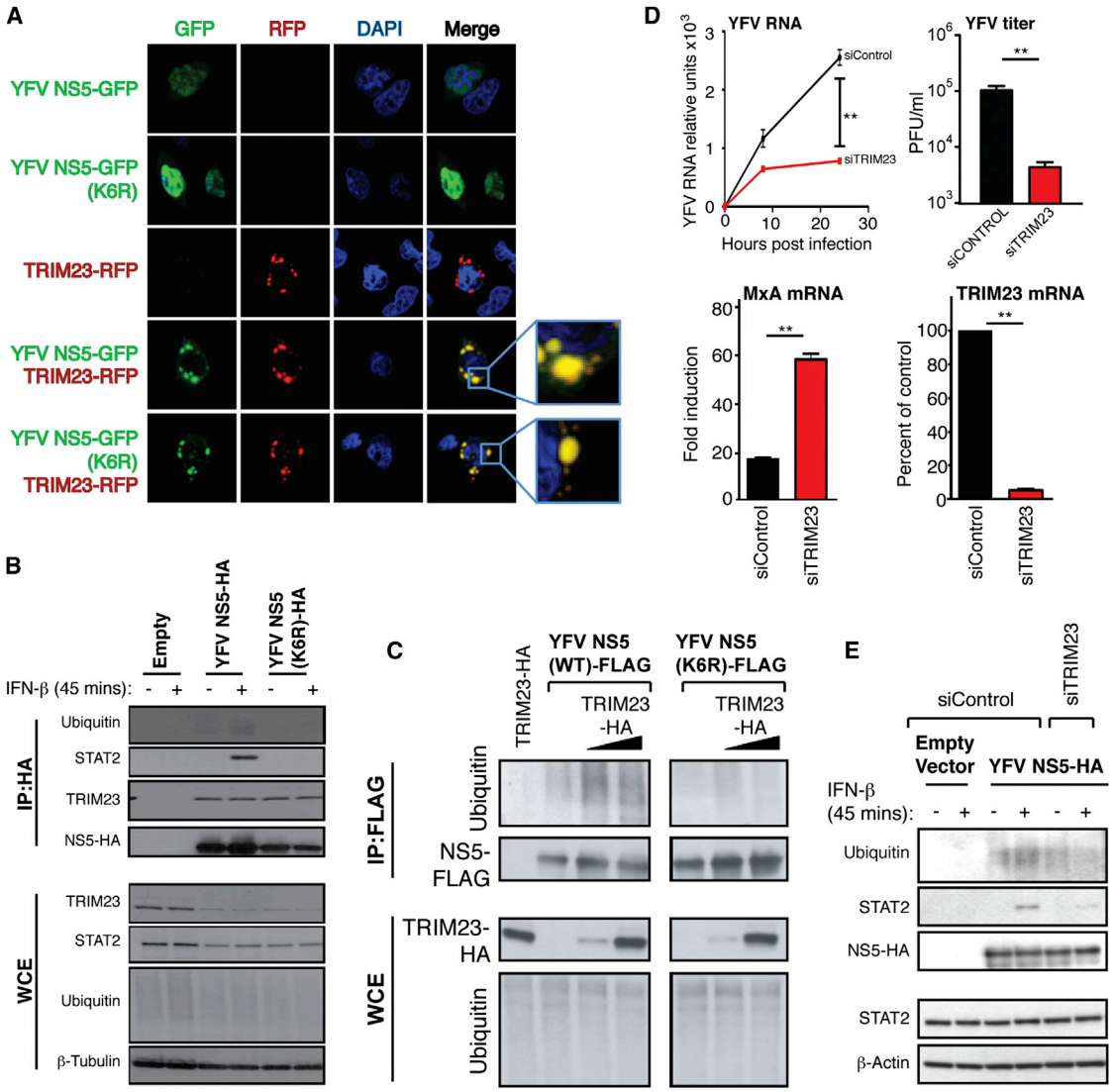

Figure 6. TRIM23 Ubiquitinates YFV NS5

(A) Confocal microscopy images showing subcellular localization of the indicated proteins in HeLa cells before or after treatment with IFN-β for 30 min.

(B) 293T cells were transfected with YFV NS5. At 24 hr, cells were mock stimulated or stimulated with IFN-β then lysed followed by immunoprecipitation. Western blot analysis (WB) was done with the indicated antibodies.

(C) Ubiquitination assay conducted in 293T cells with increasing amounts of TRIM23. Cells were transfected with FLAG-tagged wild-type YFV NS5 or YFV NS5 K6R with increasing amounts of HA-tagged TRIM23. Lysis and immunoprecipitation against the FLAG epitope was followed by western blot analysis with the indicated antibodies.

(D) siRNA-expressing A549 cells were infected with YFV-17D WT at an moi of 10. At 8 hpi, the cells were mock-treated or treated with 100 U/ml IFN-β. Virus was harvested at 24 hr and viral RNA, TRIM23 and MxA RNA quantified by qPCR. Viral titers were quantified by plaque assay on BHK-21 cells.

(E) siRNA-expressing 293T cells were transfected with empty plasmid or YFV NS5 and mock treated or treated with IFN-I for 45 min. Lysis and immunoprecipitation were followed by WB with the indicated antibodies. Statistical analyses were conducted using the unpaired t test in Prism 4 (GraphPad Software, USA). ns = no significance, where $p > 0.05$; *** = $p < 0.0001$; ** = $p < 0.001$.

containing TRIM23 (Figure 6A). NS5 did not colocalize or coimmunoprecipitate with the other TRIMs tested (Figures S6A and S6B). In addition, NS5 precipitated endogenous TRIM23 (Figure 6B). Association of NS5 with TRIM23 was, in contrast to association of NS5 with STAT2, independent of IFN-I treatment (Figure 6B). Confocal immunofluorescence analysis and coimmunoprecipitation assays showed that TRIM23 associated with both wild-type and K6R NS5 (Figures 6A and 6B). We also show that overexpression of TRIM23 enhances ubiquitination of WT but not mutant NS5 (Figure 6C), and that the TRIM23 RING domain is required for its interaction with NS5 (Figure S7). In addition, silencing of TRIM23 in IFN-competent cells resulted in increased susceptibility of YFV-17D to IFN-I and increased induction of the ISG, MxA, in YFV-infected cells (Figure 6D). Furthermore, silencing TRIM23 reduced NS5 ubiquitination and binding to STAT2 (Figure 6E).

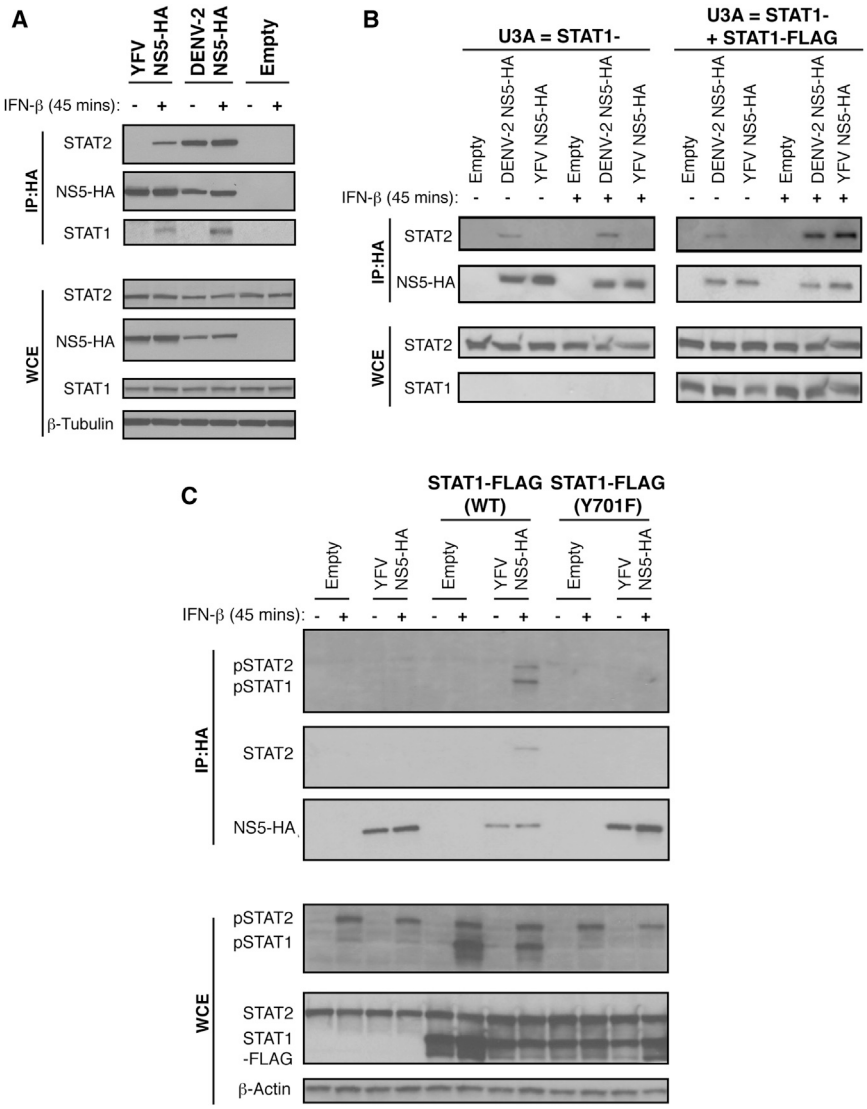

Figure 7. STAT1 Is Necessary for IFN-I-Induced YFV NS5-STAT2 Interaction

(A) 293T cells were transfected with the indicated constructs. Cells were mock stimulated or stimulated with IFN-β for 45 min at 24 hr posttransfection. Cell lysates were immunoprecipitated followed by western blot analysis with the indicated antibodies.

(B) Same experiment as in (A) done in U3A cells. Right: U3A cells were complemented with wild-type STAT1 and the same experiment was repeated.

(C) U3A cells were complemented with wild-type STAT1 or mutant STAT1 Y701F and the experimental procedure in (B) was repeated.

STAT1 Is Necessary for IFN-I-Induced YFV NS5-STAT2 Interaction

It was intriguing that STAT1 was not precipitated along with STAT2 after YFV NS5 immunoprecipitation (Figure 2D). Coimmunoprecipitation experiments using less stringent wash conditions resulted in precipitation of NS5 with both STAT2 and STAT1 (Figure 7A). These results indicate that NS5 does not compete with STAT1 for STAT2 binding after IFN-I signaling. Rather, NS5 strongly interacts with STAT2 whereas binding to STAT1 likely occurs by means of IFN-I-induced STAT1/2 dimerization. Consistent with this, coimmunoprecipitation of STAT1 with DENV-2 NS5/STAT2 complexes was also induced by IFN-I treatment.

STAT degradation by the V proteins of some paramyxoviruses requires both STAT1 and STAT2 components, even though only one of the STAT proteins is targeted for degradation (Horvath, 2004). To explore whether the IFN-I-dependent NS5-STAT2 interaction required STAT1, we examined the NS5-STAT2 interaction using U3A cells, which are STAT1-deficient (McKendry et al., 1991; Müller et al., 1993). Immunoprecipitation assays showed requirement of STAT1 for YFV NS5 to interact with STAT2 since it did not bind STAT2 in STAT1-deficient cells. However, DENV-2 NS5 bound STAT2 in STAT1-deficient cells as previously described (Ashour et al., 2010). Complementation of U3A cells with a wild-type STAT1 construct rescued the ability of YFV NS5 to interact with STAT2 after IFN-I treatment indicating that STAT1 is required for NS5-STAT2 interaction (Figure 7B). Immunoblot analysis revealed that STAT2 was phosphorylated on Y690 upon IFN-I treatment in U3A cells, which is consistent with published reports (Figure 7C; Improta et al., 1994). However, our results showed that YFV NS5 associated with STAT2 in response to IFN-I treatment when cells were reconstituted with wild-type but not mutant (Y701F) STAT1. Given that tyrosine phosphorylation induces STAT1/2 heterodimers to transform from a parallel to an antiparallel association with respect to their C- and N-termini (Lin et al., 2009), these results suggest that the NS5 binding domain is only exposed when STAT2 interacts with phosphorylated STAT1, resulting in a different STAT2 conformation. Thus, our results indicate that IFN-I promotes YFV NS5/STAT2 interaction by inducing STAT1 tyrosine phosphorylation. However, a modification in YFV NS5, i.e., ubiquitination is also required for STAT2 interaction. This modification is enhanced in the presence of IFN and this may explain why NS5 from untreated cells is unable to bind STAT2 compared to NS5 from IFN-treated cells.

DISCUSSION

The experiments described in this paper demonstrate that YFV and its NS5 protein inhibit IFN-I signaling by binding to STAT2 and preventing ISGF3 engagement with ISREs. The results also show that IFN-I triggers YFV NS5 binding to STAT2. This is a unique example of a viral IFN-I signaling antagonist that

is itself activated by IFN-I. In the absence of IFN-I treatment, NS5 is incapable of binding STAT2, but IFN-I-activated NS5 binds to both the phosphorylated and unphosphorylated forms of STAT2, indicating that the ability of IFN-I to promote NS5-STAT2 interaction is not due to IFN-I-mediated STAT2 modification. We have identified K63-linked polyubiquitination as one of the IFN-I activating modifications of NS5. In addition, IFN-I induced tyrosine phosphorylation of STAT1 is also required for NS5-STAT2 interaction, most likely by promoting conformational changes in STAT2 that trigger its association with ubiquitinated NS5.

YFV NS5 exhibits functional similarities to DENV-2 NS5; however, DENV-2 NS5 binds STAT2 regardless of IFN treatment and targets it for proteasome-mediated degradation (Ashour et al., 2009). YFV NS5, on the other hand, binds STAT2 both in the cytoplasm and nucleus only after IFN treatment, and appears to inactivate ISGF3 within the nucleus. Mapping studies identified the first ten residues of the YFV NS5 protein as essential for STAT2 interaction and IFN signaling inhibition. The first ten aa of DENV-2 NS5 are critical for mediating proteasomal degradation of STAT2 although they are dispensable for STAT2 association (Ashour et al., 2009). Therefore, the extreme N termini of both YFV NS5 and DENV-2 NS5 contain motifs required for their IFN antagonist function. Remarkably, the two N-termini initiate entirely different mechanisms that ultimately converge on STAT2 inhibition. A glycine residue and a threonine residue within the N terminus of DENV NS5 are required for DENV NS5 to bind UBR4 (a potential E3 ligase) and mediate STAT2 degradation (Morrison et al., 2013). In contrast, other E3 ligases, such as TRIM23, polyubiquitinate K6 of YFV NS5, and this, together with STAT1 tyrosine phosphorylation is required for YFV NS5/STAT2 association and inhibition of ISGF3 engagement with the ISRE.

K6 of YFV NS5 is critical for NS5-STAT2 interaction and IFN-I signaling inhibition. When a K6R mutation was placed in the context of YFV-17D, the virus displayed an enhanced sensitivity to the antiviral action of IFN-I, indicating that K6 is important for viral replication in the context of an ongoing IFN-I response. Because no replication defect was seen for the K6R mutant virus in mock-treated Vero cells, it appears that K6 may only be important for IFN antagonism. YFV-17D causes neurotropic and viscerotropic disease in immune-compromised individuals (Barrett et al., 2007; Biscayart et al., 2014; Breugelmans et al., 2013; Monath et al., 2005). Decreasing the ability of YFV-17D to antagonize IFN-I signaling may limit the replication of this vaccine in immunocompromised individuals, thereby decreasing the incidence of vaccine-associated adverse events. An YFV vaccine that is more attenuated than, but equally or more immunogenic to, the current YFV-17D vaccine would be an attractive alternative for YF vaccination.

These data show that K63-linked polyubiquitination of NS5 is required for STAT2 binding and IFN-I signaling inhibition. We have shown that a linear four-ubiquitin chain fused to the N terminus of YFV NS5 K6R binds STAT2 upon stimulation with IFN-I. A possible explanation for these results is that the fused linear chain serves as a K63-linked ubiquitin chain mimic because the structures of K63-linked and linear ubiquitin are more similar to each other than they are to the structure of K48-linked ubiquitin (Tse et al., 2011). However, we cannot rule out the possibility that the four-ubiquitin chain acts as a new scaffold for additional K63 polyubiquitination, which in turns activates NS5 binding to STAT2. It is interesting that the only requirement in the first ten aa of NS5 to bind to STAT2 and inhibit IFN signaling was the presence of a K, because all these residues could be substituted by a K without loss of function. We postulate that binding of an E3 ligase (such as TRIM23) to YFV NS5 requires a domain outside of the first ten residues, and this brings the required E2 protein into close contact with the N terminus of NS5, resulting in polyubiquitination irrespective of the precise K location within the N terminus.

We have also identified TRIM23, an E3 ligase involved in innate immune signaling (Arimoto et al., 2010; Versteeg et al., 2013), as an YFV NS5 interacting protein. TRIM23 has been shown to promote IFN-β production by ubiquitinating NEMO (Arimoto et al., 2010). Taylor and colleagues recently demonstrated that TRIM79α restricts LGTV/TBEV replication by interacting with NS5 (Taylor et al., 2011). In contrast, we show that TRIM23 promotes YFV replication by polyubiquitinating YFV NS5 at its K6 residue, a modification that is required for YFV NS5-STAT2 binding. Of note, our experiments do not rule out the possible contribution of other E3 ligases in ubiquitinating YFV NS5, because there might be redundancy in their function (Morishima et al., 2008). Nevertheless, silencing of TRIM23 results in decreased ability of YFV to inhibit IFN-induction of MxA and increased sensitivity of YFV to IFN-I, supporting a prominent role of TRIM23 in YFV-mediated inhibition of IFN signaling.

Despite the high level of amino acid conservation among flavivirus NS5 proteins, IFN antagonistic functions appear to be mediated by different mechanisms. This would suggest that IFN signaling inhibition evolved independently among flaviviruses, but that the NS5 protein acquired this function in all cases. It might be that the polyprotein-based strategy of viral protein expression that is common to all flaviviruses results in excess production of NS5, because only catalytic amounts of this protein are required for RNA synthesis, making it an ideal candidate for acquisition of additional functions.

EXPERIMENTAL PROCEDURES

Cells and Viruses

The 293T, Vero, and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. 2fTGH and 2fTGH derivatives were kindly provided by Dr. George Stark and were described previously (Leung et al., 1995). A previously described Newcastle disease virus expressing GFP (NDV-GFP) was grown in 10-day-old embryonated chicken eggs (Park et al., 2003). High titer stocks of DENV-2 16681 were obtained by passage in C6/36 cells (Diamond et al., 2000). High titer stocks of YFV-17D strain were obtained by passage in BHK-21 cells. The YFV-17D K6R mutant virus was made by PCR mutagenesis (primer sequences are available upon request) of a YFV-17D cDNA clone, pACNR-YF17D, kindly provided by Dr. Charles Rice (Bredenbeek et al., 2003; Lindenbach and Rice, 1999). The mutant virus and wild-type control virus were rescued by linearizing the cDNA clone with XhoI, and using the SP6 Cap-Scribe kit (Roche) to generate RNA. mRNA was transfected into BHK-21 cells using the Transit mRNA transfection kit (Mirus Bio), and virus was harvested 3 days later.

Plasmids

All flavivirus genes were cloned in the mammalian expression vector, pCAGGS. Primer sequences used in the generation of the constructs are available upon request. HA-NiV V plasmid was a gift from Dr. Megan Shaw. pCAGGS-Firefly luciferase and ISRE-54-CAT reporter plasmids were gifts

from Dr. Luis Martinez-Sobrido. HA-ubiquitin expressing plasmids were gifts from by Dr. Gijs Versteeg.

Virus Infections

Monolayers of cells were initially adsorbed with YFV-17D at the indicated multiplicity of infection (moi) for 1 hr at room temperature. Unbound virus was removed, and cells were maintained in Dulbecco's modified Eagle's medium 10% fetal bovine serum at 37°C. For the YFV-17D K6R versus YFV-17D growth curves, Vero cells were incubated with virus at an moi of 10 for 1 hr at 37°C. Viral replication was measured by plaque assay on BHK-21 cells.

Transfections

Lipofectamine 2000 (Invitrogen) was used for all transfections. The 293T cells were transfected using a 1:1 ratio of plasmid DNA to Lipofectamine 2000. Vero cells were transfected using a 1:2 ratio. 2fTGH and 2fTGH derivatives were transfected using a 1:3 ratio.

Antibodies and Cytokines

The following antibodies were used in this study: TRIM23 monoclonal antibody (M01; Abnova), rabbit polyclonal anti-STAT1 (BD Biosciences), anti-STAT2 and anti-YFV E (Santa Cruz Biotechnology), antiphospho-STAT1 (Tyr 701; Cell Signaling Technology), antiphospho-STAT2 (Tyr 689; Upstate Biotechnology), anti-HA, anti-FLAG, anti-GFP, antitubulin and anti-actin (Sigma-Aldrich), anti-ubiquitin (Enzo Life Sciences), anti-PKR (Abcam), anti-MDA5 (Enzo AT113), and anti-DENV-2 E (Hybridoma Facility of Icahn School of Medicine at Mount Sinai). Rabbit antibody against DENV-2 NS5 was generated in our lab and previously reported (Ashour et al., 2009). Anti-YFV NS5 antibody (YF17D NS5 C7) was kindly provided by Dr. Charles Rice (Chambers et al., 1990b). Universal IFN-I, human IFN-β, human IFN-λ, and human IFN-γ (PBL Interferon Source) were used at 1,000 U/ml unless otherwise specified.

Interferon Signaling Inhibition Assays and Antibody-Based techniques

Reporter assay, NDV GFP assay, immunoprecipitation and immunoblot assay, immunoflourescence, and subcellular fractionation assays are described in the Supplemental Experimental Procedures.

Quantitative RT-PCR

Total RNA, isolated from cells using RNeasy (QIAGEN), was subjected to DNase digestion with Turbo DNase (Ambion). Reverse transcription was performed with a cDNA reverse transcription kit (Applied Biosystems). qPCR was done in triplicates using SYBR green I master mix (Roche) in a Roche LightCycler 480. Relative mRNA values were calculated using ddCt method with 18S as an internal control and shown as fold change by normalizing to mock-control. The primers were YFV NS5 forward: GAGCTCATTGGGAGAG GAAG; reverse: AGGCAAGCTGTTTCCTTGAT; all other primers used were published previously (Rajsbaum et al., 2014).

siRNA Knockdown

TRIM23 siRNA-mediated knockdown experiments are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.07.015.

AUTHOR CONTRIBUTIONS

J.M. and M.L.-R. performed all aspects of this study. R.R., J.M.L.M., G.P., A.P., J.A., L.M., and C.M.-R. performed experiments. B.R.T. provided reagents and advice. M.L.-R., J.M., and A.G.-S. organized the study and prepared the manuscript. All authors discussed the results and commented on the manuscript.

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