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TRIM79α, an interferon-stimulated gene product, restricts tick-borne encephalitis virus replication by degrading the viral RNA polymerase

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Abstract

In response to virus infection, type I interferons (IFNs) induce several genes, most of whose functions are largely unknown. Here we show that the tripartite motif (TRIM) protein, TRIM79α, is an IFN-stimulated gene (ISG) product that specifically targets tick-borne encephalitis virus (TBEV), a *Flavivirus* that causes encephalitides in humans. TRIM79α restricts TBEV replication by mediating lysosome-dependent degradation of the flavivirus NS5 protein, an RNA-dependent RNA polymerase essential for virus replication. NS5 degradation was specific to tick-borne flaviviruses as TRIM79α did not recognize NS5 from West Nile virus (WNV) or inhibit WNV replication. In the absence of TRIM79α, IFN-β was less effective in inhibiting tick-borne flavivirus infection of mouse macrophages, highlighting the importance of a single virus-specific ISG in establishing an antiviral state. The specificity of TRIM79α for TBEV reveals a remarkable ability of the innate IFN response to discriminate between closely related flaviviruses.

INTRODUCTION

Flaviviruses have an essentially global distribution and represent a tremendous disease burden to humans, causing millions of infections annually. Significant members of this group include dengue virus (DENV) and yellow fever virus (YFV) that cause hemorrhagic fevers, as well as tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) that cause severe encephalitides (Gould and Solomon, 2008; Robertson et al., 2009). Of significant threat to public health, flaviviruses routinely emerge beyond their known geographical range, including the spread of DENV and WNV in the Americas and the increased recognition of various members of the TBEV serocomplex throughout Europe, Asia and North America (Ebel, 2010; Mackenzie et al., 2004).

The flavivirus single-stranded RNA genome is translated as one open reading frame; the resulting polyprotein is cleaved into at least ten proteins that include three structural (capsid

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[C], membrane [M] and envelope [E]), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Virus replication proceeds in association with modified membranes derived from the endoplasmic reticulum (ER) of host cells (Fernandez-Garcia et al., 2009). NS5 is the largest and most conserved of the flavivirus proteins containing approximately 900 amino acids. It encodes a methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) and associates with NS3 (the viral protease) to form the functional unit of the viral replication complex (RC) (Davidson, 2009; Kapoor et al., 1995).

In addition to its central role in RNA replication, NS5 is also the most potent interferon (IFN) antagonist encoded by the flaviviruses. NS5 inhibits IFN-α/β-dependent responses by preventing JAK-STAT signaling and thus suppressing IFN-stimulated gene (ISG) expression (Ashour et al., 2009; Best et al., 2005; Laurent-Rolle et al., 2010; Lin et al., 2006; Mazzon et al., 2009; Werme et al., 2008). Additionally, flaviviruses use NS5 to impede ISG function by 2'O methylation of the viral mRNA cap. This disguises viral RNA from recognition by the IFIT family of proteins (Daffis et al., 2010). Despite efficient antagonism of IFN responses by NS5 and other flavivirus proteins, type I IFN is effective in preventing flavivirus replication and in restricting tissue tropism and mortality in mouse models of infection (Diamond, 2009). However, the molecular mechanisms by which IFN and ISG expression suppress flavivirus replication are incompletely understood.

Members of the tripartite motif (TRIM) family of proteins are increasingly recognized as ISGs that mediate antiviral responses (Nisole et al., 2005). TRIM proteins contain at least three distinct domains, an N-terminal RING domain, one or two B-boxes and a central coiled-coil domain. In addition, the C-terminus of TRIM proteins often contains a B30.2/SPRY domain that mediates specific protein-protein interactions, although not all TRIM proteins contain this domain (Ozato et al., 2008). An example of the highly specific antiviral nature of TRIM proteins can be observed in the case of TRIM5α restriction of retrovirus replication (Towers, 2007). Old World monkeys (OWM) are not susceptible to productive infection with human immunodeficiency virus (HIV)-1. TRIM5α proteins from OWM bind and degrade incoming HIV capsids thereby accelerating uncoating and compromising virus infectivity (Stremlau et al., 2004; Stremlau et al., 2006). However, restriction of HIV replication by human TRIM5α is weak, likely contributing to human susceptibility to infection. Species-specific restriction of retrovirus replication is determined by amino acid differences in the SPRY domain of different TRIM5α molecules; amino acid divergence in viral capsid proteins determines viral sensitivity to restriction (Johnson and Sawyer, 2009; Sawyer et al., 2005). Thus, co-evolution of TRIM proteins and viruses can influence host tropism and virus pathogenesis.

The current study identifies a TRIM protein as an IFN-inducible flavivirus restriction factor. This protein, denoted TRIM79α (also known as TRIM30-3 or TRIM30D), interacted with NS5 from Langat virus (LGTV; a member of the TBEV serogroup) and TBEV, and suppressed the replication of these viruses. However, TRIM79α did not interact with WNV NS5 nor could it restrict WNV replication, indicating a high degree of specificity. The molecular mechanism of restriction was the direct targeting of NS5 for degradation by lysosomes. Importantly, the antiviral effects of IFN-β on tick-borne flavivirus replication were ameliorated by suppressing TRIM79α expression. Thus, TRIM79α is an essential mediator of the IFN response specific to TBEV infection, through targeted destruction of the viral RNA polymerase and major IFN antagonist.

EXPERIMENTAL PROCEDURES

Cell culture and reagents

HEK293 (293), L929, Vero and RAW 264.7 (RAW) cells (ATCC) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) in an atmosphere of 5% CO_2 at 37°C. Cell culture grade MG132, ammonium chloride (NH_4Cl), 3-methyladenine (3-MA), N-ethylmaleimide (NEM), puromycin, G418, polybrene, cycloheximide (CHX) and dimethyl sulfoxide (DMSO) were purchased from Sigma. Murine IFN-β and granulocyte macrophage-colony stimulating factor (GM-CSF) were obtained from R&D Systems. The generation and culture of mouse bone marrow derived dendritic cells (DCs) and mouse embryo fibroblasts (MEFs) is described in Supplemental Experimental Procedures.

Antibodies

The following antibodies were used: α-actin (A5441, Sigma); α-GFP (JL-8, Clontech); α-dsRed (632496, Clontech); α-V5 (R960-25, Invitrogen); α– HA (16B12, Covance); α-lysosome associated membrane protein 1 (LAMP1) (ab24170, Abcam); α-TBEV (RSSE VR79; ATCC); LGTV E (from Dr. C. Schmaljohn, USAMRIID); WNV E (from Dr. R. Tesh, World Reference Center for Emerging Viruses and Arboviruses [WRCEVA], UTMB); affinity purified chicken antibodies specific for LGTV NS5 peptides (sequence: CZ DRHDLHWELKLESSIF), NS3 (sequence: CZRDIREFVSYASGRR) and control IgY antisera (custom prepared by Aves Labs).

Virus infections

The following viruses were used in this study: LGTV strain TP21 (Dr. A. Pletnev, NIAID, NIH),TBEV strain Sofjin (also known as Russian spring summer encephalitis (RSSE) virus; from Dr. M. Holbrook, NIAID, NIH), Powassan virus (POWV) and WNV strain NY99 (from the WRCEVA); Sendai virus (SeV, Cantell strain; Charles River Laboratories). Flavivirus working stocks were propagated and titrated by immunofocus assay on Vero cells. Multiplicity of infection (MOI) for wild-type or equivalent UV-irradiated (360 mJ/cm^2 in a Stratalinker) flavivirus infections is represented as focus forming units (FFU) per cell. All procedures with WNV and POWV were performed under biosafety level-3 (BSL-3) conditions; procedures with TBEV were performed under BSL-4 conditions at the Rocky Mountain Laboratories Integrated Research Facility (Hamilton, MT). Lentivirus generation for gene knock-down studies is described in Supplemental Experimental Procedures.

Plasmids and transfections

TRIM79α (10470120) and TRIM30α (MGC-6159) cDNA clones were obtained from ATCC. LGTV NS2B/3 was derived from PCR amplification using the LGTV E5 molecular cDNA clone as template (from Dr. A. Pletnev, NIAID, NIH). Each gene was PCR amplified and directionally cloned into the Gateway entry vector pENTR/SD/D-TOPO (Invitrogen). Entry vectors derived from LGTV, WNV (strain NY99), JEV (strain Nakayama), and TBEV (strain Hypr) NS5 were previously described (Laurent-Rolle et al., 2010). Mammalian expression plasmids were then obtained by recombination into various Gateway destination vectors: pcDNA6.2/V5-DEST (C-terminal V5 tag); pcDNA-3.2/capTEV-NT/V5-DEST (N-terminal V5/AP tag); pcDNA-3.2/capTEV-CT/V5-DEST (C-terminal V5/AP tag); pDS_GFP-XB (N-terminal GFP tag, ATCC); pcDNA6.2-mCherry-C-DEST (C-terminal mCherry tag, from Dr. S. Grieshaber, University of Florida). Plasmids used in this study express N-terminal tagged TRIM proteins and C-terminal tagged NS5 and NS2B/3 proteins. Site-directed mutagenesis was performed using QuikChange XL kit (Stratagene) according

to manufacturer's protocol. All plasmids and mutations were verified by DNA sequencing. Additional plasmids used were: pRK5-HA-ubiquitin (Ub)-WT and K0 (Addgene plasmids 17608 and 17603; from Dr. T. Dawson, Johns Hopkins University) and pcDNA3-HA-SUMO1 (small Ub-like modifier 1; from Dr. M. White, UT Southwestern). Cell transfections were performed according to manufacturer's protocol in six-well plates or 8 well Lab Tek II chamber slides (Nunc) using Lipofectamine LTX and OptiMEM (Invitrogen) and allowed to recover at least 24 h prior to assay. Stable 293 cell lines were selected 24 h post transfection with G418 (400 μg/ml). Selected cell pools were serially diluted and stable clones were identified by western blot and RT-qPCR described in Supplemental Experimental Procedures.

Co-immunoprecipitation (Co-IP) and co-affinity purification (Co-AP)

293 cells were washed twice with DPBS and lysed by three freeze-thaw cycles in immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate) with protease inhibitor cocktail (Roche). Samples were subjected to centrifugation for 10 min to remove cellular debris. Cell lysates were then cleared by addition of protein G conjugated agarose beads (Roche) or PrecipHen for chicken antibodies (Aves) and rotated at 4°C for 3 h. Beads were removed by centrifugation, and antibody (2 μg) corresponding to the protein of interest was added to each lysate for 1 h with rotation at 4°C. Protein G-agarose or PrecipHen beads were again added, and lysates were incubated with rotation at 4°C overnight. Lysates were discarded after a brief centrifugation, and beads were washed twice with IP buffer and twice with RIPA buffer prior to elution by incubation at 95°C in 1X sample buffer (62.5 mM TRIS pH 6.8, 10% glycerol, 15 mM EDTA, 4% 2-ME, 2% SDS and bromophenol blue). Co-affinity purifications (Co-AP) were performed as for Co-IPs with the following exceptions. The expression plasmid (pcDNA-3.2/capTEV-NT or CT/V5-DEST) contains a V5 tag and also enables target protein biotinylation by the eukaryotic cellular machinery during expression, referred to as V5/AP tag. Samples were harvested as with Co-IP in IP buffer, precleared with unconjugated agarose (Sigma) and incubated with streptavidin-agarose (Invitrogen) overnight with rotation at 4°C. Washes were identical to Co-IP. Cell lysates were eluted by heating at 95°C for 5 min in 2X sample buffer. Co-IP and Co-AP assays were evaluated by western blotting.

Ubiquitination assays

Ubiquitination assays were modified from the Co-AP by the addition of NEM to lysis buffer to prevent deubiquitinase (DUB) activity and heating samples at 95°C for 5 min prior to affinity purification in 1% SDS to remove interacting proteins. HA-tagged Ub or SUMO1 plasmids were also co-transfected to enable efficient detection of modified proteins. Following co-AP, Ub-modified proteins were examined by western blotting.

Antibody based techniques

Immunofocus assays, ELISA and immunofluorescent confocal microscopy are described in Supplemental Experimental Procedures.

Statistical analysis

Data were analyzed by a one-tailed unpaired *t* test or Mann-Whitney U test using GraphPad Prism 5 software.

RESULTS

TRIM79α is an ISG expressed during virus infection

The flavivirus NS5 protein is essential for virus replication, but little is known about its molecular interactions with host proteins involved in normal cellular function. Thus, we employed a yeast two-hybrid analysis (Myriad Genetics, Salt Lake City, UT) to identify potential cellular binding partners for NS5. Using various baits derived from LGTV NS5, we found a potential interaction between amino acids 1–248 or 40–260 of the LGTV NS5 N-terminus and a putative mouse protein AI451617 (amino acids 95–498) from a mouse macrophage library (Figure 1A). Sequence analysis by BLAST and PatternProt revealed the protein contained RING, B-box, coiled-coil (CC) and SPRY domains and therefore belonged to the TRIM family and was designated TRIM79α, with α denoting the full-length isoform. To examine tissue distribution *in vivo*, we looked for TRIM79α mRNA by RT-qPCR in C57BL/6 mouse organs. Compared to TRIM79α mRNA levels in the skin, TRIM79α mRNA was enriched in organs involved in immune regulation, including spleen, lymph node and bone marrow, and was detectable in lung and liver (Figure 1B). This is reminiscent of the tissue distribution of TRIM30α, the murine TRIM closest to TRIM79α (Shi et al., 2008).

Many TRIM proteins are expressed in response to IFN or virus infection (Rajsbaum et al., 2008). Consistent with these observations, the TRIM79α promoter contains putative binding sites for transcription factors involved in immune responses including nuclear factor kappa B (NFκB), STAT1 and IFN regulatory factors (IRFs) (Figure 1A-iii). Thus, since we have been unsuccessful in raising TRIM79α-specific antisera, we determined TRIM79α expression in various murine cell types in response to IFN-β treatment, as well as during a productive LGTV or SeV infection by RT-qPCR. TRIM79α mRNA transcription was detected by 4 h post stimulation with 100 international units (IU)/ml IFN-β in mouse macrophage RAW cells (Figure 1C). Similar results were obtained in various mouse cells including primary DCs, L929 cells and primary MEFs (Figure S1). TRIM79α transcriptional induction was dependent on LGTV replication in all cells tested because UV-irradiated, replication-incompetent virus failed to generate a TRIM79α transcriptional response (Figure 1C and Figure S1). Additionally, TRIM79α transcription in response to LGTV infection relied upon IFN-dependent signaling, as DCs lacking the IFN-α/β receptor (IFNAR −/−) were virtually devoid of a TRIM79α response (Figure 1D), despite exhibiting higher levels of LGTV replication (data not shown). Lastly, SeV, a potent IFN inducer via IFN-β promoter stimulator-1 (IPS-1) (Kumar et al., 2006), induced TRIM79α transcription in L929 and RAW cells, confirming that a non-flavivirus infection also invokes TRIM79α expression (Figure 1E). Collectively, these data demonstrate that TRIM79α is an immune-related gene product that is upregulated by type I IFN and virus infection.

TRIM79α interacts with LGTV NS5

To confirm the interaction between LGTV NS5 and TRIM79α, we first examined the cellular distribution of TRIM79α expressed alone or with various LGTV proteins by confocal microscopy. TRIM79α-GFP was distributed predominantly in distinct cytoplasmic bodies as well as more diffusely in the cytoplasm (Figure 2A). Coexpression of TRIM79α with LGTV NS5 lead to a redistribution of NS5 from predominantly diffuse cytoplasmic localization to punctate sites containing TRIM79α (Figure 2A). This colocalization of TRIM79α with NS5 was specific, as other viral proteins tested, including LGTV C and NS4A, did not colocalize with TRIM79α (Figure 2A). To confirm a physical interaction between NS5 and TRIM79α, we performed co-IP analyses following co-transfection of TRIM79α-GFP and NS5-V5 expression plasmids. IP of NS5 with α-V5 antibody successfully co-precipitated TRIM79α but not the closely related TRIM30α (Figure 2B).

Likewise, the reciprocal experiment using α-GFP antibody specifically co-immunoprecipitated NS5 with TRIM79α, but not with TRIM30α (Figure 2B). To demonstrate this interaction during LGTV replication, 293 cells were transfected with either GFP or TRIM79α-GFP plasmids, infected with LGTV (MOI 5) and assayed by co-IP using control or NS5-specific IgY. TRIM79α co-immunoprecipitated with NS5 from LGTV infected samples using NS5-specific antibody but not with the control IgY (Figure 2C). Thus, TRIM79α can bind both ectopic and endogenously expressed LGTV NS5 protein.

TRIM79α protein turnover is regulated by proteasomal degradation

To understand the impact of TRIM79α on virus replication, we first examined the normal processing of TRIM79α. 293 cells expressing TRIM79α-GFP or GFP alone were treated with CHX to inhibit new protein synthesis (Figure 3A). Levels of TRIM79α were normalized to β-actin and quantitated following western blotting. TRIM79α had a rapid half life between 1.5–2h (Figure 3A), similar to that reported for other TRIM family members such as TRIM5α (Diaz-Griffero et al., 2006). To identify whether TRIM79α turnover was Ub-mediated, TRIM79α-V5/AP was co-expressed with either HA-Ub or the related HA-SUMO1. Cells were then treated with vehicle control (DMSO) or proteasome inhibitor MG132 for 4 h and modified TRIM79α was assessed using the ubiquitination assay. TRIM79α was conjugated to Ub, but not to SUMO1, and TRIM79α-Ub expression was stabilized by treatment with MG132 (Figure 3B). Interestingly, SUMO1 expression resulted in reduced TRIM79α levels in cell lysates, a phenomenon that was inhibited by MG132, suggesting some turnover of TRIM79α may be regulated by SUMOylation. However, there was no evidence that this was due to direct SUMO1 modification of TRIM79α (Figure 3B). Thus, normal turnover of TRIM79α is mediated by proteasomal degradation, an event that is most likely dependent on TRIM79α conjugation to Ub.

TRIM79α expression results in proteasome-independent degradation of NS5

To identify the consequence of NS5 interactions with TRIM79α, the relative stability of NS5 was determined in the presence of TRIM79α. Since TRIM79α is a rodent-specific TRIM protein not expressed in human cells, 293 cells were used to assay effects of TRIM79α in the absence of other mouse-specific proteins. Increasing TRIM79α expression relative to NS5 resulted in a dose-dependent decrease in NS5 levels (Figure 4A). However, increasing NS5 levels in the presence of constant TRIM79α expression did not markedly affect TRIM79α stability (Figure 4A), suggesting that TRIM79α facilitates the degradation of NS5.

To determine the degradation pathway used by TRIM79α, 293 cells expressing TRIM79α-GFP and NS5 were treated with DMSO, MG132, NH_4Cl (an inhibitor of lysosome acidification) or 3-MA (an autophagy inhibitor). Surprisingly, we did not observe any rescue of NS5 with MG132 treatment, whereas NH_4Cl restored NS5 to control levels (Figure 4B) suggesting a role for lysosomes. Autophagy is associated with lysosomal degradation and can also be inhibited by NH_4Cl. However, despite effective inhibition of lithium-induced autophagosome formation (data not shown), 3-MA led to a minimal rescue of NS5 degradation (Figure S2A) suggesting that autophagy is not the primary degradative pathway used by TRIM79α.

Due to the established role of the proteasome in normal turnover of TRIM79α (Figure 3B), it was necessary to further evaluate the Ub-proteasome system in NS5 degradation. Loss of NS5 through this mechanism would necessitate increased NS5 ubiquitination by TRIM79α. However, examination of NS5 ubiquitination demonstrated the exact opposite; ubiquitinated NS5 stabilized by MG132 was lost in the presence of TRIM79α (Figure 4C). Furthermore, expression of K0 Ub, which lacks all seven lysine residues making it incapable of chain

formation required for proteasome degradation (Lim et al., 2005), enhanced TRIM79α protein levels without rescuing NS5 (Figure 4D). Finally, mutation of the TRIM79α RING catalytic active site (C15,18A) did not prevent TRIM79α interaction with NS5 (Figure S2B) or NS5 degradation (Figure S2C). Taken together, these results strongly suggest that neither the proteasome nor ubiquitination of NS5 by TRIM79α is required for NS5 degradation.

To confirm a role for the lysosome in NS5 degradation, confocal microscopy was used to examine the localization of TRIM79α/NS5 aggregates. Compared to cells expressing either protein alone, LAMP1 positive lysosomes appeared to increase in prevalence and colocalize with NS5 and TRIM79α when these two proteins were co-expressed (Figure 4E). Colocalization of NS5 and TRIM79α was not inhibited by treatment with DMSO, MG132 or 3-MA. However, consistent with the requirement for lysosomes, NH_4Cl treatment reduced NS5 colocalization with TRIM79α at these sites (Figure S2D).

Lysosomes efficiently degrade large multi-protein complexes. Hence, recruitment of NS5 to the lysosome may facilitate degradation of proteins that interact with NS5. Thus stability of the NS3 protease with associated cofactor NS2B was examined in the presence of TRIM79α. NS2B/3 protein levels were slightly reduced in TRIM79α expressing cells relative to control cells. However, expression of NS5 in addition to TRIM79α resulted in a pronounced loss of NS2B/3 (Figure 4F). TRIM79α protein levels were also reduced following co-expression with both NS2B/3 and NS5, which was not observed with NS5 alone. Finally, a complex containing TRIM79α, NS5 and NS3 was confirmed during virus replication (Figure 4G). Taken together, these data demonstrate that TRIM79α facilitates proteasome-independent, lysosome-mediated degradation of viral RCs through specific interaction with NS5.

TRIM79α expression restricts LGTV replication

Flaviviruses are dependent upon NS5 for critical functions during virus replication (MTase, RdRP), as well as for its ability to interfere with the host IFN response. Degradation of NS5 may therefore impact viral replication. To assay the effect of TRIM79α expression on virus replication, we developed 293 clonal cell populations that constitutively express either GFP or the TRIM79α-GFP fusion protein. These cells were infected with LGTV for 24 h and viral protein expression was assessed by confocal microscopy. We observed a striking reduction in virus infected cells in TRIM79α expressing 293 cells compared to control cells (Figure 5A). Additionally, abundance of all viral proteins, including NS5, NS3 and E was lower in 293 cells expressing TRIM79α (Figure 5B). Single (MOI 10) or multi-step (MOI 0.1, data not shown) growth curve analyses of LGTV demonstrated that virus production was reduced in TRIM79α-expressing cells by 60 to 90% over 72 h of infection (Figure 5C, solid lines). This restriction was not dependent upon IFN expression as higher IFN-β protein levels were detected in supernatants from control cells relative to TRIM79α expressing cells (Figure 5C, dotted lines).

To confirm that the mechanism of NS5 degradation during LGTV replication was consistent with ectopic expression experiments, 293/TRIM79α or GFP cells were infected with LGTV followed by replacement of the inoculum with media containing DMSO, MG132, lactacystin (a proteasome inhibitor), NH_4Cl or 3-MA at 2 hpi. Only treatment with NH_4Cl prevented much of the loss of NS5 observed in TRIM79α cells at 48 hpi and relieved TRIM79α-mediated restriction of LGTV replication (Figure 5D). These data confirm that TRIM79α is an antiviral factor that inhibits virus replication by lysosomal targeting of the viral polymerase NS5.

TRIM79α is a restriction factor specific for the tick-borne flaviviruses

TRIM family members can recognize viral proteins in a virus and host species-specific fashion (Nisole et al., 2005) and thus it is of interest to determine if TRIM79α suppresses replication of other flaviviruses. Confocal microscopy demonstrated colocalization between TRIM79α and NS5 derived from TBEV (Figure 6A), but not with NS5 proteins from the mosquito-borne WNV or JEV. Consistent with this, TRIM79α interacted with NS5 from TBEV, but not with NS5 from WNV or JEV (Figure 6B). To determine the specificity of TRIM79α as a restriction factor, the replication of TBEV (strain Sofjin), or WNV (strain NY99) was compared in 293/TRIM79α-GFP and control cells. In agreement with the lack of interaction with NS5, replication of WNV NY99 (Figure 6C) was not impaired in TRIM79α expressing cells, whereas TBEV replication was significantly reduced at 24 and 48 hpi (Figure 6D). Similar restriction was observed for the tick-borne POWV (data not shown). Taken together, these results demonstrate that the function of TRIM79α as an antiviral molecule is specific to viruses belonging to the TBEV serocomplex, and is mediated through direct interaction with NS5.

TRIM79α expression is required for the antiviral effects of IFN-β on TBEV replication

To assess the importance of TRIM79α in the host IFN response to TBEV infection, we used replication-defective lentiviruses to deliver short hairpin RNA (shRNA) directed against TRIM79α (TRIM79sh) or a GFP-silencing control (GFPsh) into mouse macrophages (RAW cells). To examine knock-down efficiency, transduced cells were treated with IFN-β and mRNA expression corresponding to TRIM79α and TRIM30α was measured by RT-qPCR. TRIM79α knock-down was greater than 90% and was specific as it did not decrease TRIM30α mRNA expression (Figure 7A). Transduced RAW cells were infected with LGTV (Figure 7B) or TBEV Sofjin (Figure 7C), treated with 100 IU/ml IFN-β at 6 hpi and virus production was measured by immunofocus assay at 48 hpi. In the absence of exogenously added IFN-β, virus replication was not significantly affected by suppression of TRIM79α expression, consistent with low basal levels of TRIM79α mRNA. However, the antiviral effect of IFN-β treatment was abrogated following TRIM79α knock-down as evidenced by higher virus replication in the presence of IFN-β (Figures 7B-C). These results demonstrate that TRIM79α is an essential effector molecule of the IFN response to TBEV.

DISCUSSION

The current study has identified a highly virus-specific TRIM protein, TRIM79α, as a key mediator of the innate cellular response to TBEV infection. TRIM79α expression was dependent on type I IFN and was required for effective restriction of TBEV replication by IFN-β. The mechanism of TRIM79α-dependent restriction of TBEV was direct, targeting NS5, the viral polymerase and an essential component of the RC, for degradation. The few TRIM proteins previously demonstrated to have direct antiviral activity including TRIM5α and TRIM22 generally require the RING domain and may use the proteasome to restrict virus replication (Barr et al., 2008; Diaz-Griffero et al., 2007; Eldin et al., 2009; Gao et al., 2009; Maegawa et al., 2010; Wu et al., 2006). However, TRIM79α mediated degradation of NS5 through lysosomes independently of the RING catalytic site. TRIM79α-mediated restriction was specific to flaviviruses belonging to the TBEV serogroup because NS5 derived from the mosquito-borne flaviviruses WNV or JEV was not recognized by TRIM79α and WNV replication was unimpeded by TRIM79α expression. This high degree of specificity demonstrated by TRIM79α reveals a remarkable ability of the innate IFN response to discriminate between closely related flaviviruses.

Ectopic expression of TRIM79α in 293 cells resulted in a 50–90% reduction of both LGTV and TBEV replication, despite the fact that TRIM79α expression resulted in lower

expression of IFN-β. The degree of inhibition observed here is highly reminiscent of similar experiments evaluating virus restriction by proteins with dominant roles in IFN- dependent antiviral responses. Notable examples of these proteins include P56 inhibition of human papilloma virus (Terenzi et al., 2008), IRF-1 as a general antiviral molecule (Schoggins et al., 2011) and 2′-5′-oligoadenylate synthetase 1b (OAS1b), encoded by the flavivirus resistance gene *Flv*. In the latter case, ectopic expression of OAS1b in cells derived from susceptible mice resulted in approximately 50% reduction in WNV titers. However, WNV titers in resistant mice are 10^3–10^4 fold lower than in susceptible mice (Perelygin et al., 2002). While a limited number of additional gene products may contribute to flavivirus susceptibility, the *Flv* studies suggest that *in vitro* experiments examining ectopically expressed protein may underestimate the importance of individual ISGs in controlling virus replication *in vivo*.

Lysosomes are cellular organelles critical for macromolecule degradation and are the final destination of material undergoing phagocytosis, endocytosis or autophagy (Schroder et al., 2010). Thus, a switch from proteasome-dependent degradation of normal TRIM79α to lysosome-dependent degradation of NS5 observed in this study may represent an antiviral mechanism to target large protein complexes for destruction. In support of this, TRIM79α facilitated the degradation of protein complexes containing at least NS5, NS2B and NS3. However, despite the fact that NS5 is expressed on the cytosolic side of ER membranes, flavivirus RCs are shielded by virus-generated membrane proliferations thought to prevent recognition of viral replication intermediates by the host cell (Hoenen et al., 2007; Overby et al., 2010). NS5 is also anchored to membranes through its interactions with other viral NS proteins. Thus, it is unclear how TRIM79α would access NS5 in RCs and transport it to lysosomes. We did not find clear evidence that TRIM79α functions in concert with autophagy to drive destruction of the TBEV RC. Therefore, further studies will be required to elucidate the precise mechanism by which TRIM79α mediates TBEV restriction.

Although central to viral RC function, not all NS5 produced during flavivirus replication is found in membrane-bound RCs. NS5 is also present free in the cytoplasm or nucleus of cells infected with some flaviviruses (Davidson, 2009). Thus, multiple populations of NS5 exist over the course of infection that function indirectly in virus replication by modulating cellular processes such as suppression of IFNα/β-dependent signal transduction or host gene expression (Medin et al., 2005). These populations can be defined by the viral and cellular proteins bound to NS5, or by post-translational modifications such as phosphorylation and ubiquitination. We observed at least two species of NS5, a non-ubiquitinated form and a Ub-conjugated form that was stabilized by MG132. Thus NS5 degradation occurs by at least two pathways, the TRIM79α-dependent lysosome and the TRIM79α-independent proteasome. The presence of separable populations of NS5 suggests that TRIM79α may also target a population involved in functions other than in the RC. Understanding the molecular determinants required for TRIM79α recognition of TBEV NS5 would help illuminate the complexity of NS5 function in virus replication and pathogenesis.

The structure of NS5 is highly conserved between flaviviruses despite the fact that NS5 proteins share only ~40% identity at the amino acid level. The discovery of TRIM79α by yeast two-hybrid analysis of the NS5 MTase domain suggests that differences within this domain between TBEV and WNV may determine specificity and will be the subject of future studies. We also demonstrated a further level of specificity in TRIM recognition as TRIM30α shares 82% identity with TRIM79α but failed to directly interact with LGTV NS5. Taken together, these observations suggest that the NS5/TRIM79α interaction exists as a consequence of virus-host coevolution. The enzootic transmission cycle of TBEV occurs predominantly between tick vectors and their rodent hosts without causing obvious morbidity in the rodent (Bakhvalova et al., 2009; Ebel, 2010). This clearly differentiates the

evolutionary pressures of TBEV from those of WNV and JEV that cycle between mosquitoes and either birds or pigs (Mackenzie et al., 2004; van den Hurk et al., 2009). Hence, suppression of TBEV replication by the rodent-specific TRIM79α may represent an example of virus-host coevolution whereby type I IFN dampens virus replication, thereby contributing to reservoir host tropism and virus maintenance in nature.

For every antiviral measure utilized by the host, viruses have evolved strategies of evasion. TBEV delays production of type I IFN (Overby et al., 2010) and antagonizes IFN signaling (Best et al., 2005; Werme et al., 2008), strategies that would suppress TRIM79α expression. In addition, TRIM79α protein levels may be a target of virus antagonism. A loss in TRIM79α protein was evident late in infection with LGTV coincident with the detection of viral proteins by western blot (Figure 5B). A similar reduction in TRIM79α was seen in the presence of both NS5 and NS2B/3 (Figure 4F). TRIM79α does not appear to be degraded with NS5 in the lysosome as only proteasome inhibitors could stabilize TRIM79α expression and ectopically expressed NS5 did not impact TRIM79α levels. A protein complex containing TRIM79α and NS5 may simply be degraded more efficiently in the presence of NS2B/3. However, since NS2B/3 is the viral protease, TBEV may also evade restriction through the cleavage and inactivation of TRIM79α. A similar phenomenon was recently reported for TRIM56-mediated restriction of bovine viral diarrhea virus (BVDV), a *Pestivirus* and member of the *Flaviviridae* family. While the viral target of TRIM56 is unknown, expression of the BVDV small N-terminal protease (N^{pro}) was associated with reduced TRIM56 protein levels suggesting N^{pro} might directly antagonize this TRIM (Wang et al., 2011). Hence, interference of TRIM function may be an unexplored mechanism contributing to flavivirus evasion of innate immunity and virus pathogenesis.

In addition to direct roles in virus restriction, TRIM proteins are required to regulate signaling pathways such as toll-like receptors (TLRs) and RIG-I-like receptors (RLR) leading to virus detection and innate immune responses (Ozato et al., 2008). Both TRIM79α and TRIM30α have been linked to lysosomal degradation of the signaling components TAB2 and TAB3 (Shi et al., 2008; Tareen and Emerman, 2011), thus acting as negative regulators of the TLR/NFκB pathway. This function is consistent with reduced IFN-β expression observed during LGTV replication in TRIM79α-expressing cells. As has been demonstrated for influenza NS1 that binds to TRIM25 to inhibit RIG-I activity (Gack et al., 2009), the function of NS5-bound TRIM79α may provide a benefit to TBEV replication *in vivo*. For example, suppression of TRIM79α cellular function may increase production of inflammatory cytokines to recruit monocytes and macrophages to sites of infection and facilitate TBEV transmission to feeding ticks or dissemination in the vertebrate host. Alternatively, NS5 may potentiate TRIM79α function to suppress IFN expression. Experiments are currently in progress to evaluate the impact of TBEV infection and NS5 expression on the cellular roles of TRIM79α.

Despite the importance of host IFNα/β responses in the control of flavivirus infections, IFN is ineffective as a clinical therapy, likely compromised by virus-encoded antagonists of IFN-dependent JAK-STAT signaling (Robertson et al., 2009). Thus, understanding the precise antiviral mechanisms of ISGs may enable development of therapeutics effective against viruses like the flaviviruses that have evolved to target IFN-dependent signal transduction. Moreover, although hundreds of antiviral genes are expressed in response to IFN, this work demonstrates that antiviral activity can be tailored to individual pathogens by the activity of virus-specific ISGs. The fact that the TRIM proteins often target necessarily conserved structures such as the viral RNA polymerase suggests that resistance to TRIM mimetics as therapeutics may not be easily acquired through virus mutation. Therefore, further studies to identify additional TRIM molecules that specifically target flaviviruses as well as to understand TRIM mechanisms of restriction are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- TRIM79α is essential for the type I interferon antiviral response against TBEV
- TRIM79α facilitates lysosomal-mediated degradation of TBEV NS5
- Degradation of NS5 suppresses TBEV replication
- TRIM79α-dependent restriction is specific to flaviviruses in the TBEV serocomplex

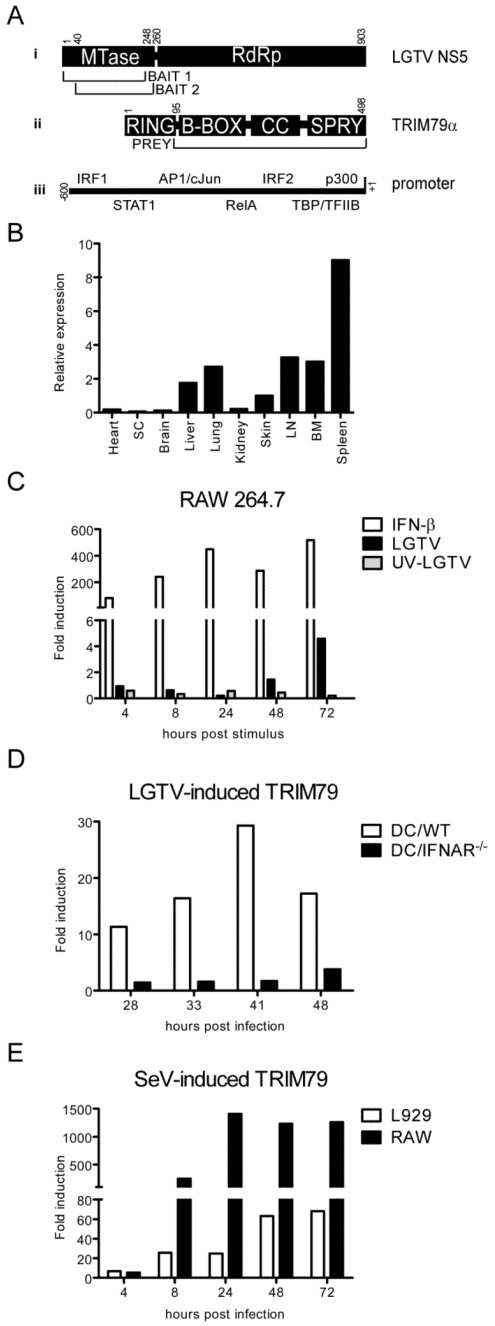

Figure 1. TRIM79α is an ISG expressed during virus infection

(A) Schematic representations of yeast two-hybrid bait derived from LGTV NS5 (i) and prey cDNA of TRIM79α (ii) identified from a murine macrophage library. (iii) Diagram of the TRIM79α promoter region with putative transcription factor binding sites. (B) TRIM79α mRNA expression in C57BL/6 mouse tissues assessed by RT-qPCR. SC, spinal cord; LN, lymph node; BM, bone marrow. Samples are expressed relative to TRIM79α mRNA level in the skin. (C) TRIM79α mRNA expression in RAW macrophages measured by RT-qPCR at indicated times and graphed as fold induction relative to untreated control. Stimuli: IFN-β (100 IU/ml), white bar; LGTV (MOI 10), black bar; UV-irradiated LGTV (MOI 10), grey bar. (D) TRIM79α mRNA expression measured by RT-qPCR in WT and IFNAR −/− DCs

infected with LGTV (MOI 25), graphed as fold induction relative to untreated control. (E) TRIM79α mRNA expression measured by RT-qPCR in mouse cells (L929 and RAW) following SeV infection (200 HA units/ml), graphed as fold induction relative to untreated control. See also Figure S1.

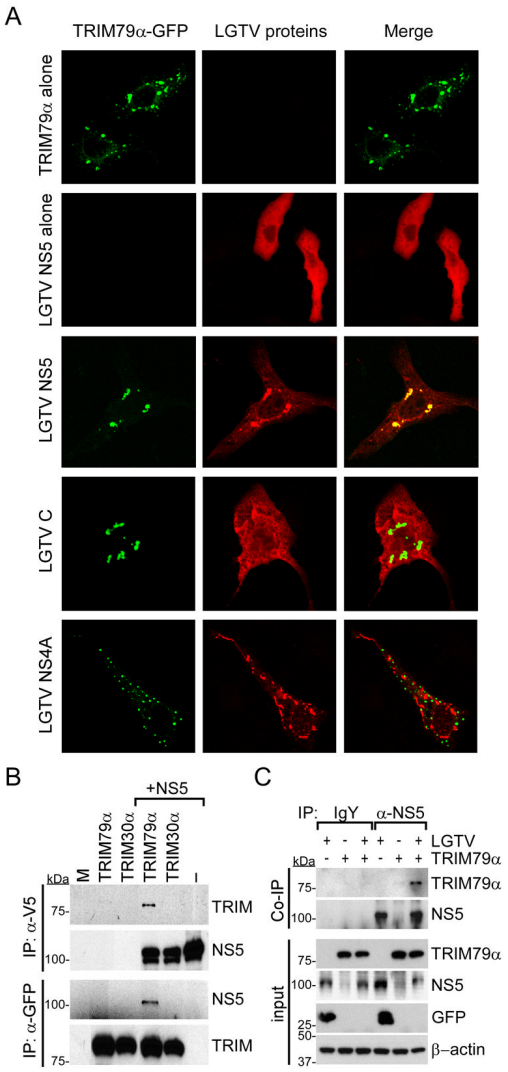

Figure 2. TRIM79α interacts with LGTV NS5

(A) Confocal microscopy of 293 cells expressing TRIM79α-GFP (green) and LGTV proteins NS5, C or NS4A (red). (B) Co-IP of lysates from 293 cells expressing TRIM79α-GFP or TRIM30α-GFP with or without LGTV NS5-V5. Reciprocal IPs were performed using α-V5 or α-GFP antibodies. M, mock transfected. (C) Co-IP of NS5 from 293 cells transfected with plasmids expressing TRIM79α-GFP or GFP and infected with LGTV for 48 h. The IP was performed with IgY or α-NS5. IP and total protein (input) blots were probed with TBEV (NS5), GFP (TRIM79α), or β-actin specific antibodies. A molecular weight marker is indicated in kDa.

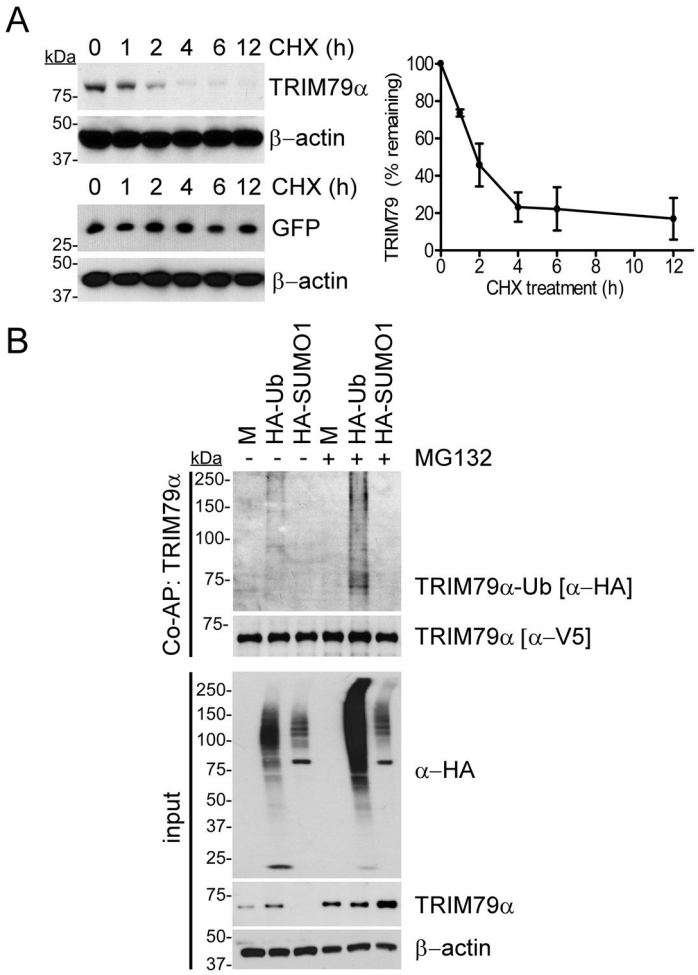

Figure 3. TRIM79α protein turnover is regulated by proteasomal degradation

(A) Western blot analysis of lysates from 293 cells transfected with TRIM79α-GFP or GFP plasmids and treated with CHX (100 μg/ml) for indicated times (h). TRIM79α-GFP blots were quantitated, normalized to β– actin, and presented as proportion of TRIM79α remaining over time. Error bars represent mean +/− SEM from three experiments. (B) Ubiquitination assay for Ub-modified TRIM79α. TRIM79α was affinity purified from lysates of 293 cells expressing TRIM79α-V5/AP, with or without HA-Ub or HA-SUMO1, and treated with DMSO or MG132 (10 μM) for 4 h. Conjugation of Ub or SUMO1 was visualized by probing blots with α-HA antibody. Western blot of input is shown to demonstrate expression levels of HA-Ub, SUMO1 and TRIM79α. M, mock transfected.

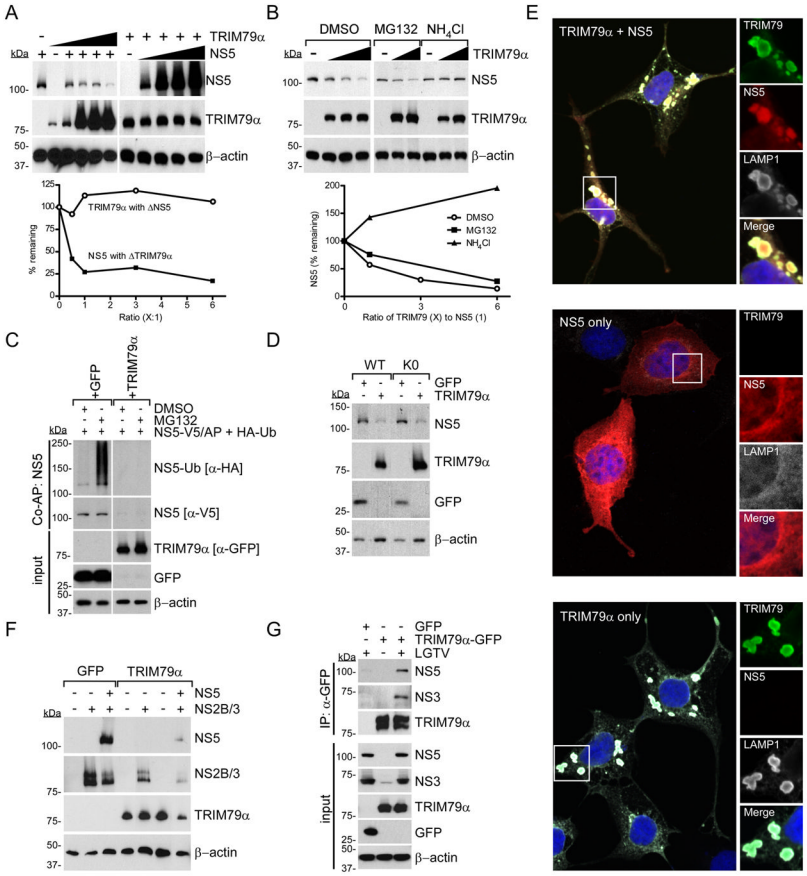

Figure 4. TRIM79α facilitates proteasome-independent degradation of NS5

(A) Western blot analysis of lysates from 293 cells transfected with a constant amount of either LGTV NS5 or TRIM79α-GFP plasmid and increasing amounts of the reciprocal plasmid. GFP vector plasmid was used to ensure equal DNA transfections. Quantitation of NS5 or TRIM79α expression normalized to actin is presented below western blots as percent remaining. (B) Western blot analysis of lysates from 293 cells transfected with a constant amount of LGTV NS5 and increasing amounts of TRIM79α-GFP plasmid. Cells were treated with DMSO, MG132 (10 μM) or NH_4Cl (20 mM) for 4 h. Quantitation of NS5 expression was normalized to actin and presented as percent remaining. (C) Ubiquitination assay and western blot analysis of lysates from 293 cells expressing LGTV NS5-V5/AP and HA-Ub with TRIM79α-GFP or GFP. Cells were treated with DMSO or MG132 for 4 h. (D) Western blot analysis of lysates from 293 cells expressing LGTV NS5-V5 with TRIM79α-GFP or GFP and HA-Ub (WT) or HA-Ub-K0 (K0). (E) Confocal microscopy of 293 cells expressing LGTV NS5 (red) and/or TRIM79α-GFP (green) and immunostained for endogenous LAMP1 (grey). Nuclei are counterstained with DAPI (blue). White box designates area shown in inset. (F) Western blot analysis of lysates from 293 cells stably expressing TRIM79α-GFP or GFP alone, and transfected with indicated combinations of LGTV NS5-V5 or NS2B/3-mCherry plasmids. (G) Co-IP of TRIM79α-GFP (using α-GFP antibody) from 293 cells expressingTRIM79α-GFP or GFP alone and infected with LGTV (MOI 5) for 48 h. Cells were treated with NH_4Cl (20 mM) for 12 h prior to harvest to routinely detect NS3. IP and total protein (input) blots were probed with TBEV (NS5), NS3, GFP or β-actin specific antibodies. See also Figure S2.

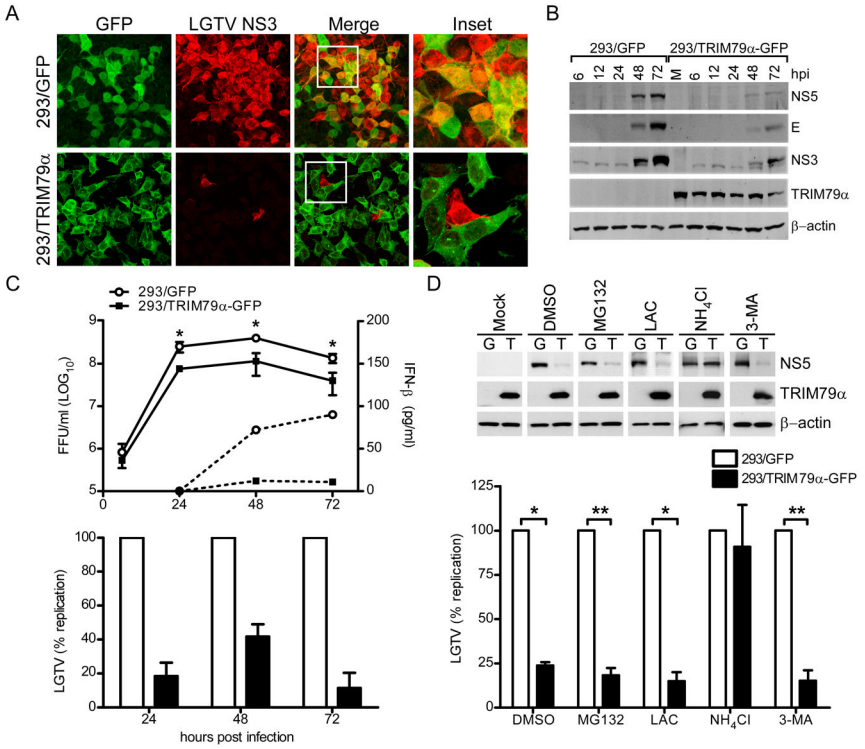

Figure 5. TRIM79α expression restricts LGTV replication

(A) Confocal microscopy of 293/TRIM79α-GFP or 293/GFP cells (green) infected with LGTV (24 hpi, MOI 0.1) and immunostained for NS3 (red). White box designates area shown in inset. (B) Western blot analysis of lysates from clonal 293/TRIM79α-GFP or 293/GFP cells infected with LGTV (MOI 10) for indicated times (h). Blots were probed with antibodies specific to LGTV NS5, NS3 and E proteins, as well as TRIM79α (GFP) and actin. (C) Titration of LGTV infectious particles in supernatants from clonal 293/TRIM79α-GFP and 293/GFP cells infected with LGTV (MOI 10) for indicated times (h,solid lines/left y-axis). Data are representative of mgultiple independent experiments performed in triplicate and plotted as mean +/− SEM. Asterisks indicate P<0.05 (*). These data are also presented as the percent replication of LGTV in 293/TRIM79α-GFP cells observed in three independent experiments plotted relative to 293/GFP control cells. IFN-β protein in supernatants from infected cells quantitated by ELISA is represented by dotted lines (right y-axis). (D) Western blot analysis of NS5 and TRIM79α expression in clonal 293/GFP cells (G) and 293/TRIM79α-GFP (T) infected with LGTV (MOI 0.1) for 48 h. Cells were treated at 2 hpi with DMSO, MG132 (0.1 μM), lactacystin (0.1 μM), NH_4Cl (20 mM), or 3-MA (1 mM) supplemented media. LGTV infectious particles in supernatants from these experiments were titrated by immunofocus assay and presented as percent replication relative to 293/GFP control cells. Data are representative of two independent experiments performed in triplicate and plotted as mean +/− SEM. Asterisks indicate P<0.05 (*) or P<0.01 (**).

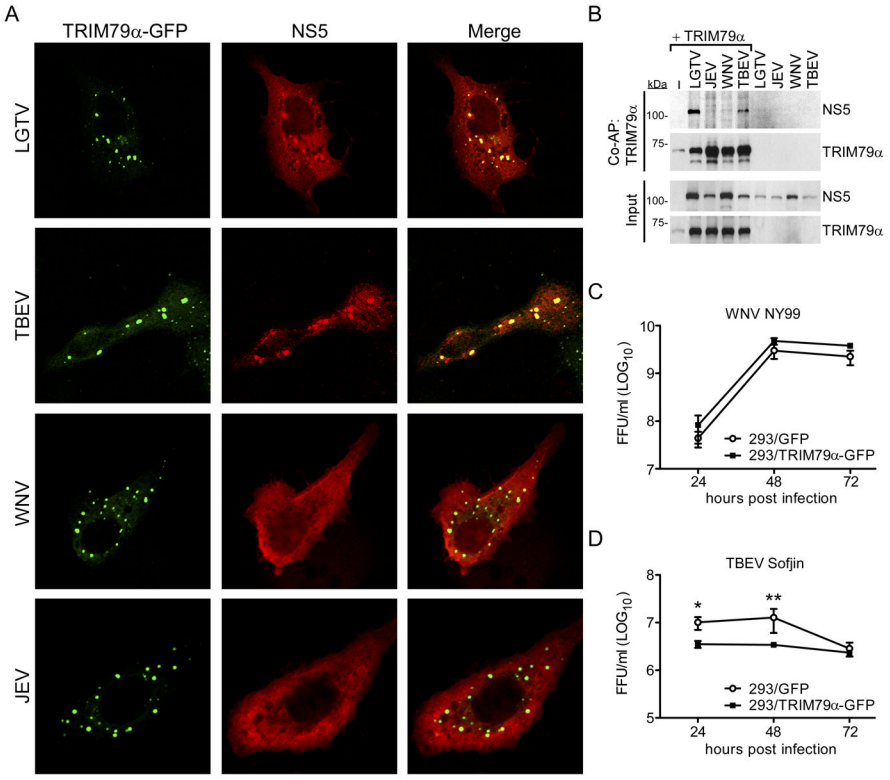

Figure 6. TRIM79α specifically interacts with NS5 from tick-borne flaviviruses and restricts TBEV replication

(A) Confocal microscopy of 293 cells expressing TRIM79α-GFP (green) and NS5 derived from LGTV, TBEV, WNV or JEV (red). (B) Co-AP of TRIM79α-AP/V5 from 293 cells transfected with TRIM79α-V5/AP and indicated flavivirus NS5-V5 plasmids. Blots were probed with α-V5 and TRIM79α-AP/V5 and NS5-V5 were differentiated by mobility. (C–D) Quantification of virus replication by immunofocus assay in clonal 293/TRIM79α-GFP or 293/GFP cells infected with WNV NY99 (C) or TBEV Sofjin (D) at an MOI of 10 FFU/cell for indicated times (h). Data are representative of three independent experiments performed in triplicate and plotted as mean +/− SEM. Asterisks indicate $P<0.05$ (*) or $P<0.01$ (**).

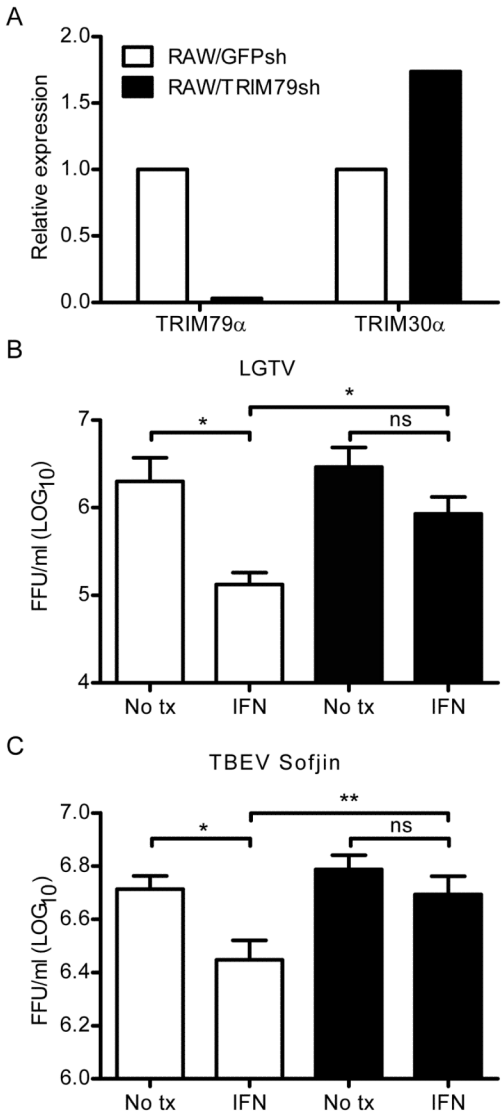

Figure 7. TRIM79α expression is required for the antiviral effects of IFN-β on TBEV replication

(A) TRIM79α and TRIM30α mRNA expression measured by RT-qPCR in RAW cells transduced with TRIM79α or GFP shRNA lentiviruses, treated with IFN-β (100 IU/ml) and normalized to HPRT mRNA. mRNA expression levels are relative to IFN-β-treated RAW/GFPsh cells. (B–C) Titration of infectious LGTV (B) or TBEV Sofjin (C) particles in supernatants from RAW cells transduced with TRIM79α or GFP shRNA lentiviruses, and either mock treated or treated with IFN-β (100 IU/ml) at 6 hpi. Virus production was measured by immunofocus assay at 48 hpi. Data are the average of three (LGTV) or two (TBEV Sofjin) independent experiments performed in triplicate and plotted as mean +/− SEM. Asterisks indicate P≤0.05 (*), P<0.01 (**) and nonsignificant (ns).