Novel antiviral host factor, TNK1, regulates IFN signaling through serine phosphorylation of STAT1

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In response to viral infection, the host induces over 300 IFN-stimulated genes (ISGs), which are the central component of intracellular antiviral innate immunity. Inefficient induction of ISGs contributes to poor control and persistence of hepatitis C virus infection. Therefore, further understanding of the hepatocytic ISG regulation machinery will guide us to an improved management strategy against hepatitis C virus infection. In this study, comprehensive genome-wide, high-throughput cDNA screening for genes regulating ISG expression identified a tyrosine kinase nonreceptor 1 (TNK1) as a unique player in the ISG induction pathway. The immune-modulatory function of TNK1 has never been studied, and this study characterizes its significance in antiviral innate immunity. TNK1 is abundantly expressed in hepatocytes and maintains basal ISG expression. More importantly, TNK1 plays a critical role in type I IFN-mediated ISG induction. We discovered that the activated IFN receptor complex recruits TNK1 from the cytoplasm. TNK1 is then phosphorylated to enhance its kinase activity. The activated TNK1 potentiates JAK-STAT signaling through dual phosphorylation of STAT1 at tyrosine 701 and serine 727 amino acid positions. Our loss-of-function approach demonstrated that TNK1 governs a cluster of ISG expression that defines the TNK1 pathway effector genes. More importantly, TNK1 abundance is inversely correlated to viral replication efficiency and is also a determinant factor for the hepatocytic response to antiviral treatment. Taken together, our studies found a critical but unidentified integrated component of the IFN-JAK-STAT signaling cascade.

hepatic immunity | nonreceptor tyrosine kinase | protein kinase C | PKC

The host response to viral infection results in the induction of IFN-stimulated genes (ISGs). ISGs are a collection of over 300 antiviral genes that play a central role in the intracellular antiviral defense program as well as in the mounting of adaptive immunity (1). Efficient ISG induction is linked to the successful clearance of viral pathogens, including hepatitis C virus (HCV). Therefore, synthesized IFN has been used as a central component of anti-HCV therapy in a clinical setting for many years (2–5). Despite its importance, our knowledge of IFN biology, including the function of individual ISGs and the host factors that regulate ISG induction pathways, is still on a steep learning curve. A recent overexpression-based, high-throughput study demonstrated that each ISG possesses a differential antiviral potency in a virus-specific manner (6). In addition, the basal and induced ISG expression exhibits a diverse profile among various cell types, thereby linking it to specific viral tropism (7). Thus, further investigation of cell type and pathogen-specific IFN biology is required to improve our management strategy of viral infectious diseases.

Virus infection of mammalian cells triggers pattern recognition receptor (PRR) signaling upon engagement with pathogen-associated molecular patterns. The PRRs, such as RIG-I–like helicases (RLHs) or Toll-like receptors (TLRs), activate IFN regulatory factors (IRFs) for the induction of ISGs (1). In addition, PRR pathways can induce the secretion of various classes of endogenous IFN to trigger the JAK-STAT signaling pathway. This leads to the amplification of ISG expression (8–10). Thus, the host possesses a variety of signaling pathways to induce grossly redundant gene sets, justifying the critical role of ISGs in an antiviral defense program. The binding of IFN to its receptor triggers the phosphorylation of JAK for the activation of the canonical IFN pathway. In addition, numerous noncanonical molecules associating with the IFN receptor complex, such as mitogen-activated protein kinase (MAPK), v-akt murine thymoma viral oncogene (AKT), protein kinase C (PKC), and IκB kinase ε, are activated to magnify the cellular response to antiviral cytokines (11–13). The activation of the canonical and noncanonical IFN signaling pathways will result in the tyrosine and/or serine phosphorylation of STAT molecules (14, 15). These activated STAT molecules undergo homo/heterodimerization and translocate into the nucleus to serve as transcription factors for ISG induction (15, 16).

This study is designed to discover novel hepatic host factors regulating ISG induction, with the ultimate aim of translating this discovery into improved management of HCV infection. Toward this goal, we conducted comprehensive genome-wide cDNA screening for the identification of host factors regulating ISG expression. The screening, using a uniquely developed stable cell line that comprises IFIT1 promoter-regulated luciferase reporter, resulted in identification of tyrosine kinase nonreceptor 1 (TNK1) as a critical regulator of the ISG induction pathway. TNK1 is a member of the Ack family of kinases and belongs to the nonreceptor tyrosine kinase (NRTK) superfamily (17). The function of TNK1 has been largely unknown, except for its modest

Significance

IFN-stimulated genes (ISGs) are the antiviral effectors and a key component of intracellular antiviral innate immunity. Inefficient induction of ISGs is linked to poor disease outcome and allows viruses to establish persistent infection. Hepatitis C virus (HCV) chronically infects over 200 million people worldwide and is a leading cause of advanced liver diseases, such as cirrhosis and liver cancer. In this work, our high-throughput cDNA screening identified a novel antiviral host factor, tyrosine kinase nonreceptor 1, that suppresses the HCV life cycle through STAT1 activation to the induction of ISGs. Our findings add an important unique player in JAK-STAT signaling and advance our understanding of IFN biology. This will contribute to the improvement of our antiviral strategy against human pathogenic infectious disease.

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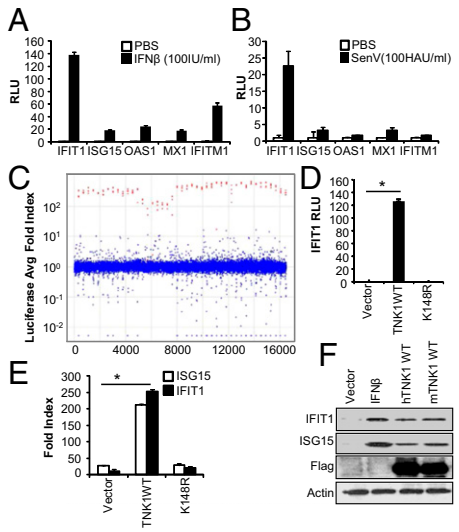

Fig. 1. Genome-wide cDNA screening for identification of unique ISG inducers. Huh7 cells cotransfected with the indicated ISG promoter firefly and with control Renilla luciferase were treated with IFN-β (*A*) or infected with SeV (*B*) for 16 h, followed by a dual luciferase assay (DLA). Data shown are mean ± SE obtained from biological triplicates. RLU, relative luciferase unit. (*C*) Results of cDNA screening. Huh7 IFIT1-luciferase cells were transfected with cDNA for the firefly luciferase assay. Each blue dot represents a single cDNA. Red dots (IPS1) serve as a positive control. Avg, average. (*D*) Huh7 cells cotransfected with the indicated expression vectors along with IFIT1 firefly luciferase for DLA similar to *A*. Data shown are mean ± SE obtained from biological triplicates. $*P < 0.01$. (*E*) Huh7 cells transfected with TNK1 expression vectors for 24 h for RT-PCR analysis of the indicated ISG. Results are shown as mean ± SE of triplicate samples. $*P < 0.01$. (*F*) Huh7 cells transfected with expression vectors for 24 h, followed by immunoblot analysis. IFN-β (100 IU/mL) serves as a positive control. Displayed data represent one of three biological triplicate experiments.

tumor suppressor phenotype (18, 19). This study determines its role in antiviral innate immunity. Here, we demonstrated that TNK1 kinase activity enhances JAK-STAT signaling largely through the serine phosphorylation of STAT1. More importantly, our loss-of-function approach defined a cluster of ISGs that are tightly regulated by TNK1. Finally, the abundance of TNK1 exhibited a significant impact on the hepatocytic susceptibility to HCV infection as well as the response to IFN-based antiviral treatment. Taken together, our results indicate that this newly identified component of JAK-STAT signaling regulates viral infection through the induction of a unique set of ISGs.

Results

Genome-Wide Comprehensive cDNA Screening. To identify novel host factors that regulate HCV through the induction of ISGs, we used a luciferase reporter cell line as a platform for the high-throughput cDNA screening. To identify the most sensitive and specific ISG promoter reporter, we first evaluated a few well-studied ISG promoters via luciferase reporter assay. Results from the testing of multiple reporter constructs, such as IFIT1, IFITM1, ISG15, MX1, and OAS1, demonstrated the expected response to both type I IFN or Sendai virus (SeV) infection, both of which were used to stimulate type I IFN-α/β receptor (IFNAR) or RIG-I signaling, respectively (Fig. 1 *A* and *B*). Of these, the IFIT1 promoter construct demonstrated prominent sensitivity to these ISG induction pathways (Fig. 1 *A* and *B*). Moreover, the IFIT1 reporter exhibited high specificity to cytokines and SeV infection that induces ISG expression (Fig. S1*A*). Additional analysis with a series of IRF constructs demonstrated a broad spectrum of responsiveness to IRFs involved in ISG induction (Fig. S1*B*). These results confirmed that the IFIT1 reporter offers high sensitivity and specificity to the ISG induction pathway and could be used as the foundation for the cDNA screening.

Given these results, the Huh7 cells, which serve as the foundation for in vitro HCV virological studies, were used to create a stable cell line harboring the IFIT1 promoter reporter construct. Upon colony selection, clone 22 demonstrated promising sensitivity and specificity to SeV infection or IFN treatment in a dose-dependent manner (Fig. S1 *C–F*). Based on these results, Huh7-IFIT1 clone 22 reporter cells were applied to genome-wide comprehensive cDNA screening.

The more than 14,000 human and mouse MGC clones comprising the cDNA overexpression library were individually transduced, followed by measurements of luciferase activity (Fig. 1*C*). This screening resulted in the identification of unique host factors that induce ISGs (Table 1) (National Center for Biotechnology Information Gene Expression Omnibus accession no. GSE49589). Of these, TNK1 was one of the most significant hits in this screening. These potent positive hits also include a few well-known molecules involved in ISG induction pathways, such as MAVS (IPS-1), IL28RA, and IRF1 (Table 1), justifying our methodology. Subsequently, an overexpression TNK1 construct showed the induction of multiple ISGs, including IFIT1 (Fig. 1 *D–F*), thus confirming the cDNA screening result.

TNK1 Stimulates the JAK-STAT Pathway for the Induction of ISGs. ISG induction is regulated through multiple types of intracellular innate immune signaling pathways, which ultimately use IRF or STAT family molecules as transcription factors. PRR signaling cascades, such as the RLH and TLR pathways, participate in the induction of ISGs mainly through the activation of IRF3 and IRF7. On the other hand, IFN-mediated signaling pathways promote the formation of STAT-containing transcription factors. These include, but not limited to, IFN-stimulated gene factor 3 (ISGF3), which is composed of STAT1, STAT2, and IRF9, and the gamma-IFN activation factor (GAF), which is a STAT1 homodimer (20).

To delineate how TNK1 induces ISGs, we first set out to determine the transcription factors regulated by TNK1. Our results showed that TNK1 overexpression did not promote activation of PRDI/III luciferase reporter, which is potently driven by IRF1, IRF3, IRF7, and IRF9C2 [mimics of ISGF3 (21)] (Fig. 2*A* and Fig. S2*A*). Accordingly, TNK1 expression did not promote IRF3 nuclear translocation, which requires the phosphorylation of IRF3 (Fig. S2 *B* and *C*). In addition, TNK1 expression did not activate reporters regulated by NFκB and IFN-β promoters or IFN-β gene transcription, which are regulated by the PRR pathways (Fig. S2 *D–F*). These results collectively indicated that the TNK1 is unlikely to cross-talk with the PRR pathway.

Table 1. Short list of potent hits from cDNA screening

Gene names	Average induction	Accession no.
IPS-1/MAVS	10.04194*	BC020006
TNK1	8.869309*	BC055303
GHR, GHBP	3.217481	BI102919
AI132321	3.09693	BC058565
MECT1, TORC1	3.041146*	BC028050
BMN, MANBA	2.972134	BC031409
NIPSNAP1	2.765203	BC010837
LGALS9	2.452722	BC003754
IFNLR1, IL-28R	2.436864*	BC057856
IRF1	2.434459*	BC003821
EPI64, TBC1D10	2.40379	BC018300
TEF	2.381422	BC017689
CD134L, TNFSF4	2.373292	BC041663
DKK4	2.313969	BC018400
BCL2L13	1.88781	BC029016

GHR, growth hormone receptor; *AI132321*, Pld4 phospholipase D family, member 4; *MECT1*; CREB-regulated transcription coactivator 1; *BMN*, mannosidase, beta A, lysosomal; *NIPSNAP1*, nipsnap homolog 1; *LGALS9*, lectin, galactoside-binding, soluble, 9; *EPI64*, TBC1 domain family, member 10A; *TEF*, thyrotrophic embryonic factor; *CD134L*, tumor necrosis factor (ligand) superfamily, member 4; *DKK4*, dickkopf WNT signaling pathway inhibitor 4; *BCL2L13*, BCL2-like 13.
$*P < 0.05$.

On the other hand, TNK1 induced activation of the IFN-sensitive response element (ISRE) (Fig. 2*B*) and, more importantly, triggered substantial activation of the IFN-gamma–activated sequence (GAS) promoter in a manner dependent on TNK1 kinase activity (Fig. 2*C*). The ISRE promoter contains a consensus IRF binding sequence, and thus responds to IRF1, IRF3, IRF7, and ISGF3 (Fig. S2*G*), whereas the GAS promoter contains a sequence specific to STAT1 homodimers (GAF). These findings suggest that TNK1-mediated ISG induction heavily involves STAT1 activation. Indeed, TNK1 expression promoted tyrosine phosphorylation of STAT1 at amino acid 701 (Y701P), which is an indispensable signature of STAT1 activation. Similarly, TNK1 activated its closest family member, STAT3, but not the more distantly related STAT2, consistent with the nuclear translocation pattern of STAT molecules after TNK1 overexpression (Fig. 2 *D* and *E* and Fig. S2*H*). Of great interest, the degree of ISRE and especially GAS reporter activation was disproportional to the level of STAT1 Y701P (Fig. 2*F*). These results led us to hypothesize that TNK1-mediated STAT1 activation may involve additional posttranslational modification of STAT1 in addition to Y701P.

It is known that serine phosphorylation of STAT1 at position 727 (S727P) supports the interaction of STAT1 with transcriptional coactivators, which leads to a significant increase in ISRE and GAS promoter activation (14, 22, 23). To test this possibility, the phosphorylation status of STAT1 S727 was examined. The results demonstrated that TNK1 expression drives S727P in a kinase activity-dependent manner (Fig. 2*G*), suggesting that TNK1-mediated STAT activation involves two distinct phosphorylations of STAT1. Because TNK1 is classified as a tyrosine kinase, it is unlikely to induce S727P directly. Therefore, this suggests that S727P is mediated by some serine/threonine (S/T) kinase that requires TNK1 for its activation. Thus, we have examined whether TNK1 activates previously reported S/T kinases involved in S727P, such as p38MAPK, AKT, ERK, or PKCδ (12, 24–29). Despite these observations, our results failed to demonstrate the activation of these S/T kinases by TNK1 (Fig. S2*I*). Of note, the involvement of PKCδ in this event is now discredited due to debunking of Rottlerin's specificity to PKCδ (12). However, a previous study also demonstrated that pan-PKC inhibitor H-7 abolished the type I IFN-mediated S727P (12). Accordingly, our study also revealed that PKC-412, a pan-PKC inhibitor, demonstrated significant reduction of TNK1-mediated S727P (Fig. 2*H*). These observations leave open the possibility that PKCs contribute to STAT1 S727P. Thus, we extended our investigation to test whether PKCs play a role in TNK1 signaling. Our result revealed that any isoforms of the PKCs that were tested enhanced TNK1-mediated STAT1 phosphorylation at S727, suggesting that PKC isoforms play a redundant role (Fig. S2*J*). Accordingly, the loss-of-function approach demonstrating the knockdown of each isoform modestly attenuated the GAS promoter activation by TNK1 (Fig. S2*K*). These results also suggest the redundant role of PKC isoforms, at least in TNK1-mediated STAT1 S727P.

TNK1 Is an Integrated Component of the IFN Response. To determine the biological significance of TNK1 in the regulation of HCV, we have analyzed TNK1 expression in primary human hepatocytes (PHHs). The protein expression analysis demonstrated an abundant expression of TNK1 in PHHs as well as in an immortalized PHH cell line (PH5CH8) (Fig. S3 *A* and *B*). In contrast, Huh7 cells expressed barely detectable levels of TNK1 (Fig. S3*C*). Thus, we established a pair of cell lines for gain- and loss-of-function approaches (Fig. S3 *C* and *D*). PH5CH8 cells stably expressing shRNA against TNK1 (PH5CH8-shTNK1) demonstrated significantly lower expression of ISGs under resting conditions (Fig. S3*E*). More importantly, TNK1 silencing dramatically attenuated ISRE promoter activation in response to type I IFN (Fig. 3*A*), which indicates that TNK1 plays a critical role in STAT1 activation. Similarly, Huh7 cells overexpressing TNK1 demonstrated an increase in type I IFN-mediated ISRE and GAS promoter activities (Fig. 3*B* and Fig. S3*F*). These results indicated that TNK1 abundance determines the level of basal and induced ISG expression and also suggested that TNK1 is an integrated component of type I IFN signaling.

Generally speaking, NRTKs are either noncovalently associated with cytokine receptors or recruited to the receptor complex upon ligand binding. Ligand binding to the receptor promotes the activation of NRTKs, typically through phosphorylation within the kinase activation loop. Based on this notion, we hypothesize that TNK1 phosphorylation is required for the regulation of ISGs. As a first step to test this hypothesis, we assessed the phosphorylation of overexpressed TNK1 with a 7.5% (mass/vol) SDS/PAGE gel, which demonstrated two distinct patterns of migration. The slower migration signal was modestly reduced in the absence of phosphatase inhibitor and diminished in the presence of alkaline phosphatase (Fig. 3*C*). This result indicates that enzymatically active TNK1 is indeed phosphorylated. Next, we tested endogenous TNK1 phosphorylation using a phospho-TNK1 (Y277P)–specific antibody. Of note, Y277 and Y287

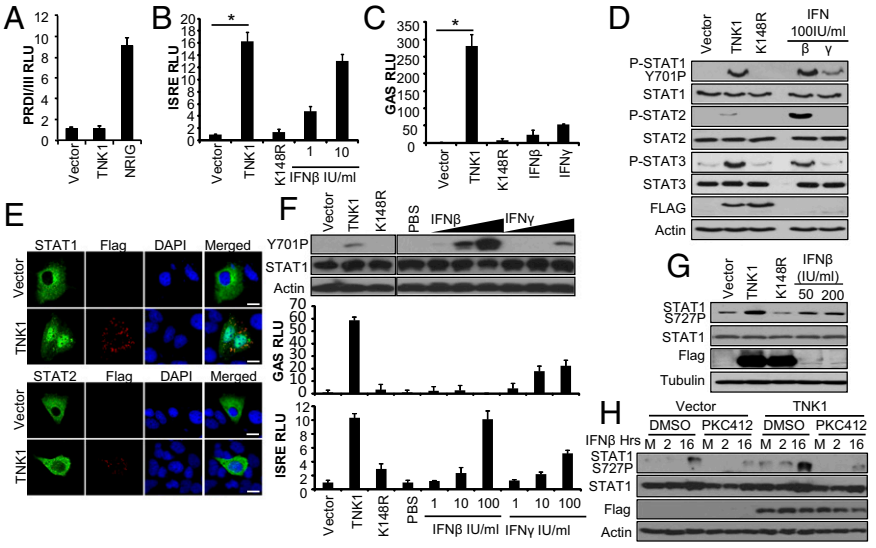

Fig. 2. ISG induction of TNK1 is mediated through STAT1 activation. Huh7 cells were transfected with TNK1 vectors along with luciferase constructs regulated by NF-κB (PRDII) (*A*), ISRE (*B*), and GAS (*C*). The active forms of RIG-I (NRIG) (*A*), IFN-β (*B*), and IFN-β or IFN-γ (100 IU/mL) (*C*) are positive controls. Results are shown as mean ± SE of triplicate samples. $*P < 0.01$. (*D*) Huh7 cells transfected with TNK1 vectors for 16 h or treated with IFN-β or IFN-γ (2 h), followed by immunoblot analysis. Displayed data represent one of the biological triplicate experiments. (*E*) Fluorescent microscopic analysis of Huh7 cells cotransfected with Flag-TNK1 and GFP-STAT [STAT1 (*Upper*) and STAT2 (*Lower*)] vectors. GFP is colored green, Flag is colored red, and DAPI is colored blue. Displayed data represent one of the biological triplicate experiments. (Scale bar: 20 μm.) (*F*, *Upper*) Huh7 cells were transfected with TNK1 vectors for 24 h or treated with IFN-β or IFN-γ (1, 10, or 100 IU/mL) for 2 h for the immunoblot analysis. (*F*, *Middle* and *Lower*) Huh7 cells were transfected with TNK1 vectors for 24 h or treated with IFN-β or IFN-γ for 8 h for the GAS or ISRE DLA. Results are shown as mean ± SE of triplicate samples. (*G*) Huh7 cells transfected with TNK1 vector were subjected to immunoblot assessment of S727P. (*H*) Huh7 cells transfected with Flag-tagged TNK1 expression vector were treated with pan-PKC inhibitor (PKC412) for 2 h at 20 μM. The cells were then treated with IFN-β at 100 IU/mL, followed by immunoblot analysis. Displayed results are from one representative experiment of the biological triplicate experiments.

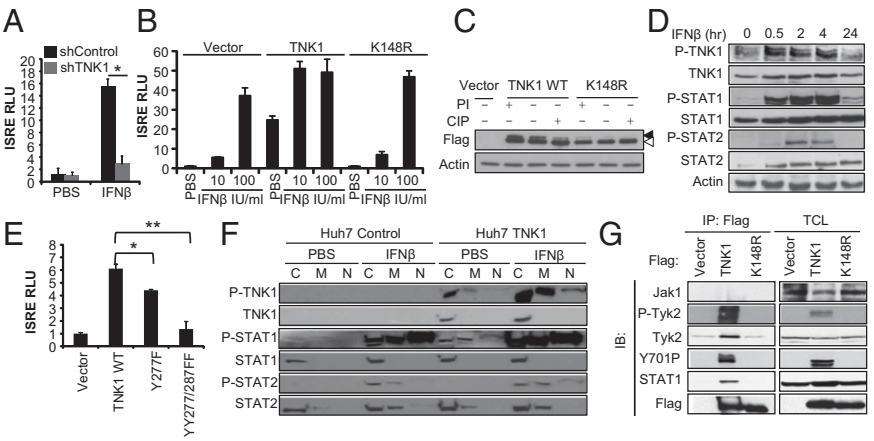

Fig. 3. TNK1, an integrated component of type I IFN-JAK-STAT signaling. (*A*) PH5CH8 shRNA cells were transfected with ISRE luciferase reporter, followed by IFN-β (100 IU/mL) for DLA. $*P < 0.005$. (*B*) Huh7 cells cotransfected with TNK1 and ISRE luciferase vectors were treated with IFN-β (16 h) for DLA. The bars represent mean ± SE of triplicate samples. (*C*) TNK1-expressing Huh7 cell lysates were treated with phosphatase inhibitor (PI) or alkaline phosphatase (1 IU/mL) at 37 °C for 30 min, followed by immunoblot analysis via 7.5% SDS/PAGE gel. CIP, calf-intestinal alkaline phosphatase. (*D*) Immunoblot analysis of PHH cells treated with IFN-β (100 IU/mL) for the detection of TNK1 (Y277P), STAT1 (Y701P), and STAT2 (Y690P). (*E*) ISRE promoter analysis of Huh7 cells transfected with TNK1 expression vectors for 24 h. $*P < 0.005$; $**P < 0.0005$. Each bar represents mean ± SE from triplicate samples. (*F*) Huh7 control cells or Huh7 TNK1 cells were treated with IFN (100 IU/mL), followed by subcellular fractionation for immunoblot analysis. C, cytoplasm; M, plasma membrane; N, nucleus. (*G*) Cells were transfected with the indicated expression vectors and subjected to immunoprecipitation (IP) with anti-Flag affinity gel, followed by immunoblot (IB) analysis of Jak1, Tyk2 (total and Y1054P), and STAT1 (total and Y701P). TCL, total cell lysate. Similar results were obtained in three independent experiments.

are located within the critical activation loop motif, and are thus proposed as activation signatures (19). We found that type I IFN treated with PHHs rapidly enhanced at least Y277P of TNK1 with similar kinetics as STAT1 Y701P but that type II IFN did so only very modestly (Fig. 3*D* and Fig. S3*G*). The significance of tyrosine phosphorylation within the activation loop was further confirmed with the point and double mutants. These Y277F and YY277/287FF mutants resulted in a substantial reduction of ISRE promoter activation (Fig. 3*E*), suggesting that phosphorylation of these tyrosine residue(s) serves as TNK1 activation markers.

To understand further how the IFN response promotes TNK1 activation, we conducted a subcellular fractionation assay. Under resting conditions, TNK1 exclusively localizes to the cytoplasm (Fig. 3*F* and Fig. S3 *B* and *H*), leading us to speculate that activated IFNAR recruits TNK1 from the cytoplasm to its receptor complex anchored on the plasma membrane (PM fraction). To test this, we have examined TNK1 localization shifts during IFN treatment in Huh7 cells stably expressing TNK1 (Huh7 TNK1). Unphosphorylated TNK1 mostly remained in the cytoplasm under resting conditions; however, TNK1 Y277P appeared in the PM fraction, especially in the cells treated with IFN (Fig. 3*F*). This result supports our hypothesis and led us to test the molecular interaction between TNK1 and IFNAR complex molecules further. Subsequent immunoprecipitation assays showed that TNK1 interacts with and activates tyrosine kinase 2 (Tyk2) and STAT1. This may explain the participation of TNK1 in the IFNAR complex for the induction of ISGs (Fig. 3*G*). Taken together, our results indicated that TNK1 is indeed a component of the activated type I IFNAR complex. Of note, our results showed that the deletion of IFNAR1 did not alter the degree of TNK1 phosphorylation (Fig. S3*I*), suggesting that an IFN receptor complex-independent mechanism(s) serves to maintain the basal TNK1 activity in the resting condition.

TNK1 Governs Hepatic Antiviral Innate Immunity Through ISG Induction. Similar to our observations with PHHs, a previous study reported that the expression of TNK1 in C57BL/6 mice is relatively high in the liver (18). Our follow-up, organ-wide expression analysis also confirmed the relatively high hepatic expression of TNK1 (Fig. S4*A*). Based on these results, we examined the role of TNK1 in ISG expression using mouse primary hepatocytes (MPHs) extracted from $TNK1^{-/-}$ and littermate control mice. Gene expression analysis of a few selected ISGs demonstrated that the genetic deletion of TNK1 reduced their basal expression (Fig. S4*B*). In addition, our gene expression analysis of a few ISGs upon IFN-β treatment demonstrated variable degrees of attenuation in $TNK1^{-/-}$ cells (Fig. 4 *A*–*D*). Moreover, MPHs lacking TNK1 demonstrated significant attenuation of STAT1 S727P, as well as modest reduction of Y701P in response to type I IFN (Fig. 4 *E* and *I*). Although S727P has been shown to augment ISG induction, the degree of its contribution differs in a gene-specific manner (14, 23). To define further the ISGs that are heavily regulated by TNK1, we conducted a quantitative PCR array analysis and found that the loss of TNK1 attenuated a cluster of ISGs (Fig. 4*F*). Next, we investigated the in vivo significance of TNK1 in the induction of hepatic ISG expression upon murine IFN-β (mIFN-β) injection. The induction of IFITs in $TNK1^{-/-}$ liver tissue showed a similar trend to our ex vivo observations (Fig. 4 *G* and *H*). Taken together, these results indicated that TNK1 regulates hepatic ISG expression through dual activation of STAT1, especially through S727P. Finally, we tested the contribution of TNK1 to ISG expression in a variety of primary cell types, such as mouse embryo fibroblasts, bone marrow-derived dendritic cells, and bone marrow macrophages. Our results demonstrated that TNK1 contributes to ISG expression in these cell types to varying degrees (Fig. S4 *C*–*E*). This suggests that TNK1 contributes to antiviral innate immunity in a cell type-specific manner.

TNK1 Regulates HCV Infection. To test the role of TNK1 in hepatic antiviral innate immunity, HCV infection assays were conducted with a set of cell lines that either constitutively express PHH-comparable levels of TNK1 (Huh7 TNK1) or shRNA against TNK1 (PH5CH8 shTNK1). We compared the susceptibility of these cells to HCV infection with that of their control cells. HCV infection in Huh7-TNK1 cells demonstrated reduced permissiveness for infection, decreased viral protein abundance, and lower levels of infectious virus production (Fig. 5 *A*–*C*). A similar experiment using PH5CH8 shTNK1 cells showed enhanced HCV replication compared with control cells (Fig. 5*D*). These data indicate that TNK1 abundance is inversely correlated to viral replication efficiency during acute HCV infection. Next, we examined whether hepatocytic TNK1 contributes to the efficacy of antiviral therapy. The introduction of TNK1 in Huh7 cells harboring an HCV subgenomic replicon (Con 1 strain) demonstrated an enhanced response to anti-HCV medications, such as type I IFN or NS5A inhibitor (Fig. 5*E*). Based on our in vitro and ex vivo study results, we concluded that TNK1 regulation of HCV is mediated through enhanced ISG expression. Taken together, these results suggest the critical role of TNK1 in regulation of both acute HCV infection and antiviral therapy during chronic infection. Moreover, we further tested the significance of TNK1 in other RNA virus. Similar to HCV, TNK1-expressing cells were quite restrictive to SeV infection (Fig. S5*A*), further confirming the antiviral activity of TNK1.

Discussion

Canonical type I IFN signaling induces STAT1 Y701P, thus leading to its incorporation into transcription factors, such as ISGF3 and GAF (20). These STAT1-containing transcription factors are key players in ISG induction. Here, our unique

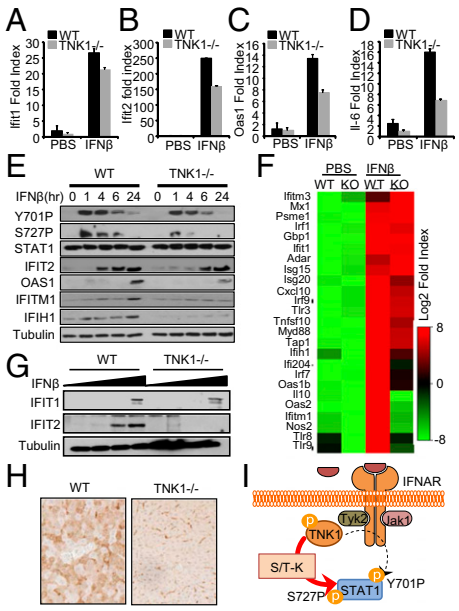

Fig. 4. ISG expression in mice with genetic deletion of TNK1: ex vivo and in vivo study. (*A–D*) Quantitative PCR analysis of WT or TNK1$^{-/-}$ MPHs treated with mIFN-β (100 IU/mL) for 8 h. All results are representative of at least three independent experiments. (*E*) MPHs extracted from WT and TNK1$^{-/-}$ mice were treated with mIFN-β (100 IU/mL), followed by IB analysis of indicated ISGs, STAT1 S727P, Y701P, and total STAT1. Displayed results represent one of three independent experiments. (*F*) WT or TNK1$^{-/-}$ MPHs treated with mIFN-β (100 IU/mL) for 8 h were applied to an ISG quantitative PCR array. The relative fold index normalized to PBS-treated WT cells (average of duplicates) was converted to a heat map at the indicated scale. (*G* and *H*) WT and TNK1$^{-/-}$ mice were injected with 10, 100, or 1,000 IU/g of mIFN-β through the i.p. route. After 24 h, the liver tissues from the mice were used for IB analysis of indicated ISGs (*G*) or immunohistochemical analysis of IFIT2 (*H*). The displayed data represent one of three independent experiments. (Scale bar: 40 μm.) (*I*) Proposed model of TNK1-mediated STAT1 activation. p, phosphorylation; S/T-K, S/T kinase.

genome-wide cDNA screening identified TNK1, which governs ISG expression. TNK1 was first discovered from CD38-negative cells with primers targeting the conserved tyrosine kinase domain (30). Subsequently, TNK1 was classified as an NRTK; therefore, it is expected to play a role in intracellular signaling. However, its biological significance has never been well determined, except for its modest function as a tumor suppressor, where it is proposed to alter Ras-Raf signaling indirectly (18, 19, 31, 32). Thus, this study delineates the function of TNK1 in antiviral innate immune signaling.

This study found that TNK1 is abundantly expressed in hepatocytes and localizes in the cytoplasm in the resting condition. TNK1 shifts its localization to the PM during the cellular response to type I IFN. This shift to the PM is accompanied by phosphorylation of the TNK1 at amino acid Y277. Our immunoprecipitation assays also demonstrated that TNK1 associates with the IFNAR complex through Tyk2. This interaction involves Tyk2 activation and is likely the explanation for phosphorylation of STAT1 Y701P by TNK1, although the exact mechanism of how this interaction preferentially activates STAT1 remains elusive. Our gain- and loss-of-function approaches collectively suggest that the interaction between TNK1 and the macromolecular IFNAR augments ISG induction through unique STAT1 at Y701 and S727. Although the degree of contribution to Y701P is modest, our study determined that TNK1 plays a critical role in STAT1 S727P, indicating the dual STAT1 activation by TNK1. It is well accepted that tyrosine phosphorylation amino acid 701 is indispensable, because it facilitates the homo- or heterodimerization required for efficient DNA binding (23). In addition to Y701P, S727P enhances ISG expression in a gene-specific manner, and this has been shown to play a certain role in the regulation of viral infection (28, 33, 34). Although the exact mechanism of how STAT1 S727P augments the expression of selective ISGs has not been fully defined, it is believed that S727P regulates the interaction between transcriptional coactivators, such as p300/CBP and STAT1 Src homology 2 domain (22, 23, 34). It is well confirmed that the S727P status confers neither Y701P nor its nuclear translocation/DNA binding (34); therefore, its selective contribution to ISG expression is likely due to each ISG's chromosomal sensitivity to p300/CBP or other coactivators. In fact, our ex vivo and in vivo studies using liver tissues from TNK1$^{-/-}$ mice also demonstrated a weakened response to IFN in a gene-specific rather than global manner. Although the link between this phenomenon and the association with the above-mentioned coactivator remains elusive, taken together, we conclude that ISG expression by TNK1 is largely regulated through STAT1 S727P.

Because TNK1 has been classified into the NRTK superfamily, it is reasonable to hypothesize that STAT1 S727P is likely mediated by S/T kinase(s), which is, in turn, presumably activated by TNK1. Many studies have proposed that various molecules are involved in STAT1 S727P, such as p38MAPK, ERK, PKC-δ, and PI3K-AKT (35–37). Of these molecules, it is thought that PKC-δ plays the most critical role in type I IFN-mediated S727P (12). However, the specificity of the PKC-δ inhibitor (i.e., Rottlerin) that was used to draw this conclusion is now discredited; therefore, the specific isoform of PKC responsible for S727P remains unknown. Our study demonstrated that pan-PKC inhibitor significantly suppressed TNK1-mediated S727P, which is similar to the previous observation using another type of pan-PKC inhibitor, H7. Moreover, our results showed that TNK1 and PKC isoforms cooperatively enhanced STAT1 S727P, suggesting the possibility that PKC isoforms are redundantly involved in this event. Although our data and those of others suggest the

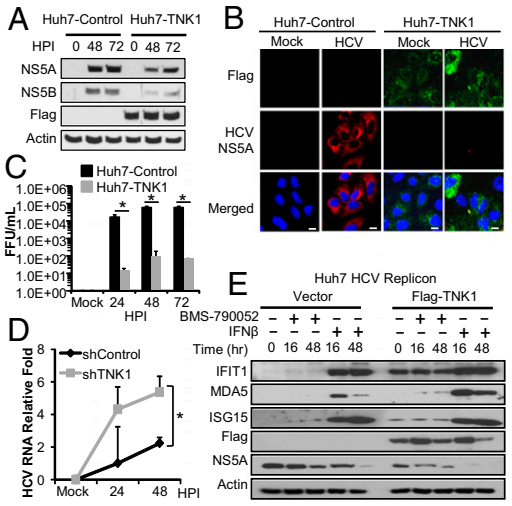

Fig. 5. TNK1 determines hepatocytic susceptibility to HCV infection. (*A–C*) Huh7 TNK1 and control cells were infected with HCV JFH-1 [multiplicity of infection (MOI) of 1] followed by IB analysis of HCV NS5A and NS5B protein (*A*) or immunofluorescent analysis of NS5A protein (red), Flag-TNK1 (green), and DAPI (blue) (*B*). (Scale bar: 20 μm.) (*C*) Culture medium collected from experiments described in *A* was assessed by HCV infection titer assay using Huh7.5 cells. FFU, focus-forming unit. *$P < 0.005$. Displayed results are from one of the biological triplicate experiments (*A* and *B*). Each bar represents mean ± SE from triplicate samples. (*D*) PH5CH8 shRNA cells were challenged with HCV JFH1 at a MOI of 5, followed by RT-PCR analysis of the HCV genome. Results are shown as mean ± SE of triplicate samples. HPI, hours postinfection. *$P < 0.05$. (*E*) HCV subgenomic replicon cells were transfected with TNK1 vector for 24 h, followed by anti-HCV treatment with IFN-β (50 IU/mL) or BMS-790052 (HCV-NS5A inhibitor; 500 pM). Cell lysates were subjected to IB analysis of indicated ISGs and HCV viral protein. Similar results were obtained from three independent experiments.

involvement of PKCs in this event, it is expected that potential off-target effects of these inhibitors on other S/T kinases may cause misinterpretation of study results. Therefore, further investigation is needed to define the intermediate S/T kinase(s) to explain how TNK1 governs S727P. An alternative explanation of how TNK1 causes S727P is that TNK1 may function as a dual kinase (both tyrosine and S/T kinase), much like ERKs (38).

Consistent with previous reports, our organ-wide expression analysis found high expression of TNK1 in the liver. In addition, PHH, the cell type where HCV replicates, expresses abundant TNK1. Therefore, we used HCV infection as a relevant disease model to test its critical role in viral regulation. Our study proved that hepatocytic TNK1 regulates ISG expression and that its abundance is inversely correlated to the permissiveness to HCV. In addition, TNK1 expression significantly influences the response to antiviral treatment in vitro and in vivo. The antiviral strategy against HCV is currently undergoing a major paradigm shift from the combination of PEG-IFN and ribavirin to an IFN-free direct antiviral agent (DAA)-based regimen. The strong motive toward this IFN-free regimen is due to its adverse effects and poor success rate. However, recent clinical trials of two DAA combinations reported that the DAA combination alone provoked the generation of a DAA-resistant variant rapidly after the initiation of treatment (39). In contrast, these two DAAs in combination with IFN completely prevented the selection of DAA-resistant variants and demonstrated substantial viral eradication. These results indicated that the antiviral effector ISGs, in the expression of which TNK1 plays a significant role, are required for the promising antiviral therapy against HCV.

In conclusion, we discovered important, but previously undefined antiviral host factor TNK1, which participates in IFN signaling through unique activation of STAT1. Since the discovery of IFN in 1957 (40), numerous host factors regulating IFN signaling pathways have been added to this critical antiviral defense pathway. Our studies proposed TNK1 as an important integrated component of IFN signaling. This will support our continued effort to improve antiviral strategies against human pathogenic viral diseases.

Materials and Methods

Genome-Wide cDNA Screening. The genome-scale screen cDNA clone collection was the Mammalian Gene Collection from Open Biosystems. Forty nanograms of each clone in the collection was plated in individual wells. Huh7-IFIT1-luciferase cells were mixed with Fugene 6 (Roche) and plated directly onto prespotted cDNA clones present in each well of the plate. After a 24-h incubation, the luciferase activity present in the cells was assayed by the direct addition of 40 μL of BriteLite reagent (PerkinElmer). Relative light activity was normalized on a per-plate basis using control wells spotted on each plate. The cDNA screening was performed in the Scripps Cell-Based Screening and Genomics core. A selected short list of potent hits is summarized in Table 1. Raw readings of the firefly luciferase activities on the cDNA array were $\log_2$-transformed. The *P* value was determined through a one-tailed Student *t* test for determination of the significance to median induction. Additional information is provided in *SI Materials and Methods*.

Statistics. Data were compared using the Student *t* test.

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