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# Glycogen Synthase Kinase 3β Regulates IRF3 Transcription Factor-Mediated Antiviral Response via Activation of the Kinase TBK1

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## SUMMARY

**Viral infection activates transcription factors IRF3 and NF-κB, which collaborate to induce type I interferons (IFNs). Here, we identified glycogen synthase kinase 3β (GSK3β) as an important regulator for virus-triggered IRF3 and NF-κB activation, IFN-β induction, and cellular antiviral response. Overexpression of GSK3β potentiated virus-induced activation of IRF3 and transcription of the *IFNB1* gene, whereas reduced expression or deletion of GSK3β impaired virus-induced IRF3 and NF-κB activation, transcription of the *IFNB1* gene, as well as cellular antiviral response. GSK3β physically associated with the kinase TBK1 in a viral infection-dependent manner. GSK3β promoted TBK1 self-association and autophosphorylation at Ser172, which is critical for virus-induced IRF3 activation and IFN-β induction. The effect of GSK3β on virus-induced signaling is independent of its kinase activity. Our findings suggest that GSK3β plays important roles in virus-triggered IRF3 activation by promoting TBK1 activation and provide new insights to the molecular mechanisms of cellular antiviral response.**

## INTRODUCTION

Host pattern-recognition receptors (PRRs) of the innate immune system recognize invading viruses and initiate a series of signaling events leading to production of type I interferons (IFNs) and proinflammatory cytokines (Akira et al., 2006; Hiscott, 2007). Type I IFNs further activate downstream signaling pathways that lead to transcriptional induction of a wide range of antiviral genes. The antiviral gene products collaborate to elicit cellular antiviral response through various mechanisms (Levy and García-Sastre, 2001; Randall and Goodbourn, 2008). Transcriptional induction of many type I IFN genes requires cooperation of transcription factors such as IFN-regulatory factor 3 (IRF3), IRF7, NF-κB, and ATF2-c-Jun, which bind to the conserved enhancer elements of the type I IFN gene promoters and initiate their transcription (Honda et al., 2006).

Understanding the signaling pathways of virus-triggered induction of type I IFNs is in progress. Viral infection and replication generates pathogen-associated molecular patterns (PAMPs) such as viral 5′-phosphorylated double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), 5′-triphosphate panhandle RNA, and viral double-stranded DNA (dsDNA), which are sensed by cellular PRRs (Koyama et al., 2008). Among the PRRs, Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) are demonstrated to be membrane associated and cytoplasmic sensors of viral RNAs, respectively. For example, TLR3, TLR7, and TLR8 recognize dsRNA or ssRNA at the membrane of endosomes. RIG-I binds to short dsRNA and 5′-triphosphate panhandle RNA, whereas MDA5, another member of RLRs, recognizes long dsRNA (Ishii et al., 2008; Kato et al., 2008; Yoneyama and Fujita, 2008). Recently, it has been reported that dA:dT-rich dsDNA is transcribed into dsRNA, which is subsequently recognized by RIG-I (Ablasser et al., 2009; Chiu et al., 2009).

Upon binding to viral RNA, RIG-I undergoes conformational change and is recruited to the mitochondrial adaptor protein VISA (also known as MAVS, IPS-1, and Cardif) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). VISA interacts with TNF receptor associated factor 6 (TRAF6) through its conserved TRAF-binding motifs to activate the IKK (IκB kinase) complex, which phosphorylates IκBα (Inhibitory kappa B alpha), leading to the release and activation of NF-κB. VISA is also associated with MITA (also known as STING) through its transmembrane domain. MITA acts as a scaffolding protein to recruit the kinase TBK1 to the VISA-associated signaling complex and promotes TBK1-IRF3 association, leading to phosphorylation and activation of IRF3 (Ishikawa and Barber, 2008; Zhong et al., 2008). However, how TBK1 itself is activated remains less clear.

Glycogen synthase kinase 3β (GSK3β) has been identified as a serine-threonine (ser-thr) kinase that phosphorylates and inactivates glycogen synthase. GSK3β is ubiquitously expressed in various tissues and organs in a constitutively active form. Previous studies have demonstrated that GSK3β is involved in various signaling pathways and regulates many cellular activities, such as metabolism, cell cycle, transcription regulation, vesicular transport, neuronal function, oncogenesis, and development (Jope and Johnson, 2004). GSK3β is also involved in TLR-mediated production of pro- or anti-inflammatory cytokines. For example, knockdown of GSK3β or treatment with GSK3β inhibitors reduces TLR4- and TLR2-mediated release of interleukin-12 (IL-12) and increases the secretion of IL-10, respectively, indicating that GSK3β is a crucial regulator for the balance between pro- and anti-inflammatory cytokines

(Martin et al., 2005). Another study demonstrates that GSK3β negatively regulates TLR4-mediated IFN-β production by regulating c-Jun expression (Wang et al., 2008). However, whether and how GSK3β is involved in virus-triggered signaling is unknown.

In this study, we found that GSK3β was essential for virus-triggered IRF3 and NF-κB activation, IFN-β induction, and cellular antiviral response. GSK3β promoted TBK1 dimerization and/or oligomerization and autophosphorylation at Ser172 independent of the kinase activity of GSK3β. Our findings provide insights to the molecular mechanisms of virus-triggered TBK1 activation, a critical step in virus-trigggered type I IFN induction and cellular antiviral response.

## RESULTS

### Overexpression of GSK3β Potentiates Virus-Triggered Activation of IRF3 and Transcription of *IFNB1*

GSK3β is involved in differential regulation of TLR-mediated production of pro- or anti-inflammatory cytokines and negatively regulates LPS-induced IFN-β production (Martin et al., 2005). Because TLR- and RLR-mediated signaling pathways share a number of key components, we examined whether GSK3β is also involved in regulation of virus-triggered and RLR-mediated signaling. In reporter assays, overexpression of GSK3β but not its homolog GSK3α significantly potentiated Sendai virus (SeV)-triggered activation of the IFN-β promoter in human embryonic 293 cells (Figure 1A). RT-PCR experiments indicated that overexpression of GSK3β potentiated transcription of virus-induced downstream genes, such as *IFNB1*, *CCL5*, and *ISG15* (Figure 1B). Because the transcription factor IRF3 is critically involved in virus-triggered induction of type I IFN genes, we determined the effect of GSK3β on virus-triggered activation of IRF3. Overexpression of GSK3β but not GSK3α potentiated SeV-induced ISRE (interferon-stimulated response element) activation (Figure 1C) and IRF3 dimerization (Figure 1D), which are hallmarks of IRF3 activation. These results suggest that GSK3β potentiates virus-triggered activation of IRF3 as well as transcription of the *IFNB1* gene.

Because the kinase activity of GSK3β is important for its functions in various signaling pathways, we next examined whether the effect of GSK3β on virus-triggered IRF3 activation is dependent on its kinase activity. Mutation of Ser9 of GSK3β to alanine (S9A) confers its constitutive kinase activity, whereas mutation of the ATP binding residue Lys85 of GSK3β to alanine (K85A) abolishes its kinase activity (Jope and Johnson, 2004). We found that both the kinase constitutively active mutant GSK3β(S9A) and the kinase inactive mutant GSK3β(K85A) enhanced SeV-induced dimerization of IRF3 (Figure 1D), as well as activation of ISRE (Figure 1E) and the IFN-β promoter (Figure 1F) to amounts comparable to wild-type GSK3β. These results suggest that the effect of GSK3β on virus-triggered signaling is independent of its kinase activity. Consistently, GSK3β and its mutants GSK3β(S9A) and GSK3β(K85A) enhanced activation of the IFN-β promoter and ISRE triggered by cytoplasmic poly(I:C) (Figure S1A available online). Overexpression of GSK3β and the two mutants also potentiated TLR3-mediated activation of the IFN-β promoter and ISRE (Figure S1B). In reporter assays, wild-type GSK3β, as well as GSK3β(S9A) and GSK3β(K85A), did not potentiate IFN-γ-triggered activation of the IRF1 promoter (Figure 1G), suggesting that GSK3β plays a specific role in virus-triggered IRF3 activation and IFN-β induction.

### Knockdown of GSK3β Inhibits IRF3 Activation and *IFNB1* Transcription

Because overexpression of GSK3β potentiated virus-triggered induction of IFN-β, we next determined whether endogenous GSK3β is required for virus-induced signaling. We constructed two GSK3β-RNAi plasmids that could efficiently inhibit the expression of tranfected and endogenous GSK3β (Figure 2A). Both of the RNAi plasmids markedly inhibited SeV-triggered activation of the IFN-β promoter and ISRE in reporter assays (Figure 2B) as well as endogenous expression of *IFNB1*, *CCL5*, and *ISG15* genes in RT-PCR experiments in 293 cells (Figure 2C). These results suggest that GSK3β plays an important role in virus-triggered induction of IFN-β. This effect is not cell type specific because knockdown of GSK3β also inhibited SeV-induced activation of the IFN-β promoter in A549 and HeLa cells (Figure S2A).

Consistently, knockdown of GSK3β markedly inhibited SeV-induced IRF3 phosphorylation and dimerization (Figure 2D). Knockdown of GSK3β did not significantly inhibit IFN-γ-induced activation of the IRF1 promoter (Figure 2E), suggesting that GSK3β is specifically involved in virus-triggered IRF3 activation and IFN-β expression.

We further determined whether GSK3β is involved in intracellular dsRNA- or dsDNA-triggered signaling. Knockdown of GSK3β markedly inhibited activation of ISRE and the IFN-β promoter triggered by poly(I:C) and poly(dA:dT) transfected into 293 cells (Figures 2F and 2G). Knockdown of GSK3β also inhibited poly(I:C)-triggered TLR3-mediated as well as LPS-induced TLR4-mediated signaling leading to IFN-β induction (Figure 2H; Figure S2B). These data suggest that GSK3β is required for both RLR-mediated and TLR3- and TLR4-mediated IRF3 activation and IFN-β induction.

### Impairment of IRF3 Activation and IFN-β Induction in GSK3β-Deficient Cells

*Gsk3b* gene deletion in mice results in embryonic lethality resulting from multifocal hemorrhagic degeneration in the livers (Hoeflich et al., 2000). However, we obtained *Gsk3b*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and examined whether virus-triggered signaling is impaired in these cells. We found that SeV-induced activation of the IFN-β promoter was severely impaired in *Gsk3b*$^{-/-}$ MEFs compared to their wild-type counterparts in reporter assays (Figure 3A). RT-PCR experiments indicated that SeV-induced expression of endogenous *Ifnb1* gene was impaired in *Gsk3b*$^{-/-}$ MEFs (Figure 3B). Consistently, SeV-induced ISRE activation (Figure 3C), IRF3 dimerization, and phosphorylation (Figure 3D) were impaired in *Gsk3b*$^{-/-}$ MEFs. Reconstitution of GSK3β but not GSK3α into *Gsk3b*$^{-/-}$ MEFs by retrovirus-mediated gene transfer significantly restored SeV-induced activation of the IFN-β promoter (Figure 3E). Similarly, reconstitution of *Gsk3b*$^{-/-}$ MEFs with GSK3β(K85A) and GSK3β(S9A) also restored SeV-induced activation of the IFN-β promoter and ISRE to degrees similar to the wild-type GSK3β (Figure 3F). Real-time PCR experiments further confirmed that SeV-induced transcription of *Ifnb1* gene was restored in

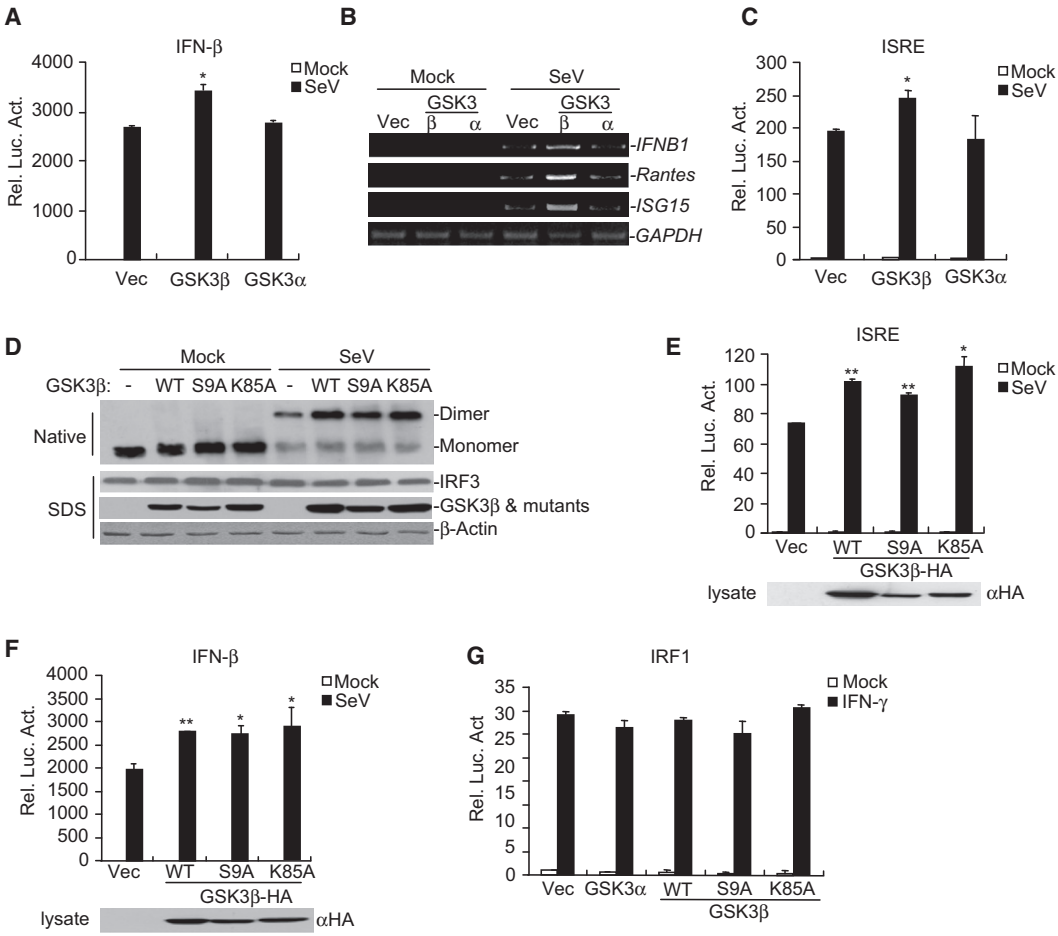


**Figure 1. Overexpression of GSK3β Potentiates Virus-Triggered Activation of IRF3 and Transcription of the *IFNB1* Gene**

(A) GSK3β but not GSK3α potentiates SeV-induced activation of the IFN-β promoter. 293 cells ($1 \times 10^5$) were transfected with the IFN-β promoter luciferase plasmid (0.1 μg) and an expression plasmid for GSK3β or GSK3α (0.25 μg). 20 hr after transfection, cells were infected with SeV or left untreated for 12 hr before luciferase assays were performed.

(B) GSK3β but not GSK3α potentiates SeV-induced transcription of endogenous *IFNB1*, *CCL5*, and *ISG15* genes. 293 cells ($2 \times 10^5$) were transfected with the indicated expression plasmids (2 μg each) for 20 hr, cells were infected with SeV or left untreated for 10 hr before RT-PCR was performed.

(C) GSK3β but not GSK3α potentiates SeV-induced ISRE activation in 293 cells. The experiments were performed as in (A) except that ISRE reporter plasmid was used.

(D) GSK3β and its mutants enhance SeV-induced dimerization of IRF3. 293 cells ($2 \times 10^5$) were transfected with the indicated plasmids. 20 hr after transfection, cells were infected with SeV or left uninfected for 8 hr. Cell lysates were separated by native (top) or SDS (bottom) PAGE and analyzed by immunoblots with the indicated antibodies.

(E and F) GSK3β and its mutants potentiate SeV-induced activation of ISRE (E) and the IFN-β promoter (F). The experiments were performed as in (A).

(G) GSK3α, GSK3β, and its mutants do not potentiate IFN-γ-induced activation of the IRF1 promoter. 293 cells ($1 \times 10^5$) were transfected with the IRF1 promoter reporter plasmid (0.1 μg) and the indicated mammalian expression plasmids (0.25 μg). 20 hr after transfection, cells were treated with IFN-γ (100 ng/ml) or left untreated. Luciferase assays were performed 12 hr after infection.

Graphs show mean ± SD, n = 3. *$p < 0.05$; **$p < 0.01$. See also Figure S1.

*Gsk3b*$^{-/-}$ MEFs after reconstitution with wild-type GSK3β, GSK3β(K85A), or GSK3β(S9A) (Figure 3G). In plaque assays with Vesicular stomatitis virus (VSV), markedly higher VSV titers were produced from *Gsk3b*$^{-/-}$ MEFs than the wild-type control cells (Figure 3H). Transfection of poly(I:C) caused antiviral response and reduced viral titers in wild-type but not *Gsk3b*$^{-/-}$ MEFs (Figure 3H). Reconstitution with GSK3β, GSK3β(K85A), or GSK3β(S9A) restored the antiviral activity of the *Gsk3b*$^{-/-}$ MEFs (Figure 3H). Thus, GSK3β is important for virus-triggered IRF3 activation, IFN-β induction, and cellular antiviral response. These results also suggest that the kinase activity of GSK3β is not required for its role in virus-triggered IFN-β induction and antiviral activity.

## GSK3β Regulates Virus-Triggered Signaling at the TBK1 Level

Various proteins have been reported to be involved in the virus-triggered IRF3 activation pathway such as RIG-I, VISA, MITA, TBK1, and IKKε. To determine the molecular order of GSK3β in the virus-triggered IRF3 activation pathway, we examined the

effects of reduced expression (knockdown with RNAi) or deletion of GSK3β on ISRE activation mediated by these molecules in reporter assays. Reduced expression or deletion of GSK3β inhibited RIG-I-, VISA-, MITA-, and TBK1-mediated ISRE activation. In the same experiments, ISRE activation mediated by IKKε or IRF3(5D), a constitutive active IRF3 mutant (Lin et al., 1998), was not markedly inhibited (Figures 4A and 4B). Because RIG-I, VISA, and MITA act upstream whereas IRF3 acts downstream of TBK1, the simplest explanation from these data is that GSK3β targets TBK1. Consistent with the notion that TBK1 acts downstream of TRIF in the TLR3-mediated IRF3 activation pathway (Fitzgerald et al., 2003; McWhirter et al., 2004), knockdown of GSK3β also inhibited TRIF-mediated activation of ISRE and the IFN-β promoter (Figure 4C). Although TBK1 and IKKε have redundant roles in certain circumstances, knockdown of GSK3β inhibited TBK1 but not IKKε-mediated ISRE activation (Figure 4A). Consistently, GSK3β potentiated TBK1- but not IKKε-mediated ISRE activation (Figure 4D). In transient transfection and coimmunoprecipitation experiments, GSK3β interacted with TBK1 but not IKKε (Figure 4E). Domain mapping experiments indicated that the kinase domain of GSK3β (aa 56–340) and the C-terminal coiled-coil containing domain of TBK1 (aa 451–729) are required for their interaction (Figure S3). Although the kinase domain of GSK3β could interact with TBK1, it was not sufficient to restore SeV-induced IFN-β production in *Gsk3b*$^{-/-}$ MEFs. In the same experiments, wild-type GSK3β but not GSK3β (aa 221–420) (which did not interact with TBK1) restored SeV-induced IFN-β production (Figure S3). These results suggest that GSK3β specifically targets TBK1 but not IKKε.

We next examined whether GSK3β is physically associated with TBK1 in untransfected cells. Endogenous coimmunoprecipitation experiments indicated that GSK3β did not interact with TBK1 under physiological condition; however, their interaction was detectable at 4 hr after SeV infection and increased to high levels at 8 and 12 hr after SeV infection. Correspondingly, IFN-β mRNA was markedly induced at 8 hr after SeV infection (Figure 4F). These results suggest that GSK3β is associated with TBK1 in a viral infection-dependent manner and this association is correlated to virus-triggered IFN-β induction.

## GSK3β Promotes TBK1 Self-Association and Autophosphorylation

We next investigated how GSK3β regulates TBK1 in the virus-triggered IRF3 activation pathway. In our earlier experiments, we demonstrated that the kinase inactive mutant of GSK3β could complement virus-triggered IFN-β induction to a level comparable to its wild-type or constitutive active counterpart (Figure 3). These results suggest that the kinase activity of GSK3β is not required for its regulation of TBK1-mediated signaling. We therefore determined whether GSK3β regulates TBK1 dimerization or oligomerization and autoactivation. In transient transfection and coimmunoprecipitation experiments, GSK3β markedly enhanced TBK1 self-association (dimerization or oligomerization) (Figure 5A). The effect of GSK3β on TBK1 self-association was independent of its kinase activity, because both GSK3β(S9A) and GSK3β(K85A) had similar effects (Figure 5A). In contrast, overexpression of GSK3β did not affect TBK1-IKKε, TBK1-MITA, or VISA-MITA interactions (Figure S4). These data suggest that GSK3β promotes TBK1 self-association independent of its kinase activity.

During our immunoprecipitation assays, we observed that transfection of GSK3β caused a shift of TBK1 but not IKKε to higher molecular weight bands (Figures 5B and 5C). Such a shift was due to phosphorylation of TBK1 as shown by the fact that calf intestine phosphatase (CIP) treatment diminished the shift (Figure 5B). Interestingly, GSK3β-mediated phosphorylation of TBK1 was independent of the kinase activity of GSK3β because both the kinase inactive mutant GSK3β(K85A) and the constitutive active mutant GSK3β(S9A) similarly enhanced phosphorylation of TBK1 (Figure 5B). Because GSK3β enhanced TBK1 self-association and phosphorylation independent of the kinase activity of GSK3β, we reasoned that GSK3β promotes TBK1 autophosphorylation. As shown in Figure 5D, overexpression of GSK3β caused phosphorylation of wild-type TBK1 but not TBK1(K38A), a kinase inactive mutant in which the ATP binding residue Lys38 was mutated to alanine. These results suggest that GSK3β mediates TBK1 dimerization or oligomerization and autophosphorylation independently of the kinase activity of GSK3β.

## TBK1 Autophosphorylation at Ser172 Is Essential for Virus-Triggered Signaling

Having established that GSK3β mediates TBK1 self-association and autophosphorylation, we next determined which amino acid residues of TBK1 are autophosphorylated. We made a series of mutants of TBK1 in which potential phosphorylation residues predicted by the NetPhos program were mutated to alanines individually (partially shown in Figure S5A). In reporter assays, mutation of Ser172 but not other serines or threonines to alanine impaired the ability of TBK1 to activate ISRE and the IFN-β promoter (Figures 6A and 6B; Figure S5A). TBK1(S172A), similar to the kinase inactive mutant TBK1(K38A), could act as a dominant-negative mutant to inhibit SeV-induced activation of ISRE and the IFN-β promoter (Figures 6A and 6B). GSK3β failed to cause phosphorylation of TBK1(S172A) whereas in the same experiments GSK3β mediated phosphorylation of wild-type TBK1 and a randomized serine mutant, TBK1(S716A) (Figure 6C). To determine whether GSK3β promotes TBK1 autophosphorylation or trans-phosphorylation at Ser172, we transfected 293 cells with Flag-tagged wild-type or mutant TBK1, HA-tagged wild-type TBK1 and GSK3β, or control plasmid. Flag-tagged TBK1 and mutants were pulled down by coimmunoprecipitation with anti-Flag. Immunoblot analysis with anti-$^{pSer172}$TBK1, an antibody that specifically recognizes Ser172-phosphorylated TBK1, indicated that GSK3β increased phosphorylation of Flag-tagged wild-type TBK1 and TBK1(S716A) but not TBK1(K38A) or TBK1(S172A) at residue 172 even in the presence of HA-tagged wild-type TBK1 (Figure 6D). These results suggest that GSK3β promotes autophosphorylation but not trans-phosphorylation of TBK1 at Ser172. TBK1 phosphorylation is not a prerequisite for its self-association as shown by the fact that TBK1(S172A) and TBK1(K38A) as well as wild-type TBK1 self-associate (Figure S5B). Although TBK1(S172A) and TBK1(K38A) acted as dominant-negative mutants to inhibit SeV-induced activation of ISRE and the IFN-β promoter (Figures 6A and 6B), they did not inhibit phosphorylation of wild-type TBK1 (Figure S5C). The simplest explanation for these observations

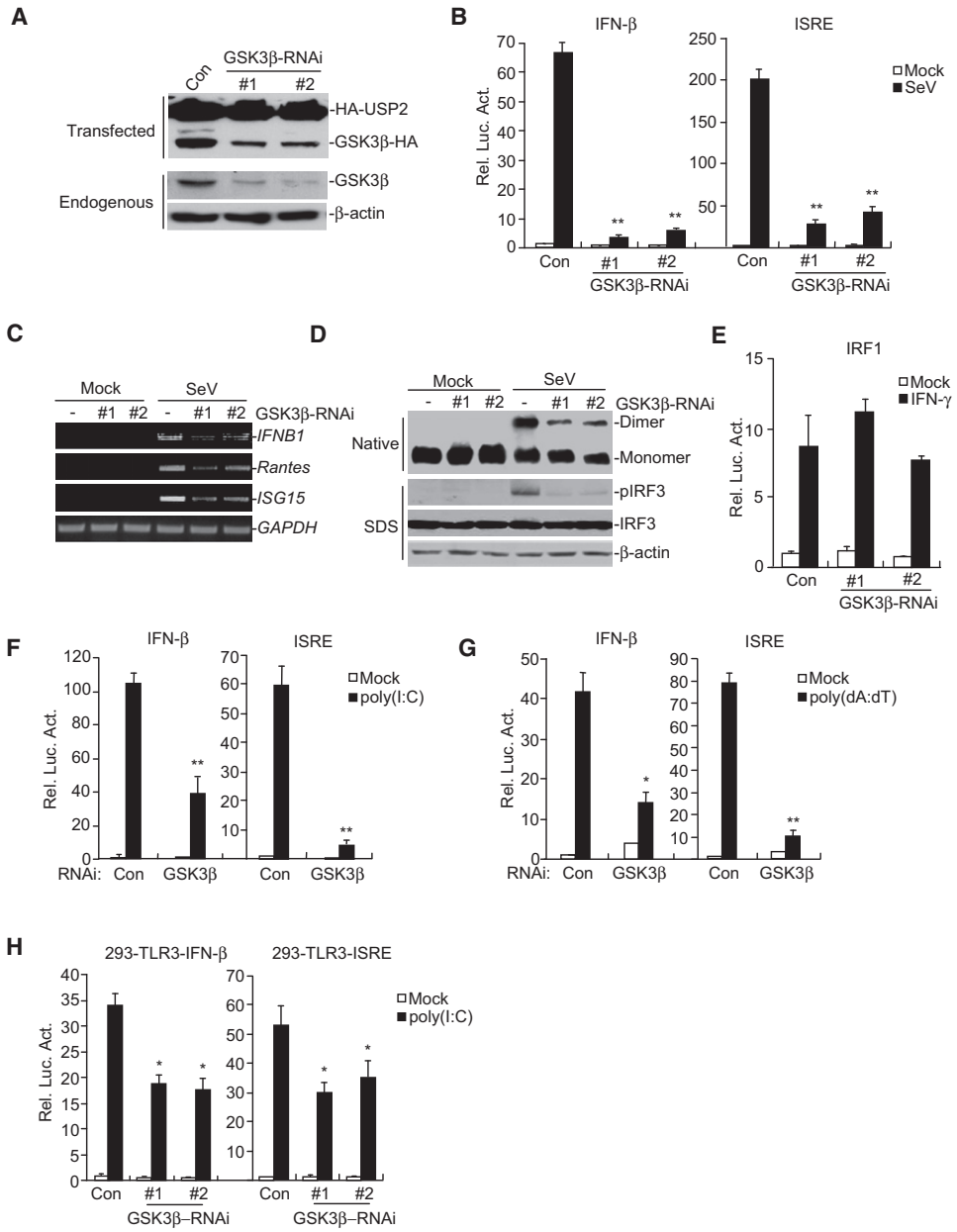


**Figure 2. Knockdown of GSK3β Inhibits Virus-Triggered Activation of IRF3 and Transcription of the *IFNB1* Gene**

(A) Effects of GSK3β RNAi plasmids on the expression of transfected and endogenous GSK3β. In the upper panel, 293 cells ($2 \times 10^5$) were transfected with expression plasmids for HA-GSK3β and HA-USP2 (0.1 μg each) and the indicated RNAi plasmids (2 μg). 24 hr after transfection, cell lysates were analyzed by immunoblot with anti-HA. In the bottom panels, 293 cells ($2 \times 10^5$) were transfected with control or the indicated GSK3β RNAi plasmids (2 μg each) for 24 hr. Cell lysates were then analyzed by immunoblots with the indicated antibodies.

(B) Effects of GSK3β RNAi plasmids on SeV-induced activation of the IFN-β promoter and ISRE. 293 cells ($1 \times 10^5$) were transfected with the indicated GSK3β RNAi (0.5 μg) and the reporter (0.1 μg) plasmids. 24 hr after transfection, cells were left uninfected or infected with SeV for 12 hr before luciferase assays were performed.

(C) Effects of GSK3β RNAi plasmids on SeV-induced expression of downstream genes. 293 cells ($2 \times 10^5$) were transfected with control or GSK3β RNAi plasmids (2 μg). 24 hr after transfection, cells were left uninfected or infected with SeV for 10 hr before RT-PCR was performed.

(D) Knockdown of GSK3β inhibits SeV-induced IRF3 dimerization and phosphorylation. 293 cells ($2 \times 10^5$) were transfected with GSK3β RNAi plasmid (2 μg). 24 hr after transfection, cells were infected with SeV or left uninfected for 8 hr. Cell lysates were separated by native (top) or SDS (bottom three panels) PAGE and analyzed with the indicated antibodies.

(E) Effects of GSK3β RNAi plasmids on IFN-γ-induced activation of the IRF1 promoter. 293 cells ($1 \times 10^5$) were transfected with the indicated GSK3β RNAi plasmids (0.5 μg) and the IRF1 reporter plasmid (0.1 μg). 24 hr after transfection, cells were left untreated or treated with IFN-γ (100 ng/ml) for 12 hr before luciferase assays were performed.

(F) Effects of GSK3β knockdown on cytoplasmic poly(I:C)-induced activation of ISRE and the IFN-β promoter. 293 cells ($1 \times 10^5$) were transfected with control or GSK3β RNAi plasmid (#1) (0.5 μg) and the indicated reporter plasmids (0.1 μg). 24 hr after transfection, cells were mock-transfected or transfected with poly(I:C) (1 μg) with Lipofectamine 2000 for 12 hr before luciferase assays were performed.

is that these TBK1 mutants bind to IRF3 and inhibit its phosphorylation by endogenous TBK1 after viral infection, thereby inhibiting virus-induced IRF3 activation and IFN-β induction.

We further examined phosphorylation of endogenous TBK1 in the absence or presence of viral infection. Although the basal phosphorylation of Ser172 of TBK1 in *Gsk3b*$^{-/-}$ MEFs was slightly higher than wild-type MEFs, SeV infection induced increased phosphorylation of TBK1 at Ser172 in wild-type but not *Gsk3b*$^{-/-}$ MEFs (Figure 6E). Taken together, these results suggest that GSK3β mediates virus-induced TBK1 autophosphorylation at Ser172 and that this phosphorylation is critical for TBK1-mediated IRF3 activation and IFN-β induction.

### GSK3β Is Required for Virus-Triggered Activation of NF-κB

Transcription induction of IFN-β requires coordinated and cooperative activation of both the transcription factors IRF3 and NF-κB. Previous studies have demonstrated that GSK3β is required for NF-κB binding to its consensus enhancer motifs (Hoeflich et al., 2000). We performed experiments to determine whether GSK3β plays a role in virus-triggered NF-κB activation. In reporter assays, knockdown of GSK3β dramatically inhibited SeV-induced NF-κB activation (Figure 7A). Knockdown of GSK3β also inhibited NF-κB activation mediated by overexpression of VISA and TRIF, suggesting that GSK3β is important for both RIG-I and MDA5 and TLR3-mediated NF-κB activation (Figure 7C). Moreover, SeV-induced IκBα phosphorylation and degradation were markedly impaired in *Gsk3b*$^{-/-}$ MEFs (Figure 7B). Previously, it has been shown that GSK3β is not required for TNF- and IL-1-induced IκBα phosphorylation and degradation and for NF-κB translocation into the nucleus but is required for NF-κB binding to its target sequences (Hoeflich et al., 2000). We confirmed these observations with *Gsk3b*$^{-/-}$ MEFs. We found that TNF- and IL-1-induced IκBα degradation were unaffected, but NF-κB-mediated transcription was impaired in *Gsk3b*$^{-/-}$ MEFs (Figures 7D and 7E). These results suggest that GSK3β is required for virus-triggered NF-κB activation through mechanisms distinct from TNF- and IL-1-induced NF-κB activation.

## DISCUSSION

GSK3β is a ser-thr kinase originally identified as a key enzyme involved in the glycogen synthesis. Subsequent studies suggest that GSK3β targets dozens of substrates involved in diverse cellular processes, such as metabolism, cell cycle, transcription regulation, vesicular transport, neuronal function, oncogenesis, and development (Jope and Johnson, 2004). Because of the diverse processes in which GSK3β is involved, efforts have been focusing on developing GSK3β inhibitors for various diseases such as diabetes, Alzheimer's disease, osteoporosis, cancer, and inflammatory diseases. In this study, we found that GSK3β is essential for virus-triggered IRF3 and NF-κB activation, IFN-β induction, and cellular antiviral response. Unlike most circumstances in which its functions are mediated by its kinase activity, the involvement of GSK3β in virus-triggered IRF3 activation and IFN-β induction is independent of its kinase activity. Instead, GSK3β mediates dimerization or oligomerization and autophosphorylation of TBK1, a kinase responsible for phosphorylating IRF3 after viral infection. The present study establishes an important role for GSK3β in virus-triggered IFN induction and cellular antiviral response and provides a mechanistic explanation on how TBK1 is activated after viral infection.

Overexpression of GSK3β potentiated virus-triggered activation of IRF3 and expression of the *IFNB1* gene. Conversely, RNAi-mediated knockdown of GSK3β markedly inhibited virus-triggered activation of IRF3 and induction of the *IFNB1* gene. Consistently, *Gsk3b*$^{-/-}$ MEFs showed impaired IRF3 activation and *Ifnb1* gene expression after viral infection, and reconstitution of GSK3β markedly restored the ability of these cells to activate ISRE and the IFN-β promoter in response to viral infection. Moreover, *Gsk3b*$^{-/-}$ exhibited increased viral replication upon VSV infection, suggesting that GSK3β is required for efficient cellular antiviral response.

Phosphorylation by GSK3β results in inhibition of many of its substrates (Jope and Johnson, 2004). For example, phosphorylation of c-Jun, AP-1, or CREB by GSK3β negatively regulates the DNA binding activities of these transcription factors (Hu et al., 2006). We therefore performed several experiments to test whether the kinase activity of GSK3β is required for its ability to potentiate virus-triggered IRF3 activation and IFN-β induction. First, both the constitutively active mutant GSK3β (S9A) and the inactive mutant GSK3β(K85A) enhanced virus-induced activation of IRF3 and the IFN-β promoter as wild-type GSK3β did in reporter assays. Second, inhibition of the kinase activity of GSK3β by its specific inhibitor SB216763 did not affect virus-triggered activation of IRF3 (data not shown). Third, reconstitution of *Gsk3b*$^{-/-}$ MEFs with kinase inactive mutant of GSK3β restored SeV-induced IFN-β expression and antiviral activity to similar degrees as with wild-type GSK3β. Fourth, overexpression of wild-type GSK3β, GSK3β(S9A), or GSK3β(K85A) caused TBK1 self-association and autophosphorylation to similar degrees. These findings suggest that the involvement of GSK3β in virus-triggered IRF3 activation and IFN-β induction is independent of its kinase activity. Although this finding is interesting and intriguing, it is not totally unexpected. GSK3β has been shown to inhibit E2F1 transcriptional activity and neural cell differentiation independent of its kinase activity (Zhou et al., 2008). Other ser-thr kinases have also exhibited kinase activity-independent functions. For example, RIP1, a ser-thr kinase required for TNF receptor I-mediated NF-κB activation, acts in a kinase activity-independent way in this process (Hsu et al., 1996).

(G) Effects of GSK3β knockdown on poly(dA:dT)-induced activation of ISRE and the IFN-β promoter. The experiments were performed as in (G) except that poly (dA:dT) was transfected instead of poly(I:C).

(H) Effects of knockdown of GSK3β on TLR3-mediated activation of ISRE and the IFN-β promoter. 293-TLR3 cells were transfected with control or GSK3β RNAi plasmid (0.5 μg) and the indicated reporter plasmids (0.1 μg). 24 hr after transfection, cells were left untreated or treated with poly(I:C) (25 μg/ml) for 12 hr before luciferase assays were performed.

Graphs show mean ± SD, n = 3. *$p < 0.05$; **$p < 0.01$. See also Figure S2.

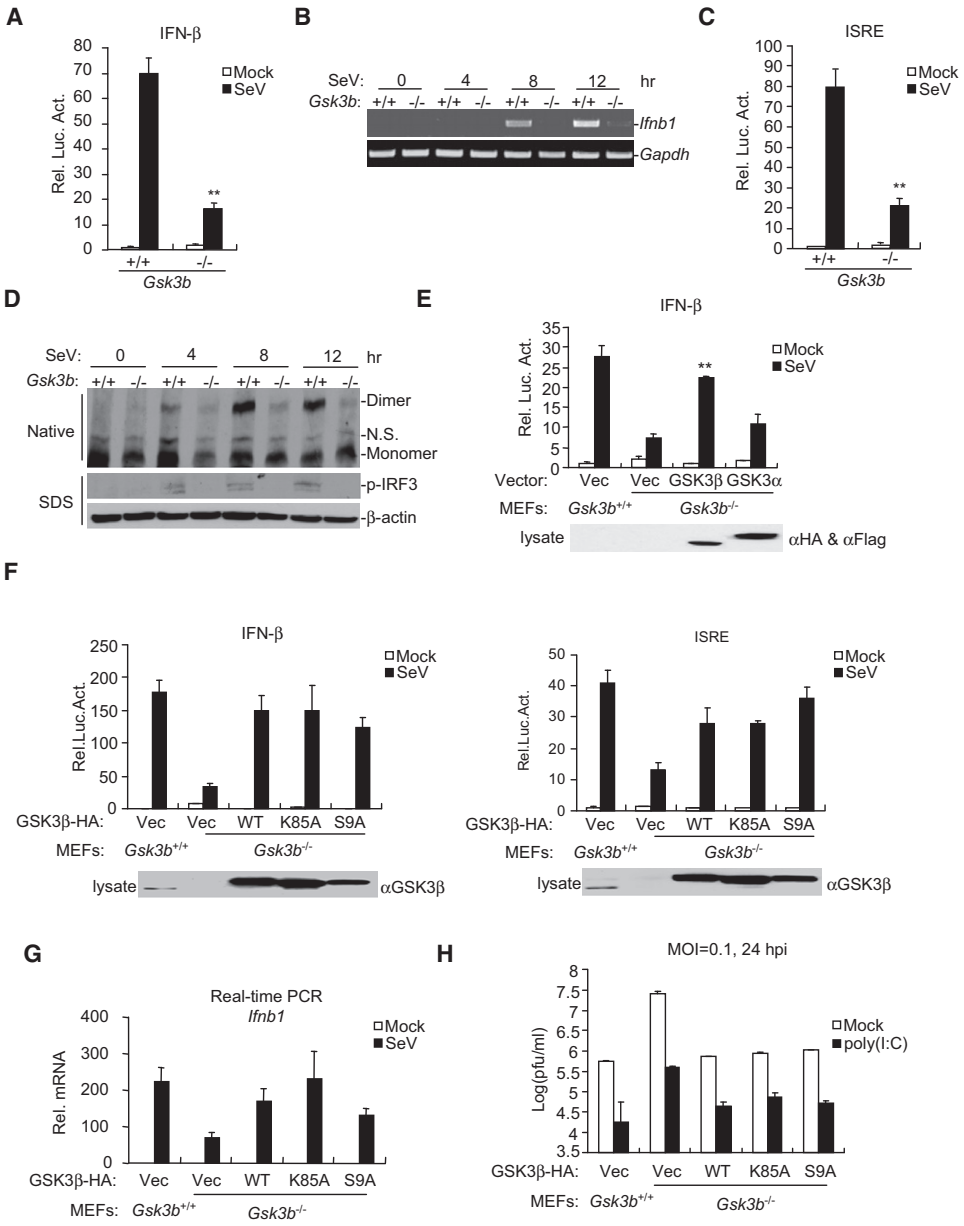


**Figure 3. GSK3β Deficiency Impairs Virus-Triggered IRF3 Activation, IFN-β Induction, and Cellular Antiviral Response**

(A) SeV-triggered activation of the IFN-β promoter is impaired in *GSK3β*$^{-/-}$ MEFs. *Gsk3b*$^{+/+}$ and *Gsk3b*$^{-/-}$ MEFs were transfected with the IFN-β promoter reporter plasmid (0.2 μg). 24 hr later, cells were left uninfected or infected with SeV for 12 hr before luciferase assays were performed.

(B) SeV-triggered transcription of the *Ifnb1* gene is impaired in *Gsk3b*$^{-/-}$ MEFs. *Gsk3b*$^{+/+}$ and *Gsk3b*$^{-/-}$ MEFs were left uninfected or infected with SeV for the indicated times before RT-PCR was performed.

(C) SeV-triggered activation of ISRE is impaired in *GSK3β*$^{-/-}$ MEFs. The experiments were done as in (A).

(D) SeV-induced IRF3 activation is impaired in *Gsk3b*$^{-/-}$ MEFs. *Gsk3b*$^{+/+}$ and *Gsk3b*$^{-/-}$ MEFs were left uninfected or infected with SeV for the indicated times. Cell lysates were separated by native (top) or SDS (bottom two panels) PAGE and analyzed with the indicated antibodies.

(E) Transduction of GSK3β but not GSK3α into *Gsk3b*$^{-/-}$ MEFs markedly restores SeV-induced activation of the IFN-β promoter. *Gsk3b*$^{-/-}$ MEFs were reconstituted with GSK3β or GSK3α by retrovirus-mediated gene transfer. The reconstituted cells were transfected with the IFN-β promoter reporter (0.2 μg) for 16 hr and then left uninfected or infected with SeV for 12 hr before luciferase assays were performed. Expression of the transduced proteins was detected by immunoblot with anti-HA (for GSK3β) and anti-Flag (for GSK3α).

(F) Complementation of *Gsk3b*$^{-/-}$ MEFs with the GSK3β mutants restores their responses to SeV-induced activation of the IFN-β promoter and ISRE. *Gsk3b*$^{-/-}$ MEFs were reconstituted with GSK3β or its mutants by retrovirus-mediated gene transfer. The reconstituted cells were transfected with the IFN-β promoter or ISRE reporter (0.2 μg) for 16 hr, and then left uninfected or infected with SeV for 12 hr before luciferase assays were performed. Expression of the transduced proteins was detected by immunoblot with anti-GSK3β.

(G) Complementation of *Gsk3b*$^{-/-}$ MEFs with GSK3β and its mutants restores their responses to SeV-induced transcription of the *Ifnb1* gene. *Gsk3b*$^{-/-}$ MEFs were reconstituted as in (E) and left uninfected or infected with SeV for 8 hr before real-time PCRs were performed.

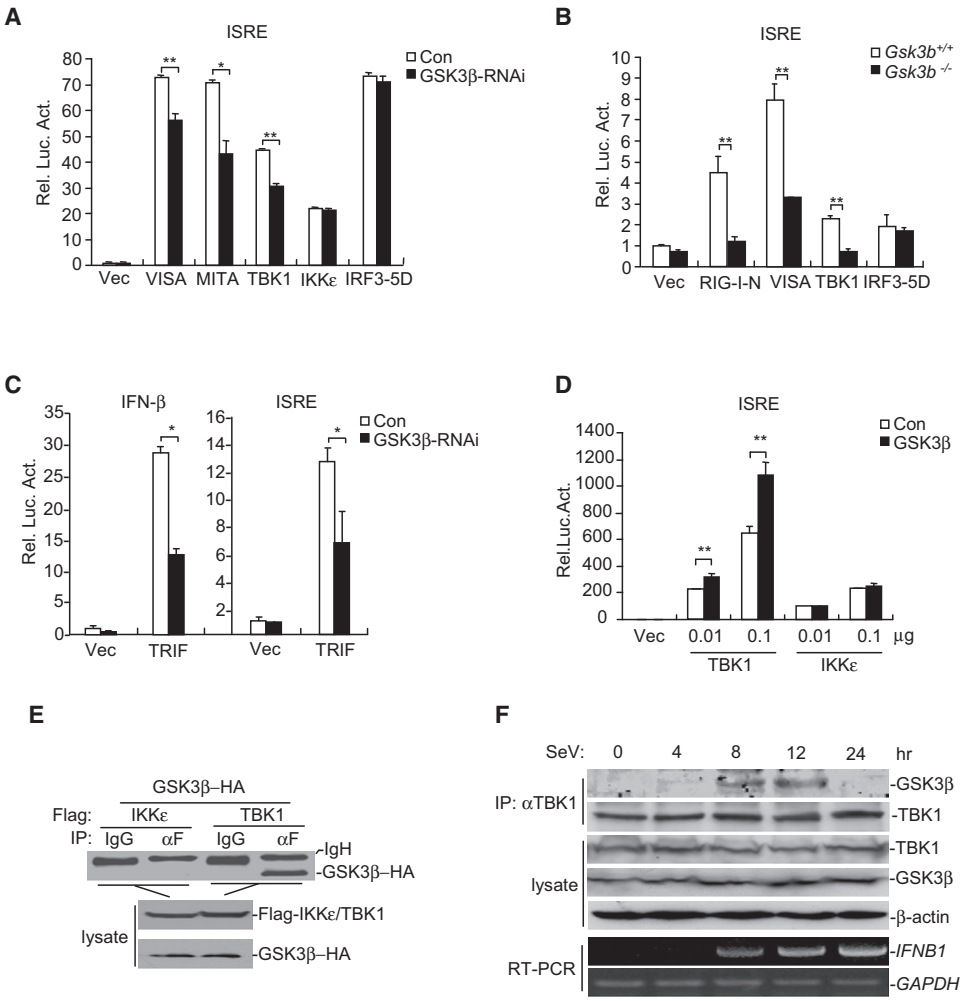


**Figure 4. GSK3β Mediates Virus-Triggered Signaling at the TBK1 Level**

(A) Knockdown of GSK3β inhibits VISA-, MITA-, and TBK1- but not IRF3(5D)-mediated ISRE activation. 293 cells ($1 \times 10^5$) were transfected with control or GSK3β RNAi plasmid (#1) (0.5 μg). 20 hr later, the cells were further transfected with the indicated plasmids (0.1 μg each). Reporter assays were performed 24 hr after transfection.

(B) Deletion of GSK3β impairs RIG-I-, VISA-, and TBK1- but not IRF3(5D)-mediated ISRE activation. *Gsk3b*$^{+/+}$ and *Gsk3b*$^{-/-}$ MEFs were transfected with the indicated plasmid (0.2 μg each) for 24 hr before reporter assays were performed.

(C) Knockdown of GSK3β inhibits TRIF-mediated activation of ISRE and the IFN-β promoter. The experiments were performed as in (A).

(D) GSK3β potentiates TBK1- but not IKKε-mediated ISRE activation. 293 cells ($1 \times 10^5$) were transfected with the indicated plasmids for 24 hr before luciferase assays were performed.

(E) GSK3β interacts with TBK1 but not IKKε. 293 cells were transfected with the indicated plasmids. Coimmunoprecipitation was performed with anti-Flag or control IgG. The immunoprecipitates were analyzed by immunoblot with anti-HA (top). The lysates were analyzed by immunoblots with anti-Flag or anti-HA (bottom).

(F) GSK3β is associated with TBK1 in a viral infection-dependent manner. 293 cells ($1 \times 10^8$) were left uninfected or infected with SeV for the indicated times. A small fraction of the cells ($1 \times 10^6$) were prepared for RT-PCR (bottom panels). The other cells were lysed and the lysates were immunoprecipitated with anti-TBK1 or preimmune serum. The immunoprecipitates were analyzed by immunoblot with anti-GSK3β or anti-TBK1 (top). The expression levels of the endogenous GSK3β, TBK1, and β-actin were detected by immunoblot analysis (middle).

Graphs show mean ± SD, n = 3. *p < 0.05; **p < 0.01. See also Figure S3.

Our experiments suggest that GSK3β is involved in virus-triggered IRF3 activation and IFN-β induction by targeting TBK1. Knockdown or deletion of GSK3β impaired IRF3 activation mediated by overexpression of TBK1 or its upstream components such as RIG-I, VISA, and MITA but not downstream component IRF3. Coimmunoprecipitation experiments indicated that GSK3β

(H) GSK3β plays an important role in cellular antiviral response. Wild-type or *Gsk3b*$^{-/-}$ MEFs were reconstituted with control, GSK3β, or GSK3β mutants as indicated. The cells were then transfected with poly(I:C) or mock transfected for 12 hr before cells were infected with VSV (MOI = 0.1). The supernatants were harvested 24 hr after infection and used for standard plaque assays.

Graphs show mean ± SD, n = 3. *p < 0.05; **p < 0.01.

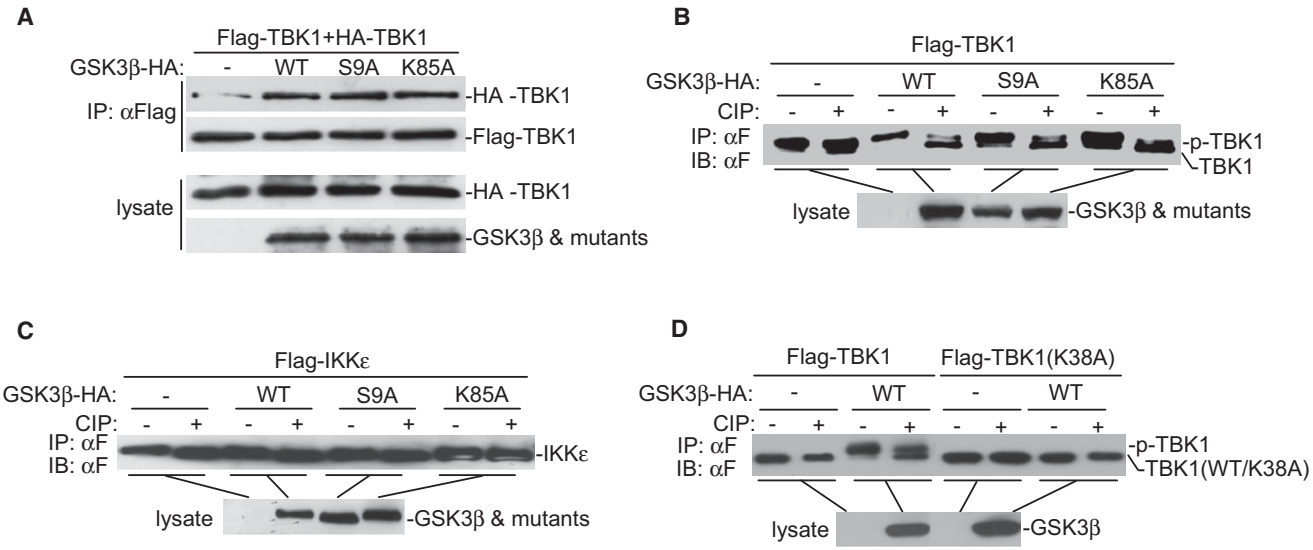


**Figure 5. GSK3β Promotes TBK1 Self-Association and Autophosphorylation**

(A) GSK3β and its mutants promote TBK1 self-association. 293 cells ($2 \times 10^6$) were transfected with the indicated plasmids (5 μg each). Coimmunoprecipitations were performed with anti-Flag. Immunoblot analysis was performed with anti-HA or anti-Flag (top). Expression levels of the transfected plasmids were confirmed by immunoblot analysis of the lysates with anti-HA (bottom).

(B) GSK3β and its mutants promote phosphorylation of TBK1. 293 cells ($2 \times 10^6$) were transfected with the indicated plasmids (6 μg each). Cell lysates were immunoprecipitated with anti-Flag. The immunoprecipitates were treated with buffer or calf intestine phosphatase (CIP) and analyzed by immunoblot with anti-Flag (top). Expression of the transfected proteins was analyzed by immunoblot with anti-HA (bottom).

(C) GSK3β and its mutants do not promote phosphorylation of IKKε. The experiments were performed as in (B).

(D) GSK3β promotes phosphorylation of wild-type but not kinase-inactive TBK1. The experiments were performed as in (B).

See also Figure S4.

was associated with TBK1 in a viral infection-dependent manner. In overexpression system, GSK3β, as well as its kinase inactive mutant GSK3β(K85A), promoted dimerization or oligomerization and autophosphorylation of TBK1 at Ser172. However, GSK3β was incapable of promoting autophosphorylation of TBK1 (K38A), the kinase inactive mutant of TBK1. Studies with *Gsk3b*$^{-/-}$ MEFs indicated that GSK3β was required for virus-triggered phosphorylation of TBK1 at Ser172. Mutation of Ser172 to alanine abolished the ability of TBK1 to activate IRF3 and this mutant acted as a dominant-negative mutant to inhibit virus-triggered activation of IRF3 and the IFN-β promoter. Taken together, our results suggest a model on the role of GSK3β in virus-triggered IRF3 activation pathway in which GSK3β is recruited to TBK1 and mediates TBK1 dimerization or oligomerization after viral infection. This step promotes TBK1 autophosphorylation at Ser172 and in turn phosphorylates IRF3. Currently, how viral infection causes association of TBK1 and GSK3β is unknown. In cell fractionation experiments, GSK3β was localized in all fractions examined, including the cytosol, mitochondria, and the ER fractions, and this distribution was not significantly affected by viral infection (data not shown). It is possible that viral infection causes recruitment of TBK1 to the VISA-associated complex, which then causes conformational changes or post-translational modifications of TBK1 and these provide high-affinity binding sites for GSK3β. Alternatively, virus infection may signal conformational changes, posttranslation modifications, or binding partner changes of GSK3β, resulting in its recruitment to TBK1-associated complex.

Endogenous coimmunoprecipitation experiments indicated that the interaction between GSK3β and TBK1 could be seen at 4 hr after SeV infection. However, the interaction reached maximum at 8–12 hr after SeV infection. Because deletion of GSK3β markedly impaired but did not completely abolish IRF3 activation and IFN-β induction after SeV infection, it is also possible that GSK3β is involved in an amplification cascade of virus-induced interferon induction through a virally induced complex rather than being required for the first wave of production of virus-induced interferons.

In addition to virus-triggered RLR-mediated IRF3 activation pathways, our study also demonstrated that GSK3β is required for TLR3- and TLR4-mediated IFN-β induction. This is consistent with the notion that IRF3 activation pathways triggered by various RLRs and TLRs converge at TBK1, which is targeted by GSK3β. Previously, it has been been shown that knockdown of GSK3β by RNAi or inhibitors decreased LPS-induced TNF production but increased LPS-induced IFN-β and IL-10 production in monocytes or macrophages (Martin et al., 2005; Wang et al., 2008). The reasons behind the discrepancy between our and the reported findings are unknown. Recently, it has been reported that the leucine-rich repeat domain-containing protein LRRFIP1, which is capable of recognizing viral and bacterial nucleic acids, mediates interferon production through a β-catenin-IRF3-dependent pathway (Rathinam et al., 2010; Yang et al., 2010). β-catenin is destabilized in an ubiquitination-proteasome-dependent manner by a destruction complex containing casein kinase Iα (CKIα), GSK3β, adenomatous polyposis coli (APC),

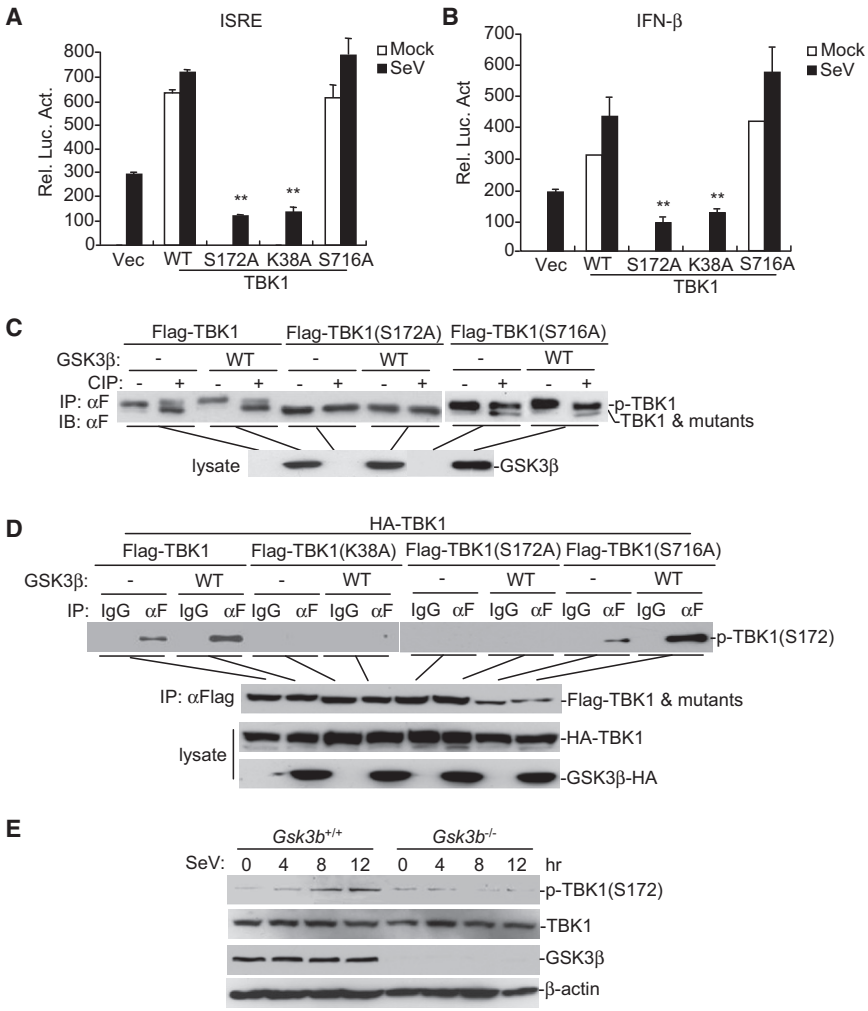


**Figure 6. GSK3β-Mediated Phosphorylation of TBK1 at Ser172 Is Required for Virus-Triggered IRF3 Activation and IFN-β Induction**

(A and B) Ser172 of TBK1 is critical for SeV-triggered activation of ISRE (A) and the IFN-β promoter (B). 293 cells ($1 \times 10^5$) were transfected with ISRE (A) or the IFN-β promoter (B) reporter plasmid (0.1 μg) and expression plasmids for TBK1 and its mutants (0.1 μg). 20 hr after transfection, cells were infected with SeV or left uninfected for 12 hr before luciferase assays were performed.

(C) GSK3β does not promote phosphorylation of TBK1(S172A). 293 cells ($2 \times 10^6$) were transfected with the indicated plasmids (6 μg each). Cell lysates were immunoprecipitated with anti-Flag. The immunoprecipitates were treated with buffer or CIP and analyzed by immunoblot with anti-Flag (top). Expression of GSK3β and its mutants were analyzed by immunoblot with anti-HA (bottom).

(D) The effects of GSK3β on phosphorylation of TBK1 and its mutants at residue 172. 293 cells ($2 \times 10^6$) were transfected with the indicated plasmids (6 μg each). Cell lysates were centrifuged at 13,000 rpm for 10 min. The supernatants were denatured by 1% SDS and heated for 5 min. The supernatants were diluted with regular lysis buffer until the concentration of SDS was decreased to 0.1%. The diluted supernatants were immunoprecipitated with anti-Flag. The immunoprecipitates were analyzed by immunoblot with anti-$p^{Ser172}$TBK1 (top). The expression levels of the transfected proteins were detected by anti-Flag and anti-HA, respectively.

(E) Impairment of virus-induced phosphorylation of TBK1 at Ser172 in GSK3β-deficient MEFs. *Gsk3b*$^{+/+}$ and *Gsk3b*$^{-/-}$ MEFs were left uninfected or infected with SeV for the indicated times. Cell lysates were analyzed with the indicated antibodies. Graphs show mean ± SD, n = 3. **p < 0.01. See also Figure S5.

and Axin (Ha et al., 2004). Whether and how the classical TBK1-IRF3 and the newly identified β-catenin-IRF3 pathways cross-talk through GSK3β remains to be investigated. One potential hypothesis is that microbial infection recruits GSK3β to TBK1 from the β-catenin destruction complex, thereby promoting activation of both the TBK1-IRF3 and the β-catenin-IRF3 pathways.

Virus-triggered transcription induction of type I IFNs requires coordinated actions of transcription factors IRF3 and NF-κB. We found that ablation of GSK3β expression severely impaired virus-triggered IκBα phosphorylation and degradation as well as NF-κB-mediated transcription. GSK3β is not required for TNF- and IL-1-induced IκBα phosphorylation and degradation and for NF-κB translocation into the nucleus, but is required for NF-κB binding to its consensus enhancer motifs (Hoeflich et al., 2000). Although our data could not tell whether GSK3β is required for NF-κB binding to its consensus DNA motifs after viral infection, they suggest that GSK3β is required for virus- and for TNF- and IL-1-triggered NF-κB activation pathways through distinct mechanisms. Although the exact mechanisms of GSK3β-mediated NF-κB activation in the virus-triggered signaling pathways require further investigations, our studies suggest that GSK3β regulates both virus-triggered IRF3 and NF-κB activation pathways. The establishment of a regulatory role for GSK3β in virus-triggered IRF3 and NF-κB activation contributes to the elucidation of the complicated molecular mechanisms of cellular antiviral response.

## EXPERIMENTAL PROCEDURES

### Reagents

Mouse monoclonal antibodies against Flag, HA, and β-actin (Sigma), phospho-TBK1(Ser-172) (BD Biosciences), and phospho-IRF3 (Ser396) (Upstate); rabbit polyclonal antibodies against IRF3 (Santa Cruz Biotechnology); and 293-TLR4-MD2-CD14 cells (Invitrogen) were purchased from the indicated manufacturers. SeV and VSV were previously described (Zhong et al., 2008, 2009). Mouse anti-TBK1 and mouse anti-GSK3β antisera were raised against recombinant human TBK1(1–320) and human full-length GSK3β, respectively. *Gsk3b*$^{-/-}$ and wild-type MEFs (J. Woodgett, Ontario Cancer Institute) and 293-TLR3 (K. Fitzgerald, University of Massachusetts) were provided by the indicated investigators.

### Constructs

NF-κB, ISRE, the IFN-β, and IRF1 promoter luciferase reporter plasmids, mammalian expression plasmids for HA- or Flag-tagged RIG-I, VISA, MITA,

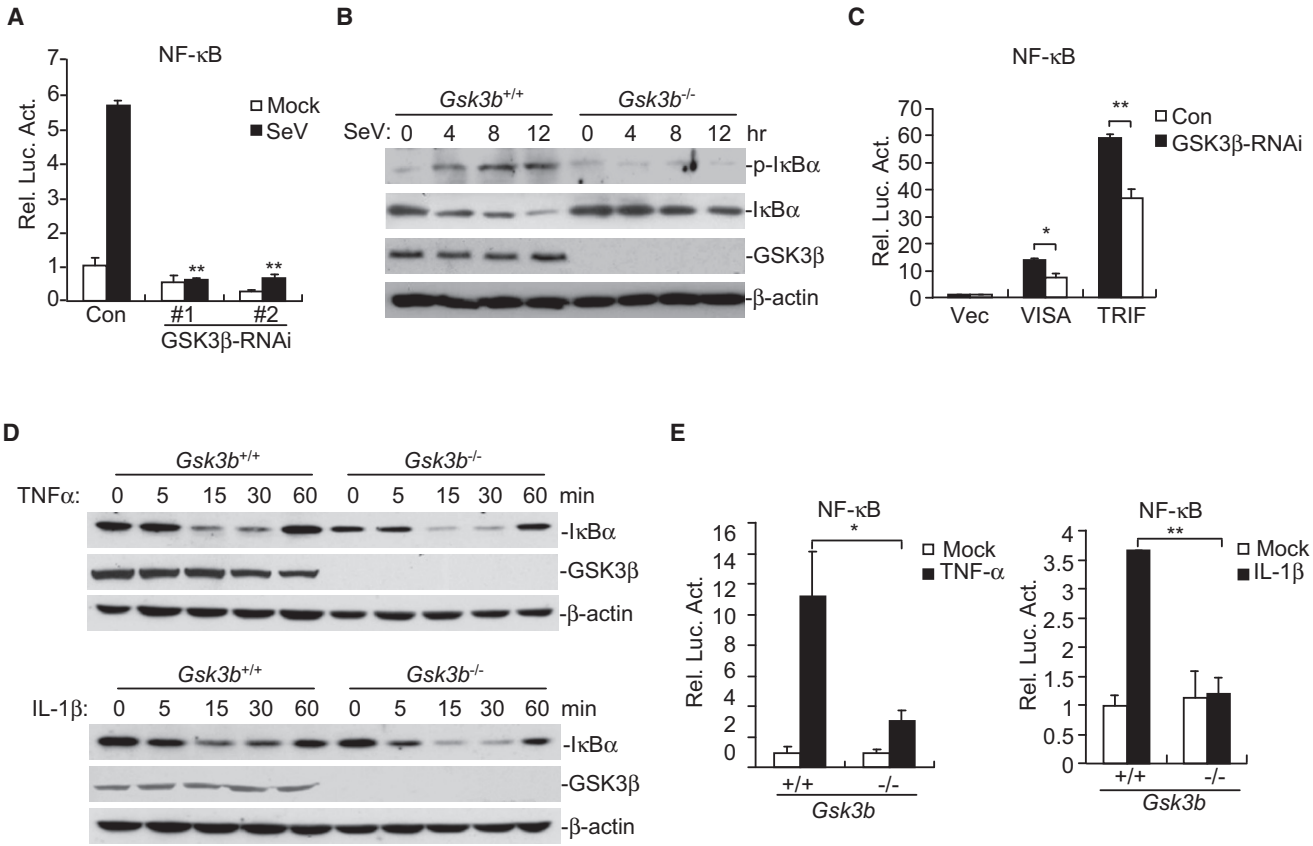


**Figure 7. GSK3β Is Required for Virus-Triggered Activation of NF-κB**

(A) Effects of GSK3β RNAi plasmids on SeV-induced NF-κB activation. 293 cells ($1 \times 10^5$) were transfected with GSK3β RNAi (0.5 μg) and NF-κB reporter (0.1 μg) plasmids. 24 hr after transfection, cells were left uninfected or infected with SeV for 12 hr before luciferase assays were performed. Graphs show mean ± SD, n = 3. **p < 0.01.

(B) SeV-induced IκBα phosphorylation and degradation was impaired in GSK3β-deficient MEFs. *Gsk3b*$^{+/+}$ and *Gsk3b*$^{-/-}$ MEFs were left uninfected or infected with SeV for the indicated times. Cell lysates were analyzed by immunoblot with the indicated antibodies.

(C) Knockdown of GSK3β inhibits VISA- and TRIF-mediated NF-κB activation. 293 cells were transfected with GFP or GSK3β RNAi construct and selected with puromycin for 2 days. The cells were then transfected with NF-κB reporter plasmid and expression plasmid for VISA or TRIF. Reporter assays were performed 24 hr after transfection.

(D) GSK3β is not required for TNF- and IL-1-induced IκBα degradation. Wild-type and *Gsk3b*$^{-/-}$ MEFs were treated with TNF (10 ng/ml) or IL-1 (10 ng/ml) for the indicated times. Cell lysates were analyzed by immunoblots with the indicated antibodies.

(E) GSK3β is essential for NF-κB-mediated transcription-induced TNF and IL-1. Wild-type and *Gsk3*$^{-/-}$ MEFs were transfected with a NF-κB reporter plasmid and then treated with TNF (10 ng/ml) or IL-1 (10 ng/ml) for 10 hr before luciferase reporter assays were performed.

TRIF, IKKε, and TBK1 were previously described (Zhong et al., 2008). Mammalian expression plasmids for human GSK3α and GSK3β were purchased from Origene company. GSK3β-HA-pcDNA and its mutants (J. Woodgett, Ontario Cancer Institute), the expression plasmid for IRF3(5D) (J. Hiscott and R. Lin, McGill University) were provided by the indicated investigators. Flag- and HA-tagged GSK3α, GSK3β and its mutants and TBK1 mutants were also constructed in the retroviral vector MIGR1 that contains a GFP or puromycin marker (provided by Z. Huang, Wuhan University).

### Transfection and Reporter Gene Assays

The 293 cells (~$1 \times 10^5$) were seeded on 24-well plates and transfected the following day by standard calcium phosphate precipitation. MEFs were transduced by Lipofectamine 2000 (Promega). In the same experiment, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, 0.01 μg of pRL-TK *Renilla* luciferase reporter plasmid was added to each transfection. Luciferase assays were performed with a dual-specific luciferse assay kit (Promega). Firefly luciferase activities were normalized based on *Renilla* luciferase activities.

### Retrovirus-Mediated Gene Transfer

The packaging cell line Plat-E was transfected with the retroviral vectors by calcium phosphate precipitation. 12 hr later, cells were washed and new medium without antibiotics was added for 24 hr. The supernatant was filtered and used to infect wild-type or *Gsk3b*$^{-/-}$ MEFs in the presence of 4 μg/ml polybrene. The infection was repeated twice so that the transduction efficiency reached at least 90% as monitored by expression of GFP.

### Coimmunoprecipitation, Immunoblot Analysis, Native PAGE, and RT-PCR

These experiments were performed as described (Xu et al., 2005; Zhong et al., 2008).

### VSV Plaque Assays

Wild-type and *GSK3β*$^{-/-}$ MEFs were tranduced with GSK3α, GSK3β, and its mutants by retrovirus-mediated gene transfer method. The cells were grown in 24-well plate dishes and transfected by Lipofactamine with poly(I:C) (1 μg) or control buffer for 24 hr prior to VSV infection. At 1 hr postinfection, cells were washed with PBS and then fresh medium was added. The supernatant

was harvested 24 hr later and used to infect confluent BHK21 cells cultured. Plaque assays were then performed as described (Zhong et al., 2008).

### RNAi

Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine Inc.). The following sequences were targeted for human GSK3β cDNA: GSK3β-RNAi #1, aagaatcgagagctccaga; #2, aagtaatccacctctggct.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.immuni.2010.11.021.

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