Induction of an antiviral innate immune response relies on pattern recognition receptors, including retinoic acid-inducible gene 1-like receptors (RLR), to detect invading pathogens, resulting in the activation of multiple latent transcription factors, including interferon regulatory factor 3 (IRF3). Upon sensing of viral RNA and DNA, IRF3 is phosphorylated and recruits coactivators to induce type I interferons (IFNs) and selected sets of IRF3-regulated IFN-stimulated genes (ISGs) such as those for ISG54 (Ifit2), ISG56 (Ifi1), and viperin (Rsad2). Here, we used wild-type, glycogen synthase kinase 3x knockdown (GSK-3x−/−), GSK-3β−/−, and GSK-3α/β double-knockout (DKO) embryonic stem (ES) cells, as well as GSK-3β−/− mouse embryonic fibroblast cells in which GSK-3x was knocked down to demonstrate that both isoforms of GSK-3, GSK-3x and GSK-3β, are required for this antiviral immune response. Moreover, the use of two selective small-molecule GSK-3 inhibitors (CHIR99021 and BIO-ace-toxime) or ES cells reconstituted with the catalytically inactive versions of GSK-3 isoforms showed that GSK-3 activity is required for optimal induction of antiviral innate immunity. Mechanically, GSK-3 isoform activation following Sendai virus infection results in phosphorylation of β-catenin at S33/S37/T41, promoting IRF3 DNA binding and activation of IRF3-regulated ISGs. This study identifies the role of a GSK-3β-β-catenin axis in antiviral innate immunity.

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine protein kinase that is expressed ubiquitously in most cell types. In mammals, two distinct genes encode GSK-3, generating two related proteins, GSK-3α and GSK-3β. The kinase domains of these GSK-3 isoforms are 98% similar in their coding sequences and 85% similar in their amino acid sequences (12). Unlike most protein kinases, GSK-3 is active in unstimulated resting cells and can be positively or negatively regulated by phosphorylation. Kinase activity of GSK-3 is negatively regulated by phosphorylation at residue Ser9 on GSK-3β and residue Ser21 on GSK-3α, while phosphorylation of a tyrosine residue located in the T loop (Tyr279 in GSK-3α and Tyr216 in GSK-3β) allows increased and sustained phosphotransferase activity toward a large number of substrates (13, 14). One key substrate of GSK-3 is β-catenin, the intracellular cytoplasmic levels of which are kept low in resting cells by degradation via a multiprotein destruction complex containing GSK-3, casein kinase 1 (CK1), axin, adenomatous polyposis coli, protein phosphatase 2A (PP2A), and the E3-ubiquitin ligase β-transducin repeat-containing proteins (β-TrCP) (15). β-Catenin is phosphorylated by CK1 at Ser45, creating a priming site for phosphorylation by GSK-3, which in turn phosphorylates β-catenin at Thr41, Ser37, and Ser33 in a sequential manner. Phosphorylation of Ser37 and Ser33 creates a binding site for β-TrCP, resulting in proteosomal degradation of β-catenin (16). Therefore, these GSK-3 phosphoacceptor sites are part of a domain of β-catenin often qualified as the phosphodegron motif.
Recently, it was shown that β-catenin phosphorylation by GSK-3 does not inevitably lead to its degradation but may have important regulatory functions (reviewed in references 17 and 18).

GSK-3 influences a multitude of cellular activities, such as glucose metabolism, transcriptional regulation, oncogenesis, the cell cycle, and immunity, and dysregulation of the kinase has been linked to the initiation and progression of diseases such as Alzheimer's disease, diabetes, and cancer. The isoforms GSK-3α and GSK-3β have redundant functions in Wnt/β-catenin signaling (19, 20), early stages of chondrocyte differentiation (21), and mixed-lineage leukemia cell proliferation and transformation (22). However, their roles do not entirely overlap, as the ablation of each isoform in the mouse has a distinct consequence. GSK-3β knockout mice die before or at birth because of liver apoptosis or malformation of the heart (23, 24), while animals lacking GSK-3α are viable but sensitized to insulin (25). Indeed, GSK-3α and GSK-3β have been shown to have different functions in metabolism, cell differentiation, and cardiovascular development (26–28). At the molecular level, both isoforms play important roles in various signaling pathways, including the Wnt, Notch, Hedgehog, nuclear factor κB (NF-κB), Ras/mitogen-activated protein kinase (MAPK), cyclic AMP, transforming growth factor β (TGF-β)/activin, phosphatidylinositol-3′ kinase, jun kinase/stress-activated protein kinase (JNK/SAPK), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (29). These protein kinases influence multiple components of the innate and adaptive arms of the immune system, where they act as important regulators of the fine balance between pro- and anti-inflammatory cytokine production (30). Interestingly, most of the functions of GSK-3 isoforms in immune systems are mediated through control of transcription factors mediating cytokine production (reviewed in reference 31).

More recently, GSK-3β and β-catenin have been shown to play important roles in the context of antiviral innate immunity and the type I IFN response. In the case of TLR4 stimulation, GSK-3β negatively regulates (32) while β-catenin promotes IFN-β production in response to lipopolysaccharides (7). However, in RLR signaling pathways, a consensus picture has not yet emerged, likely because of the use of different experimental strategies, non-selective GSK-3 inhibitors, overexpression conditions, and in vitro assays. In fact, GSK-3 was shown to either negatively (33) or positively (34) regulate the antiviral type I IFN response. Interestingly, the latter study proposed that the GSK-3β isoform (but not GSK-3α) plays positive roles in virus-triggered IRF3 activation and IFN-β induction by promoting TBK1 activation in a manner independent of its kinase activity. As for the GSK-3 substrate β-catenin, it was reported to act as a coactivator in IRF3-mediated *ifnb* gene activation in response to Sendai virus (SeV) and vesicular stomatitis virus (VSV) infections, but the role of a GSK-3/β-catenin axis was not addressed (7, 8). Therefore, despite efforts to ascertain the role of GSK-3β and β-catenin involvement in the antiviral responses, a comprehensive study addressing both isoforms of GSK-3 and the role of the GSK-3/β-catenin axis in antiviral innate immunity was lacking.

Here, we report the essential roles of both GSK-3α and GSK-3β in antiviral innate signaling, indicating that GSK-3 is an important regulator of the cellular antiviral response. In addition, by using molecular and pharmacological approaches, we demonstrate that functions of GSK-3 are dependent upon its kinase activity through the phosphorylation of β-catenin, facilitating IRF3 DNA binding.

**MATERIALS AND METHODS**

**Antibodies, reagents, and plasmids.** Anti-GSK-3α (catalog no. 9338), anti-GSK-3β (catalog no. 9315), anti-p-GSK-3α/β (Thr279/216) (catalog no. G5791), anti-p-IRF3 Ser396 (4D4G; catalog no. 4947), anti-p-TBK1/NAK Ser172 (D52C2; catalog no. 5483), and anti-p-β-catenin Ser33/377-745 (catalog no. 9561) antibodies were purchased from Cell Signaling (Danvers, MA). Anti-human IGSF4 (catalog no. NBPI-31164), anti-human IGSF5 (catalog no. NBPI-32329), and anti-TBK1 (72B587; catalog no. IMG-270A) antibodies were purchased from Novus Biologicals (Littleton, CO). Anti-CBP (A-22; catalog no. sc-369/sc-369X) and anti-IRF3 (FL-425 [catalog no. sc-9082/sc-9082X] and C-20 [catalog no. sc-15991]) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG (catalog no. F7425), anti-β-actin (AC-74; catalog no. A2282), and anti-α-tubulin (DM1A; catalog no. T6199) antibodies were purchased from Sigma, Oakville, ON, Canada. β-Catenin (catalog no. 2337-1; Epitomics, Burlingame, CA), mouse β-catenin active (catalog no. 05-666; Millipore), and vimentin (AT131; catalog no. ALX-210-956; Enzo life Sciences, Plymouth Meeting, PA) antibodies were from the companies indicated. Anti-mouse IGSF4 and anti-mouse IGSF5 were kindly provided by Ganes C. Sen (The Lerner Research Institute, Cleveland, OH). Poly(C) was from GE HealthCare (Waukesha, WI) and was transfected with Lipofectamine 2000 (Invitrogen) at a final concentration of 1.0 μg/ml. CHIR99021 (CHIR; catalog no. 1748-5) was purchased from BioVision (Milpitas, CA), and BIO-actinomycin (Bio-ac; catalog no. 361551) was obtained from EMD Millipore Chemicals (Billerica, MA). Polybrene and puromycin were purchased from Sigma (St. Louis, MO).

Reporter plasmids pGL3-IFN-β-Luc and pGL3-ISRE-Luc have been described previously (35). pNF-κB-Luc was from Stratagene (La Jolla, CA), and Renilla reporter plasmid pRL-TK was from Promega (Madison, WI). Plasmids encoding FLAG-TBK1, FLAG-IKKi, and FLAG–IRF3-5D were provided by Rongtuan Lin (McGill University, Montreal, Quebec, Canada), and a plasmid encoding FLAG–β-catenin was provided by Daniel Lamarre (Université de Montréal, Montreal, Quebec, Canada). Plasmid pGL3-OT (TOPFlash) was a kind gift from Sylvain Meloche (Université de Montréal, Montreal, Quebec, Canada). Lentiviral plasmid pLent6-VS-LargeT was kindly provided by Sylvain Meloche (Université de Montréal, Montreal, Quebec, Canada) with kind permission from Bernard Thorens (University of Lausanne, Lausanne, Switzerland). A phosphodeﬁcient mutant form of β-catenin (S33A S37A T41A; referred to here as β-catenin 3A) was generated with the QuickChange Multi site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA).

**Cell culture and infections.** Human cervical carcinoma (HeLa), human endometrial carcinoma 1B (HEC-1-B), and human embryonic kidney (HEK) 293T cell lines and primary human fetal lung fibroblasts (MRC-5) were purchased from the American Type Culture Collection (Manassas, VA), and immortalized GSK-3β knockout mouse embryonic fibroblasts (MEFs) have been described previously (23). All cell lines were maintained in Dulbecco's modified Eagle medium (Multicell, Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (Multicell, Wisent). SeV strain Cantell was obtained from Specific-Pathogen-Free Avian Supply (Charles River Laboratories, North Franklin, CT) and used at 200 hemagglutinating units (HAU)/ml. Green fluorescent protein (GFP)-expressing SeV (SV-SV515-GFP; kindly provided by Benjamin ten Oever, Mount Sinai Hospital, New York, NY) was propagated in vero cells and quantified by standard plaque assay.

**ES cell lines and culture.** Wild-type (WT), GSK-3α/−/−, GSK-3β/−/−, and GSK-3α/−β/−/− double-knockout (DKO) mouse embryonic stem (ES) cell lines have previously been described (19, 36). Cell lines generated from DKO mouse ES cells that stably express WT or kinase-dead (K148A) GSK-3α and WT or kinase-dead (K148A) GSK-3β have been described earlier (19, 36).

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All of the ES cell lines were maintained in Dulbecco's modified Eagle medium (Multicell, Wisent) supplemented with 15% ES cell-tested fetal bovine serum (Multicell, Wisent), 100 μM 2-mercaptoethanol (catalog no. M7522; Sigma-Aldrich), 1 mM modified Eagle medium nonessential amino acids (Wisent), 2 mg/ml t-glutamine, 1 mM sodium pyruvate (Wisent), and 1,000 U/ml leukemia inhibitory factor (ESGRO; Millipore). Stable cell lines were maintained in medium supplemented with 250 μg/ml hygromycin (Invitrogen) to maintain selective pressure for transgene expression. Hygromycin was removed from the culture 24 h before each experiment to rule out possible interference.

Cre-mediated deletion of exon 3 of the mouse β-catenin gene. Non-immortalized CATNB<sup>ex3/flox/+</sup> MEFs were infected with the adenoviral Cre recombinase expression system Ad(RGD)-GFP-icre (Vector Bios labs, Philadelphia, PA) at a multiplicity of infection (MOI) of 500 to delete exon 3 of the mouse β-catenin gene. Cells were maintained for a further 4 days to ensure turnover of the residual β-catenin protein before infection with SeV for 8 h, and RNA was isolated for reverse transcription-quantitative PCR (RT-qPCR). CATNB<sup>ex3/flox/+</sup> MEFs were immortalized by lentiviral-basised large T antigen expression and used for electrophoretic mobility shift assays (EMASs) and VSV-GFP-based antisense viral assays. Immortalized CATNB<sup>ex3/flox/+</sup> MEFs were infected with the Cre recombinase expression system Ad(RGD)-GFP-icre or Ad(RGD)-CMV-icre (Vector Bios labs) as described above or, as controls, infected with Ad(RDG)-GFP or Ad(RDG)-CMV-Luc (Vector Bioslabs), respectively.

Lentiviral vector production and transduction. The RNAi Consortium (TRC)/Mission shRNA lentiviral vectors targeting GSK-3α (TRCN0000010340 and TRCN0000038681) and nontargeting control (SHC002) short hairpin RNA (shRNA) were purchased from Sigma (St. Louis, MO). Lentiviral vector production was conducted as described previously (37). Briefly, 293T cells (3.5 × 10<sup>6</sup> in a 100-mm dish) were transfected with 6 μg of a nontargeting control, specific shRNA, or pLent6-VS-Large T along with 1.5 μg of pMDLp/PRRE, 1.5 μg of pRSVL REV, and 3 μg of pVSVG. The medium was replaced with fresh medium at 16 h posttransfection. On the following day, medium containing lentivirus was harvested and filtered through a 0.45-μm filter before storage at −80°C until use. Lentiviral titers were determined by limiting-dilution assay with HeLa cells as described previously (38). GSK-3B<sup>+/−</sup> or CATNB<sup>ex3/flox/+</sup> MEFs were infected with lentivirus for 24 h in the presence of 8 μg/ml Polybrene and then subjected to puromycin or blasticidin (1 μg/ml). Following selection, all of the ES cell lines were maintained in Dulbecco's modified Eagle medium (Multicell, Wisent) supplemented with 250 μg/ml Polybrene and then subjected to puromycin or blasticidin (1 μg/ml).Following selection, the immortalized CATNB<sup>ex3/flox/+</sup> MEFs were maintained in culture in the presence of blasticidin (1 μg/ml).

Western blot and coimmunoprecipitation analyses. Western blot and coimmunoprecipitation analyses were accomplished according to previously described procedures (35, 39). Briefly, whole-cell extracts (WCEs) were prepared in Triton X-100 lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 50 mM NaF; 5 mM EDTA; 10% glycerol; 1 mM Na<sub>2</sub>VO<sub>4</sub>; 40 mM β-glycerophosphate; 0.1 mM phenylmethylsulfonyl fluoride; 5 μg/ml of leupeptin, pepstatin, and aprotinin; 1% Triton X-100) and resolved by 7.5 or 10% SDS-PAGE with an SE400 electrophoresis apparatus (GE Healthcare). The proteins were electrotransferred onto a nitrocellulose membrane (BioTrace NT, Pall Gelman Laboratory, Ann Arbor, MI) with a Trans-Blot electrophoretic transfer cell (Bio-Rad). The membranes were probed with primary antibodies, followed by a horseradish peroxidase-conjugated (HRP)-conjugated secondary Ig raised against the appropriate species (KPL, Gaithersburg, MD), and bands were detected with the Western Lightning ECL kit (Perkin-Elmer, Waltham, MA). For coimmunoprecipitation assays, WCEs were incubated with 1 μg of antibody at 4°C overnight. Immune complexes were captured with 40 μl of a protein A-Sepharose suspension and washed five times with Triton X-100 lysis buffer complemented with protease inhibitors, and bound proteins were eluted with 50 μl of 2× sample buffer.

RNA isolation and RT-qPCR analysis. Relative mRNA expression was performed by RT-qPCR analysis as previously described (35). After stimulation, total RNA was extracted from ES cells with TRIzol reagent (Invitrogen). RNA was quantified with NanoPhotometer (Imlpen GmbH, Munich, Germany), and samples were evaluated for integrity with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA (2 μg) was reverse transcribed into cDNA with the High Capacity cDNA reverse transcription kit with random primers (Applied Biosystems), and qPCR analysis was performed by TaqMan technology on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) as described by the manufacturer. Reaction mixtures contained 1.5 μl of cDNA (diluted 1:5), 5 μl of 2× TaqMan Fast qPCR master mix (Applied Biosystems), and 0.5 μl of 20× TaqMan gene expression assay (Applied Biosystems) in a final volume of 10 μl in a 384-well plate. A no-template control (in which the cDNA was replaced with water) was included. Amplification conditions were 95°C for 3 min, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. All qPCRs were done in triplicate for each sample and all of the genes. Relative mRNA expression was calculated according to the comparative threshold (CT) formula 2<sup>−ΔΔCT</sup> where ΔΔCT = ΔCT<sup>samples</sup> − ΔCT<sup>calibrator</sup> sample and ΔCT<sup>samples</sup> = CT<sup>target</sup> − CT<sup>endogenous control</sup>. Hprt (hypoxanthine phosphoribosyltransferase gene) and Tbp (TATA binding protein gene) were used as endogenous control genes. The sequences of the primers and Universal Probe Library (UPL) probes used are listed in Table 1.

Reporter gene assays and ELISA. Subconfluent 293T cells (0.4 × 10<sup>6</sup>) were transfected with 40 ng of pGL3-IFN-β-Luc or pGL3-ISRE-Luc or pGL3-AGT (TOPFlash) along with 10 ng of the pRL-TK reporter (internal control expressing Renilla luciferase) by the CaPO<sub>4</sub> transfection method in 48-well plates. Cells were harvested 24 h posttransfection and lysed with lysis buffer (Promega). Extracts were assayed with a Dual-Luciferase reporter assay kit (Promega) according to the manufacturer’s instructions, and data were expressed as firefly luciferase values divided by Renilla luciferase values. IFN-β production in supernatants was determined with the VeriKine mouse IFN-β enzyme-linked immunosorbent assay (ELISA) kit (PBL Assay Science, Piscataway, NJ) according to the manufacturer’s instructions.

VSV-GFP-based antiviral assay and flow cytometry analysis. The antiviral state of cells following GSK-3β-β-catenin gene was measured by VSV-GFP reporter virus replication as described previously (40). Briefly, cells were infected with VSV-M51R-GFP and monolayers were examined and photographed with an inverted fluorescence microscope (Zeiss, Goettingen, Germany) at 16 h postinfection. Moreover, VSV-M51R-GFP-infected cells were trypsinized, fixed with 2% paraformaldehyde in phosphate-
buffered saline (PBS), and analyzed by FACS caliber (BD Bioscience) with BD FACSDiva software.

**EMSA.** To measure IRF3 DNA binding activity, an EMSA was performed as previously described (41). Briefly, 15 μg of nuclear extract was incubated with 500,000 cpm of a γ-32P-labeled double-stranded oligonucleotide containing the human ISG51 ISRE (IFN-stimulated response element) at room temperature for 15 min in a DNA binding buffer containing 20 mM Hepes (pH 7.0), 40 mM KCl, 20 mM NaCl, 10 mM NaF, 1 mM MgCl2, 1 mM β-glycerophosphate, 1 mM dithiothreitol, 0.1 mM EDTA, 4% (vol/vol) Ficoll, 0.08% (vol/vol) Triton X-100, and 2 μg of poly(dI-dC) in a final volume of 25 μL. Included in parallel were controls in which reaction mixtures were preincubated with 1 μL of an anti-IRF3 antibody, a homologous unlabelled oligonucleotide (10-fold), or a mutated unlabelled oligonucleotide (10-fold) for 15 min at 4°C before the addition of the radiolabeled oligonucleotides. In the case of MEF cells, the EMSA reaction was performed with 15 μg of WCE at 4°C for 15 min, and as controls, selected samples were preincubated with 2.5 μL of an anti-IRF3 antibody for 60 min at 4°C. Reaction products were resolved at 150 V for 3 h on a 5% acrylamide gel (42). The dried gels were exposed for autoradiography with Typhoon scanner 9410. The sequence of the double-stranded oligonucleotides used (sense orientation) was 5'-GATCGGGAAAGGGAAACCGAAACTGAAGCCA-3'. The mutated oligonucleotide was identical except for the substitution of a C for the underlined G (42).

**Chromatin immunoprecipitation (ChIP).** HeLa cells (1 × 107) or transfected 293T cells (3 × 107) were cross-linked with 1% formaldehyde for 10 min at room temperature, 125 mM glycine was added, and the mixture was incubated for 5 min. Cells were then washed in ice-cold PBS and lysed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 50 mM NaF; 40 mM β-glycerophosphate; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.05% sodium deoxycholate; 0.1 mM phenylmethylsulfonyl fluoride; 5 μg/mL leupeptin, pepstatin, and aprotinin). Cellular extracts containing chromatin were sonicated on ice to shear the DNA to ~500 bp with a Fisher Sonic Dismembrator 500 (eight cycles of 15 pulses at 30% amplitude). After centrifugation at 12,000 × g for 5 min at 4°C, soluble chromatin fractions were preclotted with protein A/G magnetic beads (Dynabeads; Invitrogen) for 1 h and 5 μg of IRF3 antibody (FL-425; Santa Cruz sc-9082X) or FLAG antibody (Sigma F7425) was incubated with 5 to 25 μg of chromatin overnight at 4°C. Rabbit IgG was used in parallel as a negative control. Protein-DNA complexes were pulled down with protein A/G magnetic beads for 1 h, washed sequentially with cold low-salt buffer (20 mM Tris-HCl, pH 8.1; 150 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% Triton X-100), high-salt buffer (20 mM Tris-HCl, pH 8.1; 500 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% Triton X-100), LiCl wash buffer (20 mM Tris-HCl, pH 8.1; 1 mM EDTA; 250 mM LiCl; 1% NP-40; 1% sodium deoxycholate), and TE buffer (10 mM Tris-HCl, pH 8.1; 1 mM EDTA) and then eluted with fresh elution buffer (1% SDS, 0.1 M NaHCO3) at room temperature. Eluted DNA was reverse cross-linked at 65°C overnight, treated with RNase A (3 μg/mL, 30 min at 37°C) and proteinase K (200 μg/mL), extracted with phenol-chloroform, and then ethanol precipitated with linear polyacrylamide as the carrier (43). Input and purified DNA was analyzed by qPCR with the EvaGreen-2X kit from ABM (Richmond, BC, Canada) on a Rotor-Gene 2000 apparatus. Each sample was normalized to the input material and shown as fold induction versus dimethyl sulfoxide (DMSO)-treated samples or mock-infected cells. The primers used have been described earlier (8) and were as follows: ISG56 ISRE, 5'-GAATTC CGCTAGCTTTAGTTTCAC-3' and 5'-CCCATAGACAGGTTATATA AGGG-3'; ISG56 Exon 2, 5'-CTCTCCTGGTTGTGCTTACAA-3' and 5'-AATGAAATGTTGAAATGGTTG-3'.

**Statistical analyses.** Statistical analyses were performed with Prism version 5.0 (GraphPad Software, San Diego, CA). Comparison of two groups was carried out with a two-tailed unpaired t test, and comparison of more than two groups was carried out by one-way analysis of variance. Differences were considered significant at P values of <0.05.

**Identification numbers of genes and proteins mentioned in the text.** The identification numbers of the human and mouse genes and proteins mentioned here are as follows: GSK-3α, 2931/6064961; GSK-3β, 2932/56637; MAVS, 57506/228607; ISG56, 3434/15957; ISG54, 3433/15958; TBK1, 29110/56480; RIG-I, 23586/230073; IRF3, 54131/56611; IKKα, 9641/56489; IFN-β, 3456/15977; β-catenin (CTNNBI1), 1499/12387; Cxcl10, 3627/15945; viperin, 9154/58185.

**RESULTS**

Both isoforms of GSK-3, GSK-3α and GSK-3β, are required for the induction of representative IRF3-regulated genes. To clarify the role of GSK-3α and GSK-3β in antiviral signaling, we used an allelic series of mouse undifferentiated ES cells lacking GSK-3 isoforms (WT, GSK-3α−/−, GSK-3β−/−, or GSK-3α/β DKO). As reported, high levels of β-catenin were found in DKO cells. However, we observed that expression of ISG54 protein and IFN-β production in response to SeV infection were decreased in the absence of either isoform of GSK-3 and further decreased in DKO cells (Fig. 1A and B). Consistently, mRNA induction of IRF3-regulated ISGs Ifnb1, Rsad2 (viperin), Ifi22 (ISG54), and Ifit1 (ISG56) (44–46) in response to infection with SeV or transfection of synthetic RLR ligand poly(I-C) was decreased in the absence of either isoform of GSK-3 and was severely compromised in DKO cells (Fig. 1C). In comparison to a previous study (34), these findings suggest that, in addition to GSK-3β, GSK-3α also has a role in antiviral immunity.

To further confirm the involvement of GSK-3α in a differentiated cell model, we employed shRNA to knock down GSK-3α in GSK-3β−/− MEFs. Of the two shRNA constructs used, only one had 100% alignment with the target sequence, which corresponded to the extent of GSK-3α knockdown (Fig. 2A). Knockdown of GSK-3α resulted in an increase in β-catenin expression and severely compromised induction of ISG54, ISG56, and viperin activation in response to SeV. Consistently, IFN-β production in supernatant and induction of IRF3-regulated genes Ifit1b, Rsad2, Ifi22, and Ifit1 in response to SeV infection were decreased following the knockdown of GSK-3α (Fig. 2B and C). Moreover, induction of mRNA for the IRF3-dependent chemokines Cxcl10 and Ccl5 was also decreased (data not shown).

Next, to confirm the role of GSK-3α in a biological context, GFP-expressing VSV was used to measure the antiviral state of GSK-3β−/− MEFs depleted of GSK-3α. The VSV mutant (VSV-M51R-GFP) is a useful indicator to assay the antiviral status of MEFs (41). We observed that phosphotransferase activity is required for their antiviral functions. We first used the selective GSK-3 inhibitors (48, 49). The inhibitory
activity of these two structurally unrelated ATP competitors was assessed first by treatment of cells transfected with the canonical TOPFlash reporter construct, a luciferase reporter containing three T-cell factor (TCF) response elements that is activated in response to β-catenin elevation (50). Used at concentrations as low as 2 μM, both inhibitors showed activation of the TOPFlash reporter (Fig. 3A), correlating with the stabilization of β-catenin (Fig. 3C), reflecting the high potency of their GSK-3 phosphotransferase inhibiting activity. The effect of pharmacological inhibition of GSK-3 on the antiviral innate immune response was addressed next. Both inhibitors significantly decreased the SeV-induced activation of the ISRE and IFN-β promoters, two IRF3-regulated promoters (Fig. 3B). However, they were less effective in inhibiting the SeV-induced activation of the NF-κB pathway (data not shown). We consistently observed decreases in SeV-induced ISG54 and ISG56 activation in 293T cells treated with BIO-ac or CHIR compared to that in DMSO-treated cells (Fig. 3C). Of note, treatment with both inhibitors resulted in the decreased phosphorylation of Tyr279 in GSK-3α and Tyr216 in GSK-3β, a commonly used indicator of GSK-3 phosphotransferase activity (51), which resulted in β-catenin accumulation (Fig. 3C).

To further substantiate the role of GSK-3 kinase activity, we used DKO ES cells stably reconstituted with either V5-GSK-3α (WT or kinase-dead K148A mutant form) or FLAG–GSK-3β (WT or kinase-dead K85A mutant form). Validation of the model with the different constructs was verified at the level of β-catenin phosphorylation on S33/S37/T41, where a phosphosignal was observed only in lysates from WT ES cells and from DKO ES cells complemented with WT V5-GSK-3α and FLAG–GSK-3β (Fig. 3D, lanes 1, 3, and 5). Conversely, total β-catenin expression was increased in ES cells lacking GSK-3 or expressing kinase-dead versions in DKO cells (Fig. 3D, lanes 2, 4, and 6). Using these validated reconstituted models (19, 36), we demonstrate that the mRNA induction of IRF3-regulated genes was partially (Rsad2) or totally (Ifit1 and Ifit2) restored following the expression of either WT V5-GSK-3α or FLAG–GSK-3β. However, expression of kinase-dead mutants failed to rescue the induction of these ISGs in response to SeV infection (Fig. 3E).

The GSK-3–β-catenin pathway positively regulates antiviral innate immunity downstream of IRF3 activation. The above data strongly suggest a role for catalytically active GSK-3 isoforms in the regulation of RLR signaling events. Thus, to evaluate at what level of IRF3 signaling GSK-3 might act, we analyzed the effects of GSK-3 inhibitors on ISRE and IFN-β activation mediated by multiple RLR signaling pathway effectors, including the RNA sensor RIG-I, mitochondrial antiviral signaling protein (MAVS), the IRF3 kinases TBK1 and IKKi, and IRF3-5D, a constitutively active form of IRF3 (52). Both GSK-3 inhibitors (BIO-ac and CHIR) resulted in statistically significant inhibition of ISRE and IFN-β activation by RIG-I and MAVS (not shown), as well as TBK1, IKKi, and IRF3-5D (Fig. 4A). In line with this, both inhibitors decreased ISG54 and ISG56 induction following the transfection of IRF3-5D (Fig. 4B). Likewise, SeV-induced TBK1 and IRF3 phosphorylation on activating phosphoacceptor sites (11, 53) was not decreased in either 293T cells exposed to GSK-3 inhibitors (Fig. 4C) or GSK-3β−/− MEF cells in which GSK-3α was knocked out in lysates from WT ES cells and from DKO ES cells complemented with either V5-GSK-3α and FLAG–GSK-3β (Fig. 3D, lanes 1, 3, and 5). Conversely, total β-catenin expression was increased in ES cells lacking GSK-3 or expressing kinase-dead versions in DKO cells (Fig. 3D, lanes 2, 4, and 6). Using these validated reconstituted models (19, 36), we demonstrate that the mRNA induction of IRF3-regulated genes was partially (Rsad2) or totally (Ifit1 and Ifit2) restored following the expression of either WT V5-GSK-3α or FLAG–GSK-3β. However, expression of kinase-dead mutants failed to rescue the induction of these ISGs in response to SeV infection (Fig. 3E).
down (data not shown). Similarly, the activation of TBK1 and IRF3 and its subsequent nuclear translocation were not impaired in DKO ES cells (Fig. 4D and E). These data support the notion that GSK-3 kinase activity acts downstream of TBK1/IKK activation, C-terminal domain phosphorylation of IRF3, and its nuclear translocation. Interestingly, IRF3 DNA binding activity was, however, greatly decreased in DKO cells compared to that in WT cells (Fig. 5A). The IRF3 signal was specific, as pretreatment of the binding reaction mixture with an anti-IRF3 antibody interfered with the signal. As opposed to the use of an excess (10-fold) of an ISRE-mutated unlabeled oligonucleotide, the inclusion of a homologous unlabeled oligonucleotide resulted in undetectable IRF3 DNA binding activity. Accordingly, the DNA binding activity of IRF3 was verified in DKO ES cells stably reconstituted with either (WT or kinase-dead K148A mutant) V5-GSK-3α/H9251 or (WT or kinase-dead K85A mutant) FLAG–GSK-3β, as validated in Fig. 3D. As suspected, a partial rescue of IRF3 DNA binding to the ISRE was observed only in ES cells reconstituted with the catalyt-
ically active versions of GSK-3 isoforms (Fig. 5B). This shows that GSK-3 kinase activity likely acts at the transcriptional level, regulating the binding activity of IRF3.

Recent studies have demonstrated a role for β-catenin, the canonical substrate of GSK-3, in IRF3 transcriptional activation (7–9). However, as observed here, an increase in its expression level rather correlates with a decrease in the induction of IRF3-regulated genes (Fig. 1 to 3). To further determine the role of the GSK-3/β-catenin axis, we used the well-described gain-of-function mutant form of β-catenin in which exon 3, which encodes the crucial Ser/Thr residues for priming by CK1 (Ser45) and phosphorylation by GSK-3 (Ser33, Ser37, and Thr 41), is flanked by two LoxP sites (CATNBex3-flox/flox MEFS). Deletion of exon 3 precludes the phosphorylation of β-catenin by GSK-3, resulting in its stabilization and nuclear accumulation and the transcriptional activation of β-catenin/TCF-responsive genes (34, 55). As ex-
expected, excision of exon 3 of the gene for β-catenin by Cre recombinase expression in MEFs resulted in the stabilization of β-catenin (Fig. 6A). Although largely expressed, the generation of the stabilized form of β-catenin correlated with a decrease in DNA binding activity of IRF3 as part of its holocomplex (6) (Fig. 6B). Accordingly, this effect is followed by diminished induction of the *Ifnb1*, *Rsad2*, *Ifti2*, and *Ifti1* mRNAs in response to SeV (Fig. 6C). Moreover, induction of the *Cxcl10* and *Ccl5* chemokine mRNAs was also decreased (data not shown). Furthermore, excision of exon 3 of the β-catenin gene by Cre recombinase expression resulted in a dramatic reduction in the ability of infected cells to mount an antiviral response against VSV (Fig. 6D and E). These results demonstrate that the phosphodegron motif of β-catenin is required for induction of the antiviral innate immune response.

Thus, we speculate that β-catenin phosphorylated by GSK-3 (p-β-catenin S33/S37/T41) may act to stimulate IRF3 transcriptional activity. If p-β-catenin S33/S37/T41 is required for this function, it should be recruited to the IRF3-CBP/p300 holocomplex following virus infection. This also implies that β-catenin should be phosphorylated on GSK-3 phosphoacceptor sites following virus infection. Indeed, we observed an increase in the phosphorylation of the β-catenin phosphodegron, which follows IRF3 phosphorylation in virus-infected ES cells. As expected, the β-catenin phosphosignal was completely absent from DKO cells (Fig. 7A). In addition, we observed an increase in the β-catenin phosphosignal level following virus infection of HEC-1-B cells, an IFN-unresponsive cell line (56) (Fig. 7B). Importantly, by using coimmunoprecipitation assays with HEC-1-B cells and primary MRC-5 fibroblasts, we documented an accumulation of p-β-catenin S33/S37/T41 in IRF3 immunocomplexes following virus infection.

FIG 4 Absence of GSK-3 does not affect IRF3 activation. (A) HEK 293T cells were cotransfected with pGL3-IFN-β or pGL3-ISRE (40 ng of each) along with pRL-TK (10 ng) and the plasmids indicated (10 ng of each). On the next day, cells were incubated with DMSO and a specific GSK-3 inhibitor (BIO-ac or CHIR) for 24 h. Relative luciferase activity was measured as described in Materials and Methods. Mean values ± the standard deviations of triplicates are shown. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001. (B) HEK 293T cells were transfected with FLAG–IRF3-5D. On the next day, cells were incubated with DMSO and a specific GSK-3 inhibitor (2 μM BIO-ac or CHIR) for 24 h. Whole-cell lysates were subjected to immunoblot analysis with the antibodies indicated. One representative experiment out of three independent experiments with similar results is shown. (C) HEK 293T cells were incubated with DMSO and a specific GSK-3 inhibitor (2 μM CHIR or BIO-ac) for 2 h, followed by SeV for the times indicated in the continuous presence of inhibitors. Whole-cell lysates were subjected to immunoblot analysis with the antibodies indicated. (D) WT and GSK-3α/β DKO ES cells were left uninfected or infected with SeV for the times indicated and then assayed for IRF3 nuclear translocation by immunoblot analysis. These results are representative of at least two independent experiments with similar results.
active (Fig. 7F), it was unable to associate with the ISG56 gene promoter although it was strongly active in the TOPFlash reporter assay.

Catenin 3A) lacking the GSK-3 phosphoacceptor sites showed that interaction when using an antibody that specifically recognizes /H9252.

More importantly, the use of a constitutively increased SeV-induced IRF3 recruitment on the ISG56 gene promoter (Fig. 7E). More importantly, the use of a constitutively increased SeV-induced IRF3 recruitment on the ISG56 gene promoter (Fig. 7E). Moreover, ChIP experiments demonstrated that GSK-3 inhibition decreased SeV-induced IRF3 recruitment on the ISG56 gene promoter (Fig. 7E). More importantly, the use of a constitutively increased SeV-induced IRF3 recruitment on the ISG56 gene promoter (Fig. 7E).

Therefore, ChIP experiments demonstrated that GSK-3 inhibition decreased IRF3 DNA binding activity by EMSA. These results are representative of at least two independent experiments with similar results.

FIG 5 GSK-3 regulates the DNA binding activity of IRF3. (A) Nuclear extracts used in the experiment shown in Fig. 4E were assayed for DNA binding activity of IRF3 by EMSA. (B) Nuclear extracts were prepared from WT and GSK-3α/β DKO ES cells, as well as DKO ES cells stably complemented with either WT or the kinase-dead forms of GSK-3α and GSK-3β, left uninfected or infected with SeV for the times indicated. Nuclear extract was assayed for IRF3 DNA binding activity by EMSA. These results are representative of at least two independent experiments with similar results.

infection (Fig. 7C and D). Moreover, we did not observe any interaction of IRF3 with the unphosphorylated form of β-catenin when using an antibody that specifically recognizes β-catenin when not phosphorylated by GSK-3 (data not shown). Accordingly, ChIP experiments demonstrated that GSK-3 inhibition decreased SeV-induced IRF3 recruitment on the ISG56 gene promoter (Fig. 7E). More importantly, the use of a constitutively active phosphodefficient mutant version of β-catenin (FLAG–β-catenin 3A) lacking the GSK-3 phosphoacceptor sites showed that although it was strongly active in the TOPFlash reporter assay (Fig. 7F), it was unable to associate with the ISG56 gene promoter following virus infection (Fig. 7G).

DISCUSSION

To our knowledge, this is the first comprehensive report showing the role of GSK-3α, as well as GSK-3B, in IRF3 signaling through the phosphorylation of β-catenin at S33/S37/T41, supporting a role for GSK-3 as an important regulator of antiviral innate immunity.

Using WT, GSK-3α−/−, GSK-3β−/−, and GSK-3α/β DKO mouse ES cells, as well as GSK-3β−/− MEF cells in which GSK-3α was silenced, we observed an essential role for GSK-3α, as well as GSK-3β, in the induction of selected sets of IRF3-regulated ISGs in response to SeV infection. Interestingly, the severely compromised antiviral response in DKO cells was partially (Rsad2) or totally (Ifit1 and Ifit2) restored following the stable expression of either WT V5-GSK-3α or FLAG–GSK-3β. Furthermore, by using molecular and pharmacological approaches, we demonstrate that the optimal induction of IRF3-regulated antiviral genes is dependent upon the phosphotransferase activity of GSK-3. Indeed, inhibition of GSK-3 kinase activity by two highly selective and structurally unrelated inhibitors (CHIR or BIO-ac) resulted in the decreased induction of ISRE and IFN-β gene promoters and antiviral genes. In line with this, inhibition of GSK-3 by BIO-ac has been reported to inhibit antiviral innate immunity in a β-catenin-dependent fashion (38). The inhibitors used in our study were shown to be quite selective. CHIR was reported to be a very selective inhibitor of GSK-3 in a study in which multiple GSK-3 inhibitors were profiled against a panel of >70 protein kinases (49). BIO-ac is a more selective analogue of 6-bromoirindirubin-32-oxime (BIO; also called GSK-3 inhibitor IX) and exhibits greater selectivity for GSK-3α/β than for cyclin-dependent kinase 5 (Cdk5)/p25, Cdk2/A, and Cdk1/B (48). As pharmacological inhibitors are prone to off-target effects, we used the GSK-3 inhibitors at low concentrations (2 to 5 μM). Other studies have used CHIR (57–61), BIO (62,63), and BIO-ac (38, 64) at concentrations as high as 10 to 20 μM. Moreover, reconstitution of DKO ES cells with kinase-dead forms of GSK-3α and GSK-3β showed decreased antiviral mRNA expression compared to that obtained by reconstitution with WT forms. Although we do not completely rule out a kinase-independent function for GSK-3 in innate immunity, our data obtained with DKO ES cells and GSK-3 inhibitors (Fig. 4) do not support the proposed role for GSK-3 in the activation of TBK1 following virus infection reported earlier (34). Therefore, our observations extend the understanding of the molecular role of GSK-3 isoforms in the antiviral response by questioning previous reports either proposing a negative role for GSK-3 (33) or suggesting that a catalytically inactive version of GSK-3β alone is sufficient for antiviral innate immunity (34). We therefore believe that the genetic and molecular approaches coupled with the use of selective pharmacological inhibitors at low concentrations favor a model where the catalytic activity of GSK-3-3 isoforms is indeed required for an optimal antiviral innate immune response.

Transcriptional regulation of IFN-β relies upon the activation and cooperative binding of multiple transcription factors, including IRF3, NF-κB, and ATF-2/c-Jun and transcriptional coactivators CBP/p300 (65). Constitutively expressed transcription factor IRF3 has a central role in the induction of immediate-early genes, including that for IFN-β (66). IRF3 activation is the result of its C-terminal phosphorylation, dimerization, and nuclear translocation. However, these steps are essential but not sufficient for transcriptional activation (67–69), and additional events, for example, coactivator recruitment, are required for optimal IRF3 transcription. β-Catenin has recently been suggested to act as a coactivator of IRF3, allowing the recruitment of the acetyltransferases CBP/p300 (7–9). However, none of these studies addressed...
FIG 6 Deletion of the phosphodegron motif of β-catenin decreases the DNA binding activity of IRF3 and the antiviral innate immune response following SeV infection. (A to C) Exon 3 of the mouse β-catenin gene was deleted from CATNB<sup>ex3-flox/flox</sup> MEFs by the expression of Cre recombinase as described in Materials and Methods. (A) WCEs were subjected to immunoblot analysis with the antibodies indicated. (B) WCEs were assayed for IRF3 DNA binding activity by EMSA 8 h after SeV infection. The same cellular extracts were also used for immunoblot (Western blot [WB]) analysis with the antibodies indicated. (C) RNA was extracted from cells left uninfected or infected with SeV for 8 h and analyzed by RT-qPCR with primers for the genes indicated. Expression of the mRNA for each gene is presented relative to that in SeV-infected MEFs that do not express Cre. (D) Cells were infected with VSV-M51R-GFP at an MOI of 10, and monolayers were examined and photographed with an inverted fluorescence microscope at 16 h postinfection. One representative experiment out of two independent experiments with similar results is shown. FL, fluorescent; BF, bright field. (E) VSV-M51R-GFP infection at the MOI indicated was determined by quantifying the percentage of GFP-positive cells by flow cytometry. The data shown were pooled from two independent experiments and are relative to those obtained with Ad(RDG)-CMV-Luc treated cells. One representative experiment out of three independent experiments with similar results is shown. **, \( P < 0.01 \); ***, \( P < 0.001 \).
the state of β-catenin phosphorylation at S33/S37/T41 (GSK-3 consensus sites) or the role of GSK-3 in this precise context. Here, we propose that activation of GSK-3 during viral infection results in increased phosphorylation of β-catenin at S33/S37/T41 and recruitment of p-β-catenin to IRF3, which likely acts as a coactivator of IRF3 DNA binding (Fig. 8). (i) Indeed, inhibition of GSK-3 kinase activity abrogated the induction of IRF3-regulated genes and IRF3 promoter recruitment without affecting its activation. (ii) In addition to the fact that β-catenin was shown to accumulate in the nuclear compartment in response to infection (8, 38), we further show that GSK-3-phosphorylated β-catenin is enriched in IRF3-CBP immunocomplexes following virus infection. (iii) Phosphodeficient mutant β-catenin lost the ability to associate with the ISG56 gene promoter following virus infection.
 Known yet. Indeed, β-catenin could be phosphorylated after its recruitment to IRF3 by nuclear GSK-3 or a fraction of phosphorylated β-catenin could be freed from the destruction complex by activated IRF3, allowing their translocation to the nucleus. Our unpublished observation of GSK-3 and IRF3 interaction supports these scenarios. As S33/S37/T41 phosphorylation leads to the eventual degradation of β-catenin, it is possible that IRF3 interaction with the phosphorylated form of β-catenin sequesters it from being recognized by the E3 ubiquitin ligase β-TrCP. A role for β-catenin phosphorylation at S33/S37/T41 in antiviral innate immunity is not unexpected. Recently, β-catenin phosphorylation by GSK-3 has emerged as a mechanism through which GSK-3 exerts effects on multiple cellular processes, including mitosis (70, 71), cell migration (72), neuroepithelial integrity (73), and neuronal excitability (74). Phosphorylation of β-catenin at Ser552 in response to VSV infection was previously reported (7); however, we did not observe any increase in the basal signal level at this phosphorylation site following SeV infection in a previous study (38).

Increased phosphorylation of β-catenin at S33/S37/T41 (Fig. 7A and B) and glycogen synthase at S641 (data not shown) following viral infection is indicative of increased GSK-3 kinase activity. Through the use of immunocomplex in vitro kinase assays, we have also observed an increase in the phosphotransferase activity of GSK-3 following virus infection (data not shown). In addition to SeV, activation of GSK-3 during infection with coxsackievirus, an RNA virus (75), and in response to HIV-1 Tat protein (76) has previously been reported. However, it is still unknown how GSK-3 becomes activated following virus infection. We did not observe any changes in the level of T-loop tyrosine phosphorylation (Tyr279 in GSK-3β and Tyr216 in GSK-3α) following SeV infection (Fig. 3C). Several alternative scenarios are possible, including the inhibition of kinases such as V-Akt (from the murine thymoma viral oncogene; AKT) and p90 ribosomal S6 kinase (RSK; data not shown), as well as Ca2+/calmodulin-dependent protein kinase cyclic-AMP-dependent protein kinase (CAMKII), and p70 S6 kinase (13, 77), that target negative phosphoacceptor sites Ser9 and PP2A could also be involved (78), considering the ability of IRF3 to interact with both of them (79, 80) and GSK-3 (data not shown). As GSK-3 is important for the induction of an antiviral immune response, it is likely that it is a target of viral proteins. The NS5A protein of hepatitis C virus (81, 82), the hepatitis B virus X protein (83), Epstein-Barr virus (84), and the latency-associated nuclear antigen of Kaposi’s sarcoma-associated herpesvirus (85) have been reported to inhibit GSK-3 and lead to the stabilization of unphosphorylated β-catenin. The utilization of GSK-3 by the innate immune system is not surprising, as this kinase is constitutively active and thus no other intermediate steps are required before its utilization in rapid innate immune responses.

Altogether, our results suggest that GSK-3 activates the antiviral innate immune response in part through phosphorylation of the phosphodegron motif of β-catenin, which regulates subsequent IRF3-DNA binding and gene expression.

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FIG 8 Model representing the implication of the GSK-3/β-catenin axis in IRF3-regulated RLR-dependent antiviral innate immunity. Virus infection activates TBK1 through MAVS, leading to IRF3 phosphorylation. In addition, it activates GSK-3 to phosphorylate β-catenin, which in turn is recruited to the IRF3-holocomplex for optimal DNA binding of IRF3 and activation of antiviral genes. The model was created with Servier Medical Art templates licensed under a CC BY 3.0 license.

(iv) Lack of p-β-catenin in DKO cells complemented with a kinase-dead form of either GSK-3α or GSK-3β correlates with abrogated DNA binding of IRF3 and decreased antiviral mRNA induction. (v) Expression of β-catenin lacking GSK-3 phosphorylation sites (or the so-called phosphodegron motif) demonstrated a decrease in IRF3 DNA binding activity affecting the induction of representative IRF3-regulated genes and reducing the establishment of a functional antiviral state following virus infection. Thus, our data suggest a positive role for β-catenin phosphorylated at S33/S37/T41 in antiviral innate immunity. Accordingly, a negative role for dephosphorylated active β-catenin in the innate immune response has also been shown (38). Recent studies have proposed models where histone deacetylase 6-mediated deacetylation of β-catenin is required for its nuclear accumulation upon infection (8, 9). Our study provides another layer of regulation where fine-tuning of the RLR/IRF3-mediated antiviral response is also dependent on the phosphorylation of β-catenin by GSK-3 isoforms. As GSK-3 is considered a negative regulator of β-catenin (16), we believe that our study reconciles this apparent paradox by demonstrating a role for GSK-3-phosphorylated β-catenin in RLR signaling. Although we show that a fraction of N-terminally phosphorylated β-catenin interacts with IRF3, the sequence of events is not
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