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 (Ifit1), and viperin (Rsad2). Here, we used wild-type, glycogen synthase kinase 3α knockout (GSK-3α-/-), 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-3α was knocked down to demonstrate that both isoforms of GSK-3, GSK-3α 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-3-β-catenin axis in antiviral innate immunity.
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 be 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 IFR3 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 IFR3-mediated ifnb gene activation in response to Sendai virus (SeV) and vesicular stomatitis virus (VSV) infections, but the role of a GSK-3β/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-3β/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 IFR3 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 (4D4; catalog no. 4947), anti-p-TBK1/NAK Ser172 (D52C2; catalog no. 5483), and anti-p-β-catenin Ser33/37/Thr41 (catalog no. 9561) antibodies were purchased from Cell Signaling (Danvers, MA). Anti-human ISG54 (catalog no. NB1-P11164), anti-human ISG56 (catalog no. NB1-P13229), 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. A2228), 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 virepin (AT131; catalog no. ALX-210-956; Enzo Life Sciences, Plymouth Meeting, PA) antibodies were from the companies indicated. Anti-mouse ISG54 and anti-mouse ISG56 were kindly provided by Ganes C. Sen (The Lerner Research Institute, Cleveland, OH). Poly(1-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-acetoxime (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). NF-κB-Luc was from Stratagene (La Jolla, CA), and Renilla reporter plasmid pRL-TK was from Promega (Madison, WI). Plasmids encoding FLAG-TBK1, FLAG-IKKβ, 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 Lamarré (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-V5–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 phospho-deficient 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-S515R-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 (K85A) GSK-3β have been described earlier (19, 36).
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 mM l-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>x3-flox/flox</sup> MEFs were infected with the adenoviral Cre recombinase expression system Ad(RGD)-GFP-iCre (Vector Bioslabs, 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>x3-flox/flox</sup> MEFs were immortalized by lentivirus-based large T antigen expression and used for electrophoretic mobility shift assays (EMSAs) and VSV-GFP-based antisense assays. Immortalized CATNB<sup>x3-flox/flox</sup> MEFs were infected with the Cre recombinase expression system Ad(RGD)-GFP-iCre or Ad(RGD)-CMV-iCre (Vector Bioslabs) as described above or, as controls, infected with Ad(RGD)-GFP or Ad(RGD)-CMV-Luc (Vector Bioslabs), respectively.

**Lentival vector production and transduction.** The RNAi Consortium (TRC)/Mission shRNA lentivector targets generating GSK-3α (TRCN0000010340 and TRCN0000038681) and nontargeting control (SHC002) short hairpin RNA (shRNA) were purchased fromSigma (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-V5-VS-Large T along with 1.5 μg of pMDLg/pRRE, 1.5 μg of pRSV-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-3β<sup>−/−</sup> or CATNB<sup>x3-flox/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, the immortalized CATNB<sup>x3-flox/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-mercaptoethanol; 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 (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. All of the ES cell lines were extracted from ES cells with TRIzol reagent (Invitrogen). RNA was quantified with NanoPhotometer (I muepn 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 the Applied 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 ([C<sub>T</sub>]) formula 2−<sup>ΔΔC<sub>T</sub></sup>, where ΔΔC<sub>T</sub> = C<sub>T</sub> test sample − C<sub>T</sub> calibrator sample and ΔC<sub>T</sub> = C<sub>T</sub> (target) − C<sub>T</sub> (endogenous control). 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-OT (TOPFlash) along with 10 ng of the pRL-TK reporter (internal control expressing Renilla lucerase) by the CaPO<sub>4</sub> transfection method in 48-well plates. Cells were harvested 24 h posttransfection and lysed with passive 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 lucerase values divided by Renilla lucerase values. IFN-β production in supernatants was determined with the VeriKine mouse IFN-β enzyme-linked immunosorbent assay (ELISA) kit (PBL Essay 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-3α knockdown or deletion of exon 3 of the 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-

### Table 1 RT-qPCR probes and primers used in this study

| Gene      | RefSeq accession no. | UPL probe | Forward                     | Reverse                     |
|-----------|----------------------|-----------|-----------------------------|-----------------------------|
| Ifnb1     | NM_010510.1          | 18        | CTGGCTTCCATCATGAAACA        | AGAGGGCTGTGGTGAGAAGA        |
| Rsd2      | NM_021384            | 100       | GAGATTTCGCAAGGAGAGCTTA     | TGGGCGGAGGATAGACAGA         |
| Ifi1      | NM_008331.3          | 6         | TCTAACCAGGGGCTTGCAG        | GCAGAGCCCTTTTTGTAATAGT     |
| Ifi2      | NM_008332.3          | 42        | CAATTGCTAGGGGAGGCTGA       | TGATTTTCATCGGGAGATGTC       |
| Hprt      | NM_013556.2          | 95        | TCCTCCTCAGAGCCGTATT        | CTCCTGTCATCGCTAATC         |
| Tbp       | NM_013684.3          | 107       | CGGTTGCGTCATTTTCTC         | GGGTTATTCCTACACCACTAGA     |
buffered saline (PBS), and analyzed by FACScalibur (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 ISG15 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 unlabeled oligonucleotide (10-fold), or a mutated unlabeled 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 in 0.25× Tris-borate-EDTA running buffer. The gels were dried and 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 aproptin). 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 precleared 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 (10 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’-GGATTC CCCTAGGTTTATGTTCA-3’ and 5’-CCCCAAGACAGTGTTATATA AGGG-3’; ISG56 Exon 2, 5’-CCTCCTTGGTGCCTGCTACAA-3’ and 5’-AATGGAATGTGAAATGGCTGTA-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, 3434/159577; ISG54, 3433/15958; TBK1, 29110/56480; RIG-1, 23586/230073; IRF3, 54131/5661; IFI16, 9641/56489; IFN-β, 3456/15977; β-catenin (CTNNB1), 1499/12387; Cxcl10, 3627/15945; viperin, 91543/58185.

**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), Ifit2 (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 Ifnb1, Rsad2, Ifit2, 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 Cxcl5 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 cells in vitro, as the mutation renders it more sensitive to the inhibitory action of type I IFN (47). In agreement with the observed effect at the level of the IRF3-regulated genes described above, reduction of the GSK-3α expression level led to a significant increase in VSV-M51R-GFP replication in GSK-3β−/− MEFs (Fig. 2D and E). Altogether, our results indicate that the GSK-3 isoforms are essential antiviral signaling effectors that control the induction of IRF3-regulated ISGs.

**Phosphotransferase activity of both GSK-3α and GSK-3β is required for antiviral activity.** Having determined the essential role of the GSK-3 isoforms in the induction of IRF3-regulated genes and the establishment of a functional antiviral state, we examined whether phosphotransferase activity is required for their antiviral functions. We first used the selective GSK-3 inhibitors CHIR and BIO-ac (also called GSK-3 inhibitor X; note that these inhibitors target both isoforms equally) (48, 49). The inhibitory
activity of these two structurally unrelated ATP competitors was assessed first by treatment of cells transfected with the canonical a 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 GSK-3 α/β DKO ES cells left uninfected or infected with SeV for the times indicated. (A) Whole-cell lysates were subjected to immunoblot analysis with the antibodies indicated. (B) Supernatant was collected at the postinfection times indicated and analyzed for IFN-β production by ELISA. (C) RNA was extracted and analyzed by RT-qPCR with primers for the genes indicated following infection with SeV or transfection with poly(I·C) (1 μg/ml). The expression of each gene is presented relative to gene induction in WT cells infected with SeV. These results are representative of at least two independent experiments with similar results. ***, P < 0.001.

FIG 1 Essential roles of GSK-3α and GSK-3β in the induction of IRF3-regulated antiviral genes. WT, GSK-3α−/−, GSK-3β−/−, and GSK-3α/β DKO ES cells were left uninfected or infected with SeV for the times indicated. (A) Whole-cell lysates were subjected to immunoblot analysis with the antibodies indicated. (B) Supernatant was collected at the postinfection times indicated and analyzed for IFN-β production by ELISA. (C) RNA was extracted and analyzed by RT-qPCR with primers for the genes indicated following infection with SeV or transfection with poly(I·C) (1 μg/ml). The expression of each gene is presented relative to gene induction in WT cells infected with SeV. These results are representative of at least two independent experiments with similar results. ***, P < 0.001.

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...
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/IKKi 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-3 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 $\beta$-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/$\beta$-catenin axis, we used the well-described gain-of-function mutant form of $\beta$-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 $\beta$-catenin by GSK-3, resulting in its stabilization and nuclear accumulation and the transcriptional activation of $\beta$-catenin/TCF-responsive genes (54, 55). As ex-

FIG 3 Pharmacological inhibition of GSK-3 kinase activity blunts antiviral innate immunity. (A) HEK 293T cells were transfected with pGL3-TOPFlash along with pRL-TK. On the next day, cells were incubated with DMSO and a specific GSK-3 inhibitor (2 µM BIO-ac or CHIR) for 24 h. (B) HEK 293T cells were transfected with pGL3-IFN-$\beta$ or pGL3-ISRE along with pRL-TK. On the next day, cells were incubated with DMSO and a specific GSK-3 inhibitor (BIO-ac or CHIR) for 2 h at the concentrations indicated before infection with SeV for 16 h in the continuous presence of DMSO or an inhibitor. Relative luciferase activity was measured as described in Materials and Methods. Mean values ± the standard deviations of triplicates are shown. **, $P < 0.01$; ***, $P < 0.001$. These results are representative of three independent experiments with similar results. (C) HEK 293T cells were incubated with DMSO and a specific GSK-3 inhibitor (2 µM BIO-ac or CHIR) 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, E) GSK-3/3-β DKO ES cells were stably complemented with either WT or the kinase-dead forms of GSK-3α and GSK-3β. (D) Whole-cell lysates were subjected to immunoblot analysis with the antibodies indicated. (E) RNA was extracted from cells left uninfected or infected with SeV for the times indicated and analyzed by RT-qPCR with primers for the genes indicated. Expression of mRNA for each gene is presented relative to that in WT cells infected with SeV. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. These results are representative of at least two independent experiments with similar results.
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 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 β-catenin-/- 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.

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.
DISCUSSION

To our knowledge, this is the first comprehensive report showing the role of GSK-3α, as well as GSK-3β, 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 (Ift1 and Ift2) 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-bromoindirubin-3-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 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 transcriptional regulation of IFN-β transcription.

GSK-3/β-Catenin Pathway Fine-Tunes Antiviral Immunity
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-ex3-flox/flox 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.
Molecular and Cellular Biology

B-catenin deacetylation of have proposed models where histone deacetylase 6-mediated upon infection (GSK-3 isoforms. As GSK-3 is considered a negative regulator of response is also dependent on the phosphorylation of RLR/IRF3-mediated antiviral repression where fine-tuning of the RLR/IRF3-mediated antiviral re-

![FIG 8 Model representing the implication of the GSK-3/B-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 B-catenin, which in turn is recruited to the IRF3 holocomplex for optimal DNA binding of IRF3 and activation of antivi-

tural genes. The model was created with Servier Medical Art templates licensed under a CC BY 3.0 license.]

(iv) Lack of p-B-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 B-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 B-catenin phosphorylated at S33/S37/T41 in antiviral innate immunity. Accordingly, a negative role for dephosphorylated active B-catenin in the innate immune response has also been shown (38). Recent studies have proposed models where histone deacetylase 6-mediated deacetylation of B-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 re-

![FIG 8 Model representing the implication of the GSK-3/B-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 B-catenin, which in turn is recruited to the IRF3 holocomplex for optimal DNA binding of IRF3 and activation of antivi-

tural genes. The model was created with Servier Medical Art templates licensed under a CC BY 3.0 license.]

Increased phosphorylation of B-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-3α and Tyr216 in GSK-3β) 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 Ser21 in the N-terminal domain of GSK-3. Phosphatases PP1 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 N55A 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 B-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 antivi-

![FIG 8 Model representing the implication of the GSK-3/B-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 B-catenin, which in turn is recruited to the IRF3 holocomplex for optimal DNA binding of IRF3 and activation of antivi-

tural genes. The model was created with Servier Medical Art templates licensed under a CC BY 3.0 license.]

Although we show that a fraction of N-terminally phosphory-

![FIG 8 Model representing the implication of the GSK-3/B-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 B-catenin, which in turn is recruited to the IRF3 holocomplex for optimal DNA binding of IRF3 and activation of antivi-

tural genes. The model was created with Servier Medical Art templates licensed under a CC BY 3.0 license.]

B-catenin which regulates subsequent IRF3-DNA binding and gene expression.

ACKNOWLEDGMENTS

This work was supported by research grants from the Canadian Institutes of Health Research (CIHR) to M.J.S. (MOP-84571) and J.R.W. (MOP-74711). M.J.S. holds Canada Research chairs in inflammatory response signaling. K.A.K. received a postdoctoral fellowship from the Groupe de

No hallucination.
REFERENCES

1. Iwasaki A. 2012. A virological view of innate immune recognition. Annu Rev Microbiol 66:177–196. http://dx.doi.org/10.1146/annurev-micro-092611-150203.

2. Unterholzner L. 2013. The interferon response to intracellular DNA: why so many receptors? Immunobiology 218:1312–1321. http://dx.doi.org/10.1016/j.imbio.2013.07.007.

3. Yoneyama M, Fujita T. 2010. Recognition of viral nucleic acids in innate immunity. Rev Med Virol 20:24–22. http://dx.doi.org/10.1002/rmv.633.

4. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Goyle AJ, Liao SM, Maniatis T. 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 4:491–496. http://dx.doi.org/10.1038/ni921.

5. Sharma S, ten Oever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. 2003. Triggering the interferon antiviral response through an IKK-related pathway. Science 300:1148–1151. http://dx.doi.org/10.1126/science.1081315.

6. Suhara W, Yoneyama M, Kitabayashi I, Fujita T. 2002. Direct involvement of CREB-binding protein/p300 in sequence-specific DNA binding of virus-activated interferon regulatory factor-3 holocomplex. J Biol Chem 277:22304–22313. http://dx.doi.org/10.1074/jbc.M200192200.

7. Yang P, An H, Liu X, Wu M, Zheng Y, Rui Y, Cao X. 2010. The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. Nat Immunol 11:487–494. http://dx.doi.org/10.1038/ni.1876.

8. Zhu J, Coyne CB, Sarkar SN. 2011. PKC alpha regulates Sendai virus-mediated interferon induction through HDAc6 and beta-catenin. EMBO J 30:4838–4849. http://dx.doi.org/10.1038/embj.2011.351.

9. Chattopadhyay S, Fensterl V, Zhang Y, Veleeparambil M, Wetzel JL, Suhara W, Yoneyama M, Kitabayashi I, Fujita T. 2007. Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. J Biol Chem 282:9441–9447. http://dx.doi.org/10.1074/jbc.M209851200.

10. Hiscott J, Grandvaux N, Sharma S, Tenoever BR, Servant MJ, Lin R. 2003. Convergence of the NF-kappaB and interferon signaling pathways in the regulation of antiviral defense and apoptosis. Ann N Y Acad Sci 999:237–248. http://dx.doi.org/10.1196/annals.1299.042.

11. Servant MJ, Grandvaux N, ten Oever BR, Duguay D, Lin R, Hiscott J. 2003. Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. J Biol Chem 278:9441–9447. http://dx.doi.org/10.1074/jbc.M209851200.

12. Woodgett JR. 1990. Molecular cloning and expression of glycogen synthase kinase-3alpha and -beta. EMBO J 9:2431–2438.

13. Kaidanovich-Beilin O, Woodgett JR. 2011. GSK-3: functional insights from cell biology and animal models. Front Mol Neurosci 4:23. http://dx.doi.org/10.3389/fnmol.2011.00023.

14. Itoh S, Saito T, Hirata M, Ushita M, Ikeda T, Woodgett JR, Algl H, Schmid RM, Chung UI, Kawaguchi H. 2012. GSK-3alpha and GSK-3beta proteins are involved in early stages of chordomyocyte differentiation with functional redundancy through RelA protein phosphorylation. J Biol Chem 287:29227–29236. http://dx.doi.org/10.1074/jbc.M112.372086.

15. Westphal A, Chen X, Wang Z, Smith KS, Murphy M, Pilojo O, Somervaille TC, Cleary ML. 2008. Glycogen synthase kinase 3 in MLL leukemia maintenance and targeted therapy. Nature 455:1205–1209. http://dx.doi.org/10.1038/nature07284.

16. Hoeflich KP, Luo J, Rubie EA, Tsaio MS, Jin O, Woodgett JR. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature 406:90–90. http://dx.doi.org/10.1038/35017574.

17. Kerkela R, Kockeritz L, Macaulay K, Zhou J, Doble BW, Beham C, Greytak S, Woulfe K, Trivedi CM, Woodgett JR, Epstein JA, Force T, Huggins GS. 2008. Deletion of GSK-3beta in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. J Clin Invest 118:3609–3618. http://dx.doi.org/10.1172/JCI36245.

18. MacAulay K, Doble BW, Patel S, Hansotia T, Sinclair EM, Drucker DJ, Nagy A, Woodgett JR. 2008. Tissue-specific role of glycogen synthase kinase 3beta in glucose homeostasis and insulin resistance. Mol Cell Biol 28:6314–6328. http://dx.doi.org/10.1128/MCB.00763-08.

19. Force T, Woodgett JR. 2009. Unique and overlapping functions of GSK-3 isozymes in cell differentiation and proliferation and cardiovascular development. J Biol Chem 284:9643–9647. http://dx.doi.org/10.1074/jbc.R800077200.

20. Woodgett JR. 2012. Can a two-faced kinase be exploited for osteosarcoma? Nat J Natl Cancer Inst 104:722–723. http://dx.doi.org/10.1093/jnci/djr223.

21. Han M, Renahi K, Jope RS, Michalek SM. 2005. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol 6:777–784. http://dx.doi.org/10.1038/ni1221.

22. Beurel E, Michalek SM, Jope RS. 2010. Innate and adaptive immune responses regulated by glycogen synthase kinase-3 (GSK3). Trends Immunol 31:24–31. http://dx.doi.org/10.1016/j.it.2009.09.007.

23. Wang H, Garcia CA, Renahi K, Cekic C, Alard P, Kinane DF, Mitchell T, Martin M. 2008. IFN-beta production by TLR4-stimulated immature immune cells is negatively regulated by GSK3-beta. J Immunol 181:6797–6802. http://dx.doi.org/10.4049/jimmunol.181.10.6797.

24. Wang JT, Chang LS, Chen CJ, Doong SL, Chang CW, Chen MR. 2014. Glycogen synthase kinase 3 negatively regulates IFN regulatory factor 3 transactivation through phosphorylation at its linker region. Innate Immun 20:78–87. http://dx.doi.org/10.1177/17534539135148507.

25. Lei CJ, Zhong B, Zhang Y, Zhang J, Wang S, Shu HB. 2010. Glycogen synthase kinase 3beta regulates IRF3 transcription factor-mediated antiviral response via activation of the kinase TBK1. Immunity 33:878–889. http://dx.doi.org/10.1016/j.immuni.2010.11.021.

26. van Zuylen WJ, Doyon P, Clément JF, Khan KA, D’Ambrosio LM, Do F, St-Amant-Verret M, Wissani T, Emerguy G, Ginsaras AC, Meloche S, Savagner P. 2012. Coordination of the TRAF3 protein family reveals a new role for the ER-to-Golgi transport compartments in innate immunity. PLoS Pathog 8:e1002747. http://dx.doi.org/10.1371/journal.ppat.1002747.

27. Kelly NF, Ng DY, Jayakumaran G, Wood GA, Koide H, Doble BW. 2011. Beta-Catenin enhances Oct-4 activity and reinforces pluripotency via a TGFB-independent mechanism. Cell Stem Cell 8:214–227. http://dx.doi.org/10.1016/j.stem.2010.12.010.

28. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. 1998. A third-generation lentivirus vector with a conditional packaging system. J Virol 72:8463–8471.

29. Baril M, Es-SAad S, Chatel-Chaix L, Fink K, Pham T, Raymond VA, Audette K, Guenier AS, Duchaine J, Servant M, Bilodeau M, Cohen E, Grandvaux N, Lamarre D. 2013. Genome-wide RNAi screen reveals a new role of a WNT/CTNNB1 signaling pathway as negative regulator of Wnt signaling/activation via frizzled, dishevelled and axin functions. Development 135:367–375.
virus-induced innate immune responses. PLoS Pathog 9:e1003416. http://dx.doi.org/10.1371/journal.ppat.1003416.
39. Khan KA, Coquelle A, Davrinche C, Herbein G. 2009. Bcl-3-regulated transcription from major immediate-early promoter of human cytomegalovirus in monocyte-derived macrophages. J Immunol 182:7784–7794. http://dx.doi.org/10.4049/jbiolchem.2009.3080.
40. Sun Q, Sun L, Liu HH, Chen X, Seth RB, Forman J, Chen ZJ. 2006. The specific and essential role of MAVS in antiviral innate immune responses. Immunity 24:633–642. http://dx.doi.org/10.1016/j.immuni.2006.04.004.
41. Gravel SP, Servant MJ. 2005. Roles of an IkappaB kinase-related pathway in human cytomegalovirus-infected vascular smooth muscle cells: a molecular link in pathogenesis-provoked proatherosclerotic conditions. J Biol Chem 280:7477–7486. http://dx.doi.org/10.1074/jbc.M410932200.
42. Preston CM, Harman AN, Nicholl MJ. 2001. Activation of interferon response factor-3 in human cells infected with herpes simplex virus type 1 or human cytomegalovirus. J Virol 75:8909–8916. http://dx.doi.org/10.1128/JVI.19.8909-8916.2001.
43. Gaillard G, Strauss F. 1990. Ethanol precipitation of DNA with linear polyacrylamide as carrier. Nucleic Acids Res 18:378. http://dx.doi.org/10.1093/nar/18.2.378.
44. Andersen J, VanScy C, Cheng TF, Gomez D, Reich NC. 2008. IRF-3-dependent and augmented target genes during viral infection. Genes Immun 9:168–175. http://dx.doi.org/10.1038/sj.gene.6364449.
45. Dixit E, Boulant S, Zhang Y, Lee AS, Ondellad C, Shum B, Hacohen N, Chen ZJ, Whelan SP, Fransen M, Nibert ML, Superti-Furga G, Kagan JC. 2010. Feroxosomes are signaling platforms for antiviral innate immunity. Cell 141:649–660. http://dx.doi.org/10.1016/j.cell.2010.04.018.
46. Grandvaux N, Servant MJ, tenOever B, Ser 339 generates a hyperactive form of IRF-3 through regulation of interferon regulatory factor 3 following virus particle entry. J Virol 83:e58501. http://dx.doi.org/10.1128/JVI.02069-08.
47. Stoilov DF, Lichte BY, tenOever BR, Paterson JM, Power AT, Knowles S, Marius R, Reynard J, Poliquin L, Atkins H, Brown EG, Durbin RK, Durbin JE, Hiscott J, Bell JC. 2003. VSV strains with defects in their activity is required for lithium to modulate mood-related behaviors in mice. Neuropsychopharmacology 36:1397–1411. http://dx.doi.org/10.1038/npp.2011.24.
48. Schroeder JH, Bell LS, Janss ML, Turner M. 2013. Pharmacological inhibition of glycosyn thase kinase 3 regulates T cell development in vitro. PLoS One 8:e53850. http://dx.doi.org/10.1371/journal.pone.0053850.
49. Sumi T, Oki S, Kitajima K, Menc O. 2013. Epiblast ground state is controlled by canonical Wnt/beta-catenin signaling in the postimplantation mouse embryo and epiblast stem cells. PLoS One 8:e63378. http://dx.doi.org/10.1371/journal.pone.0063378.
50. Chen L, Salinas GD, Li X. 2009. Regulation of serotonin 1B receptor by glycosyn thase kinase 3-m. Mol Pharmacol 76:1128–1161. http://dx.doi.org/10.1124/mol.109.056994.
51. Grumolato L, Liu G, Mong P, Mudharry R, Biswas R, Arroyave R, Vijayakumar S, Economides AN, Aaronson SA. 2010. Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. Genes Dev 24:2517–2530. http://dx.doi.org/10.1101/gad.1957710.
52. Selmo M, Suemori H, Naka suji N, Suemori H. 2008. Defining early signaling specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. Development 135:2969–2979. http://dx.doi.org/10.1242/dev.021121.
53. Ford E, Thanos D. 2010. The transcriptional code of human IFN-beta gene expression. Biochim Biophys Acta 1798:328–356. http://dx.doi.org/10.1016/j.bbabio.2010.01.010.
54. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T. 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity 13:539–548. http://dx.doi.org/10.1016/S1074-7613(00)00053-4.
55. Clément JF, Bibeau-Poirier A, Gravel SP, Grandvaux N, Bibeau JF, Meloche S, Servant MJ. 2008. Phosphorylation of IRF-3 on Ser 339 generates a noncatalytic form of IRF-3 through regulation of dimerization and CBP association. J Virol 82:3978–3996. http://dx.doi.org/10.1128/JVI.02526-07.
56. Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC. 2004. Novel roles of TLR3 tyrosine phosphorylation and PIK3 kinase in double-stranded RNA signaling, Nat Struct Mol Biol 11:1060–1067. http://dx.doi.org/10.1038/nsmb847.
57. Nooye RS, Collins SE, Mossman KL. 2009. Differential modification of interferon regulatory factor 3 following virus particle entry. J Virol 83:4013–4022. http://dx.doi.org/10.1128/JVI.02069-08.
58. Hadjiathanas MV, Bruckner M, Behrens J. 2010. Conductin/axin2 and Wnt signalling regulates centrosome cohesion. EMBO Rep 11:317–324. http://dx.doi.org/10.1038/embor.2010.23.
59. Huang P, Senga T, Hamaguchi M. 2007. A novel role of phospho-beta-catenin in microtubule regrowth at centrosome. Oncogene 26:4357–4371. http://dx.doi.org/10.1038/sj.onc.1210217.
60. Faux MC, Coates JL, Kershaw NJ, Layton MJ, Burgess AW. 2010. Novel phosphospecific antibody for phospho-beta-catenin with E-cadherin at cell-cell contacts and APC at cell protrusions. PLoS One 5:e14127. http://dx.doi.org/10.1371/journal.pone.0014127.
61. Chikov D, Sinjushina N, Rita H, Taketo MM, Makela TP, Partanen J. 2011. Phosphorylated beta-catenin localizes to centrosomes of neuronal progenitors and is required for cell polarity and neurogenesis in developing midbrain. Dev Biol 357:259–268. http://dx.doi.org/10.1016/j.ydbio.2011.06.029.
62. Tapia M, Del Puerto A, Puime A, Sanchez-Ponce D, Fonranzoloi-Molinieres L, Pallas-Bazarra N, Carlier E, Giraud P, Debanne D, Wan-
dosell F, Garrido JJ. 2013. GSK3 and beta-catenin determines functional expression of sodium channels at the axon initial segment. Cell Mol Life Sci 70:105–120. http://dx.doi.org/10.1007/s00018-012-1059-5.

75. Yuan J, Zhang J, Wong BW, Si X, Wong J, Yang D, Luo H. 2005. Inhibition of glycogen synthase kinase 3beta suppresses coxsackievirus-induced cytopathic effect and apoptosis via stabilization of beta-catenin. Cell Death Differ 12:1097–1106. http://dx.doi.org/10.1038/sj.cdd.4401652.

76. Maggirwar SB, Tong N, Ramirez S, Gelbard HA, Dewhurst S. 1999. HIV-1 Tat-mediated activation of glycogen synthase kinase-3beta contributes to Tat-mediated neurotoxicity. J Neurochem 73:578–586.

77. Song B, Lai B, Zheng Z, Zhang Y, Luo J, Wang C, Chen Y, Woodgett JR, Li M. 2010. Inhibitory phosphorylation of GSK-3 by CaMKII couples depolarization to neuronal survival. J Biol Chem 285:41122–41134. http://dx.doi.org/10.1074/jbc.M110.130351.

78. Hernández F, Langa E, Cuadros R, Avila J, Villanueva N. 2010. Regulation of GSK3 isoforms by phosphatases PP1 and PP2A. Mol Cell Biochem 344:211–215. http://dx.doi.org/10.1007/s11010-010-0544-0.

79. Long L, Deng Y, Yao F, Guan D, Feng Y, Jiang H, Li X, Hu P, Lu X, Wang H, Li J, Gao X, Xie D. 2014. Recruitment of phosphatase PP2A by RACK1 adaptor protein deactivates transcription factor IRF3 and limits type I interferon signaling. Immunity 40:515–529. http://dx.doi.org/10.1016/j.immuni.2014.01.015.

80. Gu M, Zhang T, Lin W, Liu Z, Wang X. 2014. Protein phosphatase PP1 negatively regulates the Toll-like receptor- and RIG-I-like receptor-triggered production of type I interferon by inhibiting IRF3 phosphorylation at serines 396 and 385 in macrophage. Cell Signal 26:2930–2939. http://dx.doi.org/10.1016/j.cellsig.2014.09.007.

81. Park CY, Choi SH, Kang SM, Kang JS, Ahn BY, Kim H, Jung G, Choi KY, Hwang SB. 2009. Nonstructural 5A protein activates beta-catenin signaling cascades: implication of hepatitis C virus-induced liver pathogenesis. J Hepatol 51:853–864. http://dx.doi.org/10.1016/j.jhep.2009.06.026.

82. Street A, Macdonald A, McCormick C, Harris M. 2005. Hepatitis C virus NS5A-mediated activation of phosphoinositide 3-kinase results in stabilization of cellular beta-catenin and stimulation of beta-catenin-responsive transcription. J Virol 79:5006–5016. http://dx.doi.org/10.1128/JVI.79.8.5006-5016.2005.

83. Srisuttee R, Koh SS, Kim SJ, Malilas W, Boonying W, Cho IR, Jhun BH, Ito M, Horio Y, Seto E, Oh S, Chung YH. 2012. Hepatitis B virus X (HBX) protein upregulates beta-catenin in a human hepatic cell line by sequestering SIRT1 deacetylase. Oncol Rep 28:276–282.

84. Everly DN, Jr, Kusano S, Raab-Traub N. 2004. Accumulation of cytoplasmic beta-catenin and nuclear glycogen synthase kinase 3beta in Epstein-Barr virus-infected cells. J Virol 78:11648–11655. http://dx.doi.org/10.1128/JVI.78.21.11648-11655.2004.

85. Fujimuro M, Wu FY, ApRhys C, Kajumbula H, Young DB, Hayward GS, Hayward SD. 2003. A novel viral mechanism for dysregulation of beta-catenin in Kaposi’s sarcoma-associated herpesvirus latency. Nat Med 9:300–306. http://dx.doi.org/10.1038/nm829.