AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

β-Catenin, a Transcription Factor Activated by Canonical Wnt Signaling, Is Expressed in Sensory Neurons of Calves Latently Infected with Bovine Herpesvirus 1

Yilin Liu,[a] Morgan Hancock,[a] Aspen Workman,[b] Alan Doster,[a] Clinton Jones[a]*

School of Veterinary Medicine and Biomedical Sciences, Nebraska Center for Virology, Morrison Life Sciences Research Center, University of Nebraska—Lincoln, Lincoln, Nebraska, USA[a]; U.S. Department of Agriculture, Agricultural Research Service, U.S. Meat Animal Research Center, Clay Center, Nebraska, USA[b]

ABSTRACT

Like many *Alphaherpesvirinae* subfamily members, bovine herpesvirus 1 (BoHV-1) expresses an abundant transcript in latently infected sensory neurons, the latency-related (LR)-RNA. LR-RNA encodes a protein (ORF2) that inhibits apoptosis, interacts with Notch family members, interferes with Notch-mediated transcription, and stimulates neurite formation in cells expressing Notch. An LR mutant virus containing stop codons at the amino terminus of ORF2 does not reactivate from latency or replicate efficiently in certain tissues, indicating that LR gene products are important. In this study, β-catenin, a transcription factor activated by the canonical Wnt signaling pathway, was frequently detected in ORF2-positive trigeminal ganglionic neurons of latently infected, but not mock-infected, calves. Conversely, the lytic cycle regulatory protein (BoHV-1 infected cell protein 0, or bICP0) was not frequently detected in β-catenin-positive neurons in latently infected calves. During dexamethasone-induced reactivation from latency, mRNA expression levels of two Wnt antagonists, Dickkopf-1 (DKK-1) and secreted Frizzled-related protein 2 (SFRP2), were induced in bovine trigeminal ganglia (TG), which correlated with reduced β-catenin protein expression in TG neurons 6 h after dexamethasone treatment. ORF2 and a coactivator of β-catenin, mastermind-like protein 1 (MAML1), stabilized β-catenin protein levels and stimulated β-catenin-dependent transcription in mouse neuroblastoma cells more effectively than MAML1 or ORF2 alone. Neuroblastoma cells expressing ORF2, MAML1, and β-catenin were highly resistant to cell death following serum withdrawal, whereas most cells transfected with only one of these genes died. The Wnt signaling pathway interferes with neurodegeneration but promotes neuronal differentiation, suggesting that stabilization of β-catenin expression by ORF2 promotes neuronal survival and differentiation.

IMPORTANCE

Bovine herpesvirus 1 (BoHV-1) is an important pathogen of cattle, and like many *Alphaherpesvirinae* subfamily members establishes latency in sensory neurons. Lifelong latency and the ability to reactivate from latency are crucial for virus transmission. Maintaining the survival and normal functions of terminally differentiated neurons is also crucial for lifelong latency. Our studies revealed that BoHV-1 gene products expressed during latency stabilize expression of the transcription factor β-catenin and perhaps its cofactor, mastermind-like protein 1 (MAML1). In contrast to expression during latency, β-catenin expression in sensory neurons is not detectable following treatment of latently infected calves with the synthetic corticosteroid dexamethasone to initiate reactivation from latency. A viral protein (ORF2) expressed in a subset of latently infected neurons stabilized β-catenin and MAML1 in transfected cells. ORF2, β-catenin, and MAML1 also enhanced cell survival when growth factors were withdrawn, suggesting that these genes enhance survival of latently infected neurons.

Bovine herpesvirus 1 (BoHV-1) is a significant bovine pathogen that initiates infection on mucosal linings within the ocular, nasal, or oral cavity (reviewed in references 1 to 3). BoHV-1, like many *Alphaherpesvirinae* subfamily members, establishes lifelong latency in sensory neurons after an initial burst of lytic cycle viral gene expression (reviewed in references 3 to 6). Maintaining normal functions and promoting survival of latently infected sensory neurons are crucial for lifelong latency because sensory neurons are terminally differentiated. Thus, it would be beneficial for BoHV-1 to encode functions that promote neuronal survival, maintain normal axonal connections, and sustain a differentiated phenotype.

The BoHV-1-encoded latency-related (LR)-RNA is abundantly expressed in latently infected neurons in trigeminal ganglia (TG) (4, 6–10), and poly(A)$^+$ LR-RNA is alternatively spliced in TG of infected calves (11). Two open reading frames (ORF1 and ORF2) and two reading frames that lack an initiating ATG (reading frame B [RF-B] and RF-C) are present in the LR gene (8). An LR mutant virus strain with three stop codons at the amino terminus of ORF2 exhibits diminished clinical symptoms and re-

Received 23 November 2015 **Accepted** 30 December 2015

Accepted manuscript posted online 6 January 2016

Citation Liu Y, Hancock M, Workman A, Doster A, Jones C. 2016. β-Catenin, a transcription factor activated by canonical Wnt signaling, is expressed in sensory neurons of calves latently infected with bovine herpesvirus 1. J Virol 90:3148–3159. doi:10.1128/JVI.02971-15.

Editor: R. M. Longnecker

Address correspondence to Clinton Jones, clint.jones10@okstate.edu.

* Present address: Clinton Jones, Oklahoma State University, Center for Veterinary Health Sciences, Department of Veterinary Pathobiology, Stillwater, Oklahoma, USA.

duced virus shedding from the eye, TG, or tonsils of infected calves (12). Unlike wild-type BoHV-1, the LR mutant virus does not reactivate from latency following dexamethasone (DEX) treatment, in part because the LR mutant causes higher levels of apoptosis in TG neurons (13); thus, the efficiency of establishing latency is reduced (14). The ability of ORF2 to interfere with apoptosis (15, 16) is believed to enhance survival of infected neurons by maintaining a pool of latently infected neurons that has the potential to reactivate from latency. Interestingly, expression of wild-type BoHV-1 LR gene products, including ORF2, restores reactivation from latency of a herpes simplex virus 1 (HSV-1) strain that does not express crucial functions encoded within the latency-associated transcript (LAT) (17, 18). LAT (19), like ORF2, interferes with apoptosis in the absence of other viral genes.

ORF2 is a multifunctional protein that interacts with cellular transcription factor Notch1, Notch3, or C/EBP-alpha (20–22) and reduces Notch-mediated transactivation of the BoHV-1 infected cell protein 0 (bICP0) early promoter and glycoprotein C promoter. We suggest that the ability of ORF2 to interact with and influence Notch signaling is crucial during certain steps of the latency reactivation cycle because Notch3 expression and downstream targets of Notch are stimulated during DEX-induced reactivation from latency (22, 23). Notch receptor family members (Notch1 to Notch4) are membrane-tethered transcription factors that regulate many developmental and physiological processes, including neuronal maintenance, development, and differentiation (24–26). Upon interaction with its ligand, the Notch intracellular domain (NICD) is cleaved and enters the nucleus, where it interacts with a member of the CSL family of transcriptional factors [CBF1, Su(H), or Lag1; also referred to as RBP-Jκ binding proteins] and the mastermind-like protein 1 (MAML1) to activate target genes (27, 28). When CSL family members are not bound to Notch, transcription is extinguished through corepressor recruitment.

Three MAML proteins (29–31) can interact with Notch and β-catenin (32), thus linking the canonical Notch and Wnt signaling pathways. Wnt is a family of secreted glycoproteins that interacts with Frizzled and the coreceptor low-density lipoprotein receptor-related protein 5 (LRP5)/LRP6 (reviewed in reference 33). Upon Wnt binding, the signal is transduced to the transcription factor β-catenin, which is stabilized and enters the nucleus, where it interacts with T-cell factor (TCF) family members specifically bound to the consensus site AGATCAAGG (reviewed in reference 33) (Fig. 1A). Binding of β-catenin to TCF displaces bound corepressors and recruits coactivators such as MAML1 to activate Wnt target genes (31). Two demethylases in the nucleus also promote ubiquitin-mediated proteolysis of β-catenin in the nucleus (34), underscoring the importance of regulating β-catenin protein levels in normal cells. Wnt-mediated activation of β-catenin is required for embryogenesis and adult homeostasis (27, 28, 33, 35–37).

In this study, we demonstrate that β-catenin and ORF2 were frequently detected in the same neuron in calves latently infected with BoHV-1. When reactivation from latency was initiated by treatment with the synthetic corticosteroid DEX, β-catenin expression in neurons was detected in fewer neurons by 6 h, which correlated with increased expression of two Wnt antagonists, Dickkopf-1 (DKK-1) and secreted Frizzled-related protein 2 (SFRP2). The β-catenin coactivator MAML1 was weakly expressed in a subset of ORF2-positive ($ORF2^+$) neurons but was not detected in TG neurons prepared from uninfected calves. In transfected mouse neuroblastoma cells (Neuro-2A), β-catenin, MAML1, and ORF2 stabilized expression of each other and stimulated β-catenin-dependent transcription. Finally, ORF2, MAML1, and β-catenin enhanced Neuro-2A cell survival following growth factor withdrawal. We suggest that the ability of BoHV-1 to stabilize β-catenin expression in TG neurons may promote the maintenance of latency, in part by enhancing neuronal survival and preserving normal neuronal functions.

MATERIALS AND METHODS

Infection of calves and DEX-induced reactivation. All TG samples from calves used for this study were previously described (23). In brief, BoHV-1-free crossbred calves (~200 kg) were inoculated with 10^7 PFU of BoHV-1 into ocular and nasal cavities as described previously (12–14, 38–40). Calves were housed under strict isolation and given antibiotics to prevent secondary bacterial infections. At 60 days postinfection (dpi), certain calves were injected intravenously in their jugular vein with 100 mg of DEX to initiate reactivation and then transported to a veterinary diagnostic lab. Calves were anesthetized with xylazine (Rompun), followed by electrocution. One calf was decapitated at a time to ensure that samples were processed in a timely manner. Calves were decapitated in the same order in which they were injected with DEX to ensure that the time points after DEX treatment were as close as possible to the designated time point. Three calves/time point were used for these studies. Experiments were performed in accordance with the American Association of Laboratory Animal Care guidelines and University of Nebraska IACUC committee (A3459). Following euthanasia, TG were collected and minced into small pieces, and then TG were formalin fixed and paraffin embedded. The remaining TG pieces were minced into small pieces and placed into a single 50-ml conical tube, and the tube was placed in a dry-ice ethanol bath and then stored at −80°C. After decapitation, it took 5 to 10 min to collect TG, mince TG, place TG pieces in formalin or in a 50-ml conical tube, and then submerge TG pieces in 50-ml conical tubes in a dry-ice ethanol bath.

Microarray analysis. Details of the microarray study were previously described (23). In brief, 200 ng of total RNA was reverse transcribed and processed using an Affymetrix 3′ IVT Express kit and hybridized per the manufacturer's protocols for the Bovine Gene Chip (Affymetrix, Santa Clara, CA), which contains more than 23,000 genes. Following overnight hybridization, arrays were stained and scanned with the Affymetrix 450 fluidics station and the 3000 7G high-resolution scanner. After quality control assessment with respect to background and the other standardized parameters suggested by Affymetrix was performed, the CEL files were analyzed. Robust multichip averaging (RMA) was used to do background correction, quantile normalization, and median polish summarization of probe-level data (41). A preliminary filter was applied, retaining genes where at least 20% of the arrays had at least a 1.5-fold change from the median value for that gene. To determine differentially expressed genes over time, we compared latently infected calves (designated controls; 0 h) with groups of calves treated with DEX for 1.5 h, 3 h, 6 h, and 24 h using a random variance *F* test and selected genes with a false discovery rate of less than 10%. Fold changes were calculated for each differentially expressed gene relative to the level of the control group. Verification of certain highly differentially expressed genes was previously confirmed by reverse transcription-PCR (RT-PCR) and immunohistochemistry (IHC) (23).

Immunohistochemistry analysis. Immunohistochemistry (IHC) studies were performed using an ABC kit (Vector Laboratories) according to specifications of the manufacturer as previously described (41). Thin sections (4 to 5 μm) of TG were cut and mounted on glass slides. Slides were first incubated at 65°C for 20 min and then washed twice in xylene for 10 min each time, twice in 100% ethanol for 5 min, once in 90% ethanol for 5 min, once in 70% ethanol for 5 min, twice in distilled H_2O for 5 min, and three times in 1× Tris-buffered saline (TBS) for 5 min each time. To

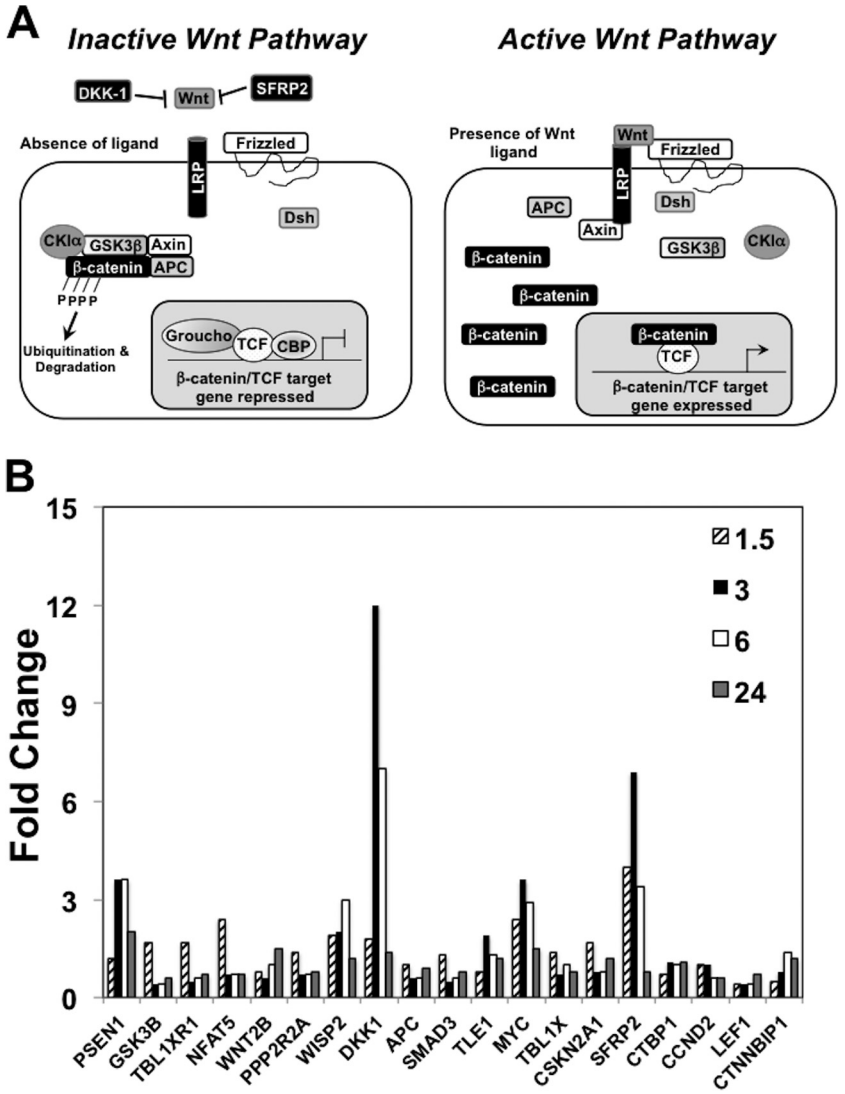

FIG 1 Schematic of canonical Wnt signaling pathway and its regulation during DEX-induced reactivation from latency. (A) In the absence of the Wnt ligand (inactive Wnt pathway), a β-catenin destruction complex, axin, adenomatous polyposis gene (APC), glycogen synthase kinase 3β (GSK3β), and CKIα, hyperphosphorylate β-catenin, which leads to polyubiquitination of β-catenin and degradation by the proteasome. Soluble Frizzled-like proteins (DKK-1 and FRP2) interact with Wnt family members and prevent Wnt binding to its true receptors. Consequently, TCF bound to DNA interacts with transcriptional repressors, resulting in silencing of promoters containing TCF binding sites. In the active Wnt pathway, binding of Wnt to LRP and Frizzled family members leads to disassembly of the β-catenin destruction complex, and hypophosphorylated β-catenin accumulates in the nucleus. Nuclear β-catenin binds to TCF family members and displaces repressors of TCF-dependent transcription. Nuclear β-catenin bound to a TCF family member also recruits additional transcriptional regulators, which leads to transcriptional activation. (B) Previously published microarray data (23) was analyzed for Wnt-responsive genes and genes that regulate the canonical Wnt signaling pathway. The 19 genes present in the final list of genes that were differentially regulated (levels with DEX treatment compared to those in latency) had at least a 1.5-fold difference, and values were significantly different from values during latency. The time points (in hours) after DEX treatment are as indicated on the figure.

block endogenous peroxidase activity, sections were incubated in hydrogen peroxide (0.03% in phosphate-buffered saline [PBS], pH 7.4) for 20 min at room temperature. Tissue sections were then washed three times in 1× TBS for 5 min at room temperature, followed by digestion with proteinase K (S3020; Dako) for 20 min at 37°C. Tissue sections were then blocked with 5% normal serum diluted in 1× TBS containing 0.25% bovine serum albumin for 45 min at room temperature in a humidified chamber. Slides were incubated with β-catenin at 1:200 (ab6302; Abcam), MAML1 at 1:200 (12166; Cell Signaling), bICP0 antibody at 1:250, or ORF2 rabbit polyclonal antibody at a 1:250 dilution overnight in a humidified chamber at 4°C. The next day, slides were washed in 1× TBS and incubated in biotinylated goat anti-rabbit IgG (PK-6101; Vector Laboratories) for 30 min at room temperature in a humidified chamber. Avidin-biotinylated enzyme complex was added to the slides for 30 min of incubation at room temperature. After three washes in 1× TBS, slides were incubated with freshly prepared substrate (SK-4800; Vector Laboratories), rinsed with distilled water, and counterstained with methyl green. The percentage of neurons that were β-catenin positive (β-catenin$^+$) was estimated by counting neurons in a blinded fashion.

Cells and plasmids. Murine neuroblastoma cells (Neuro-2A; CCL-131) were obtained from the ATCC (Manassas, VA, USA) and grown in Earle's modified Eagle's medium (EMEM) supplemented with 10% fetal calf serum (FCS), penicillin (10 U/ml), and streptomycin (100 μg/ml).

The ORF2 expression construct was generated in the vector pCMV-

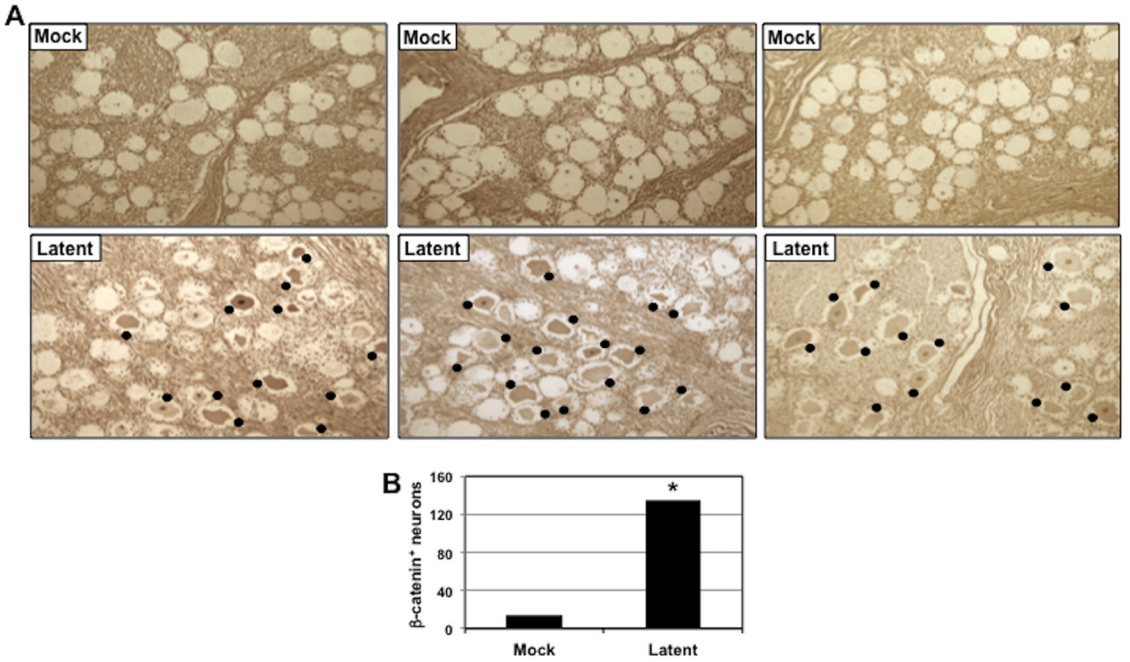

FIG 2 Detection of β-catenin during latency. (A) TG were collected from three mock-infected calves or three latently infected calves (at least 60 days postinfection). Thin sections were cut from formalin-fixed paraffin-embedded TG sections. The β-catenin antibody used for this study was purchased from Abcam (ab6302) and was diluted 1:200. Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody. Thin sections from mock-infected calves were used as controls. Closed circles denote β-catenin-positive neurons. (B) The number of β-catenin-positive neurons from 750 total neurons was estimated from sections derived from three latently infected calves or three mock-infected calves. An asterisk denotes significant differences ($P < 0.05$) in the numbers of β-catenin-positive neurons as determined by a Student *t* test.

Tag-2B (Stratagene) and was described previously (15, 42, 43). A Flag epitope is present at the N terminus of ORF2, and the human IE cytomegalovirus (CMV) promoter drives its expression. The β-catenin expression construct (S33Y) was a gift from Bert Vogelstein (plasmid 16519; Addgene) (44). M50 Super 8× TOPFlash contains a simple promoter that is stimulated by β-catenin and was a gift from Randall Moon (plasmid 12456; Addgene) (45). pHAGE-N-V5-MAML1 is an expression vector that expresses MAML1 and was a gift from Peter Howley (plasmid 37048; Addgene) (46).

A luciferase construct containing the HES1 (Hairy/Enhancer of split 1) promoter was purchased from Addgene. Notch3 intracellular domain (NICD3) constructs were kindly provided by U. Lendahl, Karolinska Institute, Stockholm, Sweden. All plasmids were transfected into Neuro-2A cells in 60-mm dishes by using TransIT Neural (MIR2145; Mirus) according to the manufacturer's instructions.

Western blot analysis. Neuro-2A cells in 60-mm dishes were transfected with plasmids denoted in the legends of the respective figure. Forty-eight hours after transfection cells were collected, washed once with PBS, and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (Thermo-Scientific). The respective samples were boiled in Laemmli sample buffer for 5 min, and all samples were separated on a 10% SDS-polyacrylamide gel. Immunodetection of ORF2 and NICD3 was performed using a mouse anti-Flag antibody (F1804; Sigma) (1:1,000) or rabbit anti-Notch3 antibody at a dilution of 1:300 (sc-5593; Santa Cruz Biotechnology), anti-V5 antibody for MAML1 (1:5,000) (600-410-378; Rockland), or anti-β-catenin antibody (1:2,000) (ab6302; Abcam).

Dual-luciferase reporter assay. Neuro-2A cells (8×10^5) were seeded into 60-mm dishes containing EMEM with 10% FCS at 24 h prior to transfection. Two hours before transfection, medium was replaced with fresh EMEM containing 0.5% FCS to lower the basal levels of promoter activity. Cells were cotransfected with a plasmid containing the firefly luciferase gene downstream of the HES1 (0.3 μg) or the TCF/LEF promoter (0.1 μg DNA), a plasmid encoding *Renilla* luciferase under the control of a minimal herpesvirus thymidine kinase (TK) promoter (40 ng), the designated NICD plasmid (0.3 μg DNA), MAML plasmid (0.1 to 0.3 μg DNA), and an ORF2 expression plasmid (0.1 to 1 μg DNA). To maintain equal plasmid amounts in the transfection mixtures, an empty expression vector was added as needed. Forty hours after transfection, cells were harvested, and protein extracts were subjected to a dual-luciferase assay by using a commercially available kit (E1910; Promega) according to the manufacturer's instructions. Luminescence was measured by using a GloMax 20/20 luminometer (E5331; Promega).

Cell survival assay. Cell survival assays were similar to the neurite development assay that was previously described (42, 43). In brief, Neuro-2A cells grown in 60-mm plates were cotransfected, either singly or in combination, with a human CMV promoter plasmid expressing the S33Y plasmid (1 μg of DNA), an ORF2 construct (1 μg of DNA), an MAML1 plasmid, and the pCMV-β-galactosidase (β-Gal) plasmid (1 μg of DNA). Twenty-four hours after transfection, cells were seeded into new plates at a low density of 4,000 cells/cm^2 and starved in medium with 0.5% serum for 3 days. Cells were then fixed and stained, aβ-Gal assay was performed, and β-Gal-positive (β-Gal$^+$) blue cells were counted. The number of blue cells expressed in the S33Y construct was arbitrarily set as 1, and all other samples were normalized to the S33Y value. The results are an average of three independent experiments.

RESULTS

Expression of Wnt antagonists is induced during DEX-induced reactivation from latency. A commercially available bovine microarray was previously used to identify cellular transcription factors induced in TG during DEX-induced reactivation from latency (23). This study also demonstrated that the Notch signaling pathway is stimulated during early stages of reactivation from la-

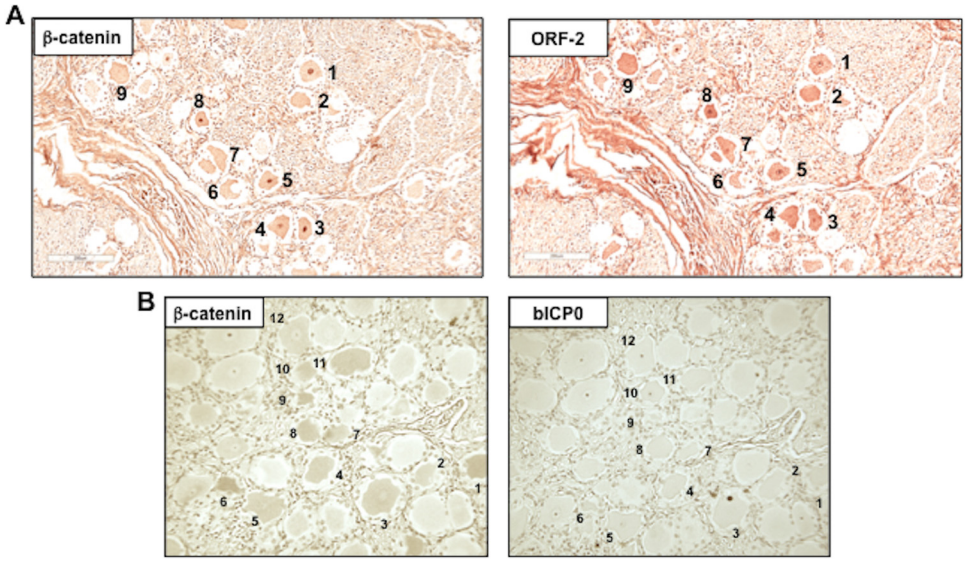

FIG 3 ORF2 and β-catenin are frequently expressed in the same neuron during latency. Consecutive sections were cut from formalin-fixed paraffin-embedded TG from latently infected calves. (A) For one section, IHC was performed using a peptide-specific ORF2 antibody (1:250 dilution) that has been previously described (16, 22, 42, 43). The adjacent section was stained with the β-catenin antibody (1:200 dilution). Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody. Neurons, indicated by numbers 1 to 9, are ORF2$^+$ and β-catenin$^+$ neurons. These results are representative of results from many sections using TG from two calves. (B) For one section, IHC was performed using the β-catenin antibody (1:200 dilution). The consecutive section was stained using a peptide-specific bICP0 antibody (1:250 dilution) that specifically recognizes the bICP0 protein (52, 53). Neurons, indicated by numbers 1 to 12, are β-catenin$^+$ neurons in one section and are also shown in the section stained with the bICP0 antibody to point out the location of the same neurons numbered in the section stained by the β-catenin antibody. These results are representative of four sections cut from TG of two latently infected calves.

tency, in part because Notch3 expression is induced (43). Since the Notch and Wnt signaling pathways have been reported to engage in cross talk (29–32, 37, 47, 48), expression levels of genes regulated in a Wnt-dependent manner was compared in TG during latency and during DEX-induced reactivation from latency. In this study, only transcripts that had at least a 2-fold difference in expression levels were examined. Of the 19 Wnt-regulated genes, only two were upregulated more than 5-fold following DEX treatment, namely, Dickkopf-1 (DKK-1) and secreted Frizzled-related protein 2 (SFRP2) (Fig. 1B). DKK-1 levels were approximately 10- and 7-fold higher at 3 and 6 h after DEX treatment, respectively, than during latency. SFRP2 levels were approximately 3- and 7-fold higher at 1.5 and 6 h after DEX treatment, respectively. Interestingly, DKK-1 and SFRP2 are both secreted proteins that antagonize the canonical Wnt signaling pathway by interfering with Wnt binding to its receptor (49, 50) (Fig. 1A). Attempts to examine DKK-1 and SFRP2 protein expression in TG neurons have been unsuccessful (i) because a commercial DKK-1 antibody recognized a bovine protein of the correct molecular weight in Western blots but yielded only background staining by IHC, perhaps because DKK-1 is a secreted protein, and (ii) because we were unable to identify a commercially available SFRP2 antibody that specifically recognizes the bovine protein (data not shown). There were no significant differences between expression levels of β-catenin and MAML1 during latency and those after DEX-induced reactivation from latency (data not shown). In nonneural cells, the primary mechanism of regulating β-catenin levels is at the protein, not mRNA, level (reviewed in reference 33) (Fig. 1A), suggesting that this mechanism is similar in sensory neurons.

Detection of β-catenin expression in TG neurons during the latency reactivation cycle. The data presented in Fig. 1 suggested that the canonical Wnt signaling pathway may be regulated during the latency reactivation cycle of BoHV-1-infected TG neurons. To test this prediction, immunohistochemistry (IHC) studies were performed to examine β-catenin expression during the latency reactivation cycle. The β-catenin antibody used for these studies (ab-6302; Abcam) recognizes the protein from many mammals, including bovine kidney cells, and works well for formalin-fixed tissue. β-Catenin protein expression was detected in a subset of TG neurons from three latently infected calves; conversely β-catenin$^+$ neurons were not readily detected in mock-infected calves (Fig. 2A). The number of β-catenin$^+$ neurons in 750 total TG neurons was significantly higher in latently infected calves than in mock-infected calves (Fig. 2B). In certain TG sections, neuronal retraction was observed. We believe this is due to the fact that fixation does not occur efficiently in bovine TG because the pieces of tissue are large and very rigid. Neuronal retraction does not appear to have resulted in high background values because β-catenin protein expression was not readily detected in uninfected TG, which had levels of neuronal retraction similar to those of TG from latently infected calves.

We previously found that ORF2 is expressed in a subset of latently infected TG neurons but that expression is reduced during DEX-induced reactivation from latency (51). In order to determine if ORF2$^+$ neurons expressed β-catenin, consecutive sections were prepared; one slide was stained with ORF2, and the other was stained with β-catenin (Fig. 3A). Out of 150 ORF2$^+$ TG neurons, 137 were β-catenin$^+$. In general, ORF2$^+$ neurons were present in patches, which correlated with β-catenin expression in TG prepared from latently infected calves. In contrast to the expression pattern of β-catenin in TG neurons, lytic cycle viral genes (bICP0, VP16, gC, and gD) (52, 53) and DEX-induced transcription factors (23, 43) are not readily detected in TG neurons by IHC during latency. However, these same antibodies recognize TG neurons

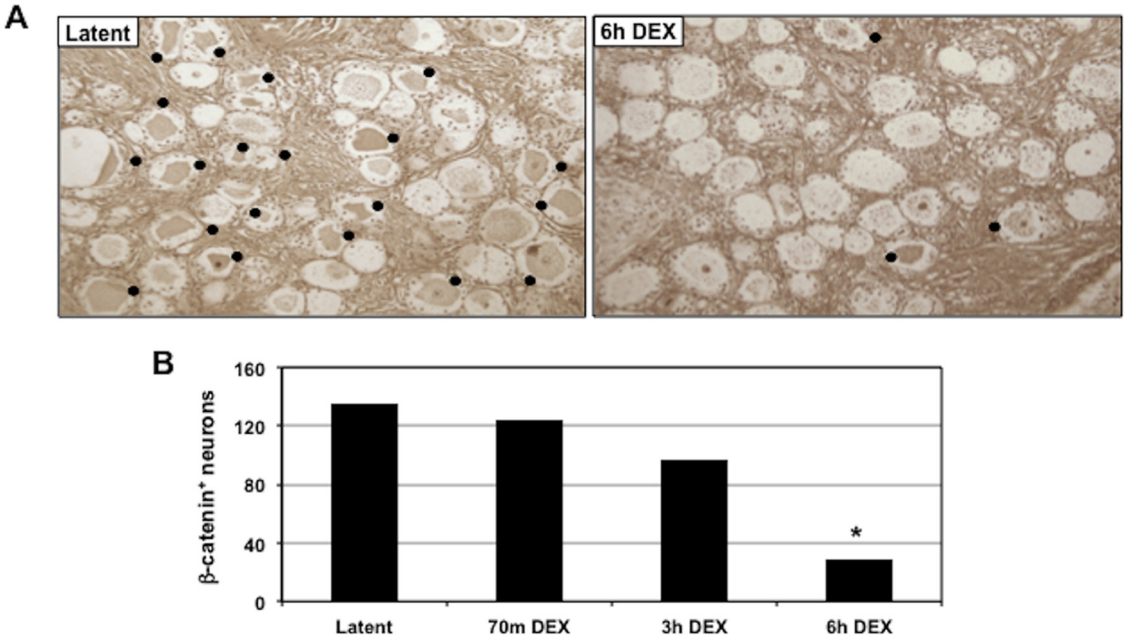

FIG 4 Decreased numbers of β-catenin$^+$ neurons during DEX-induced reactivation from latency. (A) A representative TG section collected from a calf that was latently infected (at least 60 days postinfection) or from a latently infected calf treated with DEX for 6 h to induce reactivation from latency. Thin sections were cut from formalin-fixed paraffin-embedded TG sections. The β-catenin antibody used for this study was purchased from Abcam (ab6302) and was diluted 1:200. Biotinylated goat anti-rabbit IgG (Vector laboratories) was used as a secondary antibody. Thin sections from mock-infected calves were used as controls. Closed circles denote β-catenin-positive neurons. (B) The number of β-catenin-positive neurons from 750 total neurons was estimated from TG sections from three latently infected calves and from three latently infected calves treated with DEX for 70 min, 3 h, or 6 h. An asterisk denotes significant differences ($P < 0.05$) in numbers of β-catenin-positive neurons as determined by a Student *t* test.

during DEX-induced reactivation. An additional study was performed to test whether β-catenin$^+$ neurons in TG of latently infected neurons recognize a lytic cycle viral regulatory protein, BoHV-1 infected cell protein 0 (bICP0). For this study, one slide was stained with β-catenin, and a consecutive slide was stained with a bICP0 peptide antibody. Although a subset of TG neurons was stained with the β-catenin antibody (Fig. 3B), only background staining was observed with the bICP0 antibody.

Additional studies were performed to test whether β-catenin expression changed during DEX-induced reactivation from latency. In contrast to latently infected TG, few β-catenin$^+$ neurons were detected in TG sections of calves treated with DEX for 6 h (Fig. 4A). At 70 min or 3 h after DEX treatment, β-catenin expression in TG neurons was still detected (data not shown). The percentage of β-catenin$^+$ neurons during reactivation from latency was calculated from 750 neurons from two different calves at 70 min, 3 h, or 6 h after DEX treatment. Although it was clear that significantly fewer TG neurons were β-catenin$^+$ at 6 h after DEX treatment, the differences at 70 min and 3 h after DEX treatment were not as dramatic (Fig. 4B). In summary, the findings shown in Fig. 2 to 4 indicated that β-catenin protein expression was readily detected in TG neurons during latency; conversely, β-catenin was not readily detected in TG neurons from uninfected calves or at 6 h after latently infected calves were treated with DEX.

ORF2 and MAML1 increased β-catenin steady-state levels in transfected Neuro-2A cells. The LR gene encodes multiple products, including ORF1, ORF2, various isoforms of ORF2 due to alternative splicing of LR-RNA, and two micro-RNAs (reviewed in references 4, 54, and 55). Since ORF2 has multiple functions (16, 42, 43) and appears to be a dominant virus-encoded factor that regulates the latency reactivation cycle (7, 54, 56), we tested whether ORF2 has an effect on β-catenin expression levels in mouse neuroblastoma cells (Neuro-2A). We also examined MAML1 because it is a coactivator for the Notch (29) and Wnt/β-catenin (31, 33) signaling pathways, and IHC studies suggested that low levels of MAML1 protein expression were detected in a subset of TG neurons from latently infected calves but not from mock-infected calves (data not shown). Neuro-2A cells were used for these studies for the following reasons: they can be readily transfected, ORF2 is consistently detected in these cells following transfection with an expression plasmid, ORF2 stimulates neurite formation in cells expressing Notch family members following growth factor withdrawal (42, 43), and MAML1 or β-catenin is not readily detected in nontransfected Neuro-2A cells (Fig. 5A). The β-catenin expression plasmid pCI-Neo β-catenin S33Y (S33Y) that expresses a constitutively active protein due to point mutations that alter phosphorylation sites (44) was used to transfect Neuro-2A cells because β-catenin was not detectable in Neuro-2A cells, suggesting that the negative regulatory components of β-catenin are hyperactive in these cells. β-Catenin levels were low in cells transfected with just the S33Y plasmid (referred to as β-catenin in Fig. 5), adding support to the prediction that β-catenin is efficiently degraded in Neuro-2A cells. However, we have consistently observed higher steady-state β-catenin levels in Neuro-2A cells when the S33Y construct was cotransfected with ORF2 and the MAML1 expression vector than in Neuro-2A cells transfected with any of the other combinations (Fig. 5A). Quantification of β-catenin bands revealed higher levels of β-catenin in Neuro-2A cells cotransfected with plasmids that express

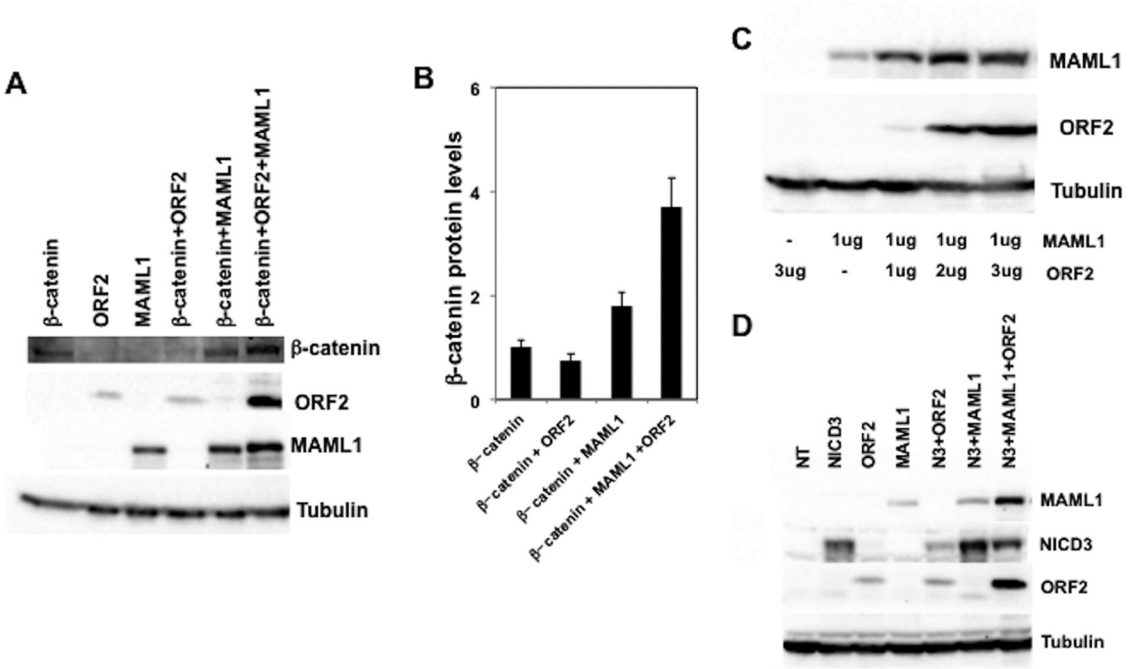

FIG 5 ORF2 and MAML1 stabilized β-catenin and NICD3 protein levels in transfected Neuro-2A cells. (A) Neuro-2A cells were transfected with the designated plasmids (2 μg of DNA for each plasmid). An empty CMV expression vector (pcDNA3.1) was added to maintain equal amounts of plasmid transfected. Forty-eight hours after transfection cells were collected and processed for Western blot analysis using a Flag antibody (1:1,000 dilution; Sigma) to detect ORF2, a β-catenin antibody (1:5000 dilution), or an anti-V5 antibody (1:5,000 dilution; Rockland) to detect MAML1. Fifty micrograms of protein in the total cell lysate was used for each lane. β-Tubulin was used as a loading control. (B) Levels of β-catenin protein in the respective samples presented in panel A were estimated by comparing levels of tubulin to those of β-catenin using a Bio-Rad molecular imager. β-Catenin protein levels in samples transfected with just the β-catenin expression plasmid were normalized to 1, and other values were compared to this result. The results shown in this graph were derived from three independent studies. (C) Neuro-2A cells were transfected with 1 μg of MAML1 expression plasmid alone or with 1 μg to 3 μg of ORF2 as indicated. Forty-eight hours after transfection cells were collected and processed for Western blot analysis using a Flag antibody (1:1,000 dilution; Sigma) or V5 antibody (1:5,000 dilution; Rockland). Fifty micrograms of protein in the total cell lysate was used for each lane, and β-tubulin was used as a loading control. (D) Neuro-2A cells were transfected with DNA constructs (2 μg of DNA for each plasmid) as indicated, and the empty expression vector was added as needed to maintain equal amounts of plasmid. Forty-eight hours after transfection, cells were collected and processed for Western blot analysis using the indicated antibodies at the same concentrations as for the experiments shown in panels A and B. The anti-Notch3 antibody (1:300) (Santa Cruz) was used to detect NICD3. The results shown in panels A, C, and D are representative of four independent experiments. NT, not treated.

β-catenin, MAML, and ORF2 than in other combinations that were tested (Fig. 5B).

Neuro-2A cells cotransfected with plasmids that express MAML1 and ORF2 also contained higher steady-state levels of each protein than cells transfected with either plasmid alone (Fig. 5C). ORF2 interacts with the Notch intracellular domain 3 (NICD3) and reduces NICD3 levels (42, 43) (Fig. 5D). Since MAML1 is known to associate with NICD3 and other Notch family members, we examined the effect that the ability of ORF2 to stabilize MAML1 had on NICD3 levels. When ORF2 was cotransfected with the MAML1 and NICD3 expression plasmids, NICD3 steady-state protein levels were increased in Neuro-2A cells compared to levels after cotransfection with NICD3 and ORF2 (Fig. 5D). For all combinations, NICD3 levels were consistently highest when MAML1 and NICD3 were cotransfected. In summary, these studies suggested that the ability of ORF2 to stabilize MAML1 protein levels led to increased β-catenin and NICD3 protein levels in transfected Neuro-2A cells.

ORF2 and MAML1 stimulate β-catenin-dependent transcription. The effect that MAML1 and ORF2 has on Wnt-dependent transcription was examined using a promoter construct containing eight TCF binding sites upstream of a minimal promoter (Super 8× TOPFlash) and S33Y. As expected, the S33Y expression construct (referred to as β-catenin in Fig. 6) stimulated promoter activity of Super 8× TOPFlash approximately 8-fold (Fig. 6A). ORF2 expression alone slightly reduced the ability of β-catenin to stimulate Super 8× TOPFlash promoter activity in transfected Neuro-2A cells (Fig. 6A). ORF2 in combination with MAML1 expression consistently enhanced the ability of β-catenin to transactivate Super 8× TOPFlash promoter activity compared to the level of activity with MAML1 and β-catenin (Fig. 6B).

Since the combination of ORF2 and MAML1 enhanced the transactivation potential of the β-catenin expression construct, it was of interest to determine if a similar effect was observed for Notch-dependent transcription. Although previous studies demonstrated that ORF2 interferes with NICD1- and NICD3-mediated transcription (16, 22, 42), the effect of ORF2 in combination with MAML1 expression on Notch-dependent transcription was not examined. We used the Hairy/Enhancer of split 1 (HES1) promoter for these studies because it is transactivated by Notch family members (reviewed in reference 57). Cotransfection of MAML1 with NICD1 stimulated HES1 promoter activity approximately 200- or 300-fold using 0.1 or 0.3 μg of MAML1, respectively, whereas NICD1 alone transactivated HES1 promoter activity only approximately 50-fold (Fig. 6C). ORF2 had a positive effect on the ability of MAML1 and NICD1 to transactivate HES1

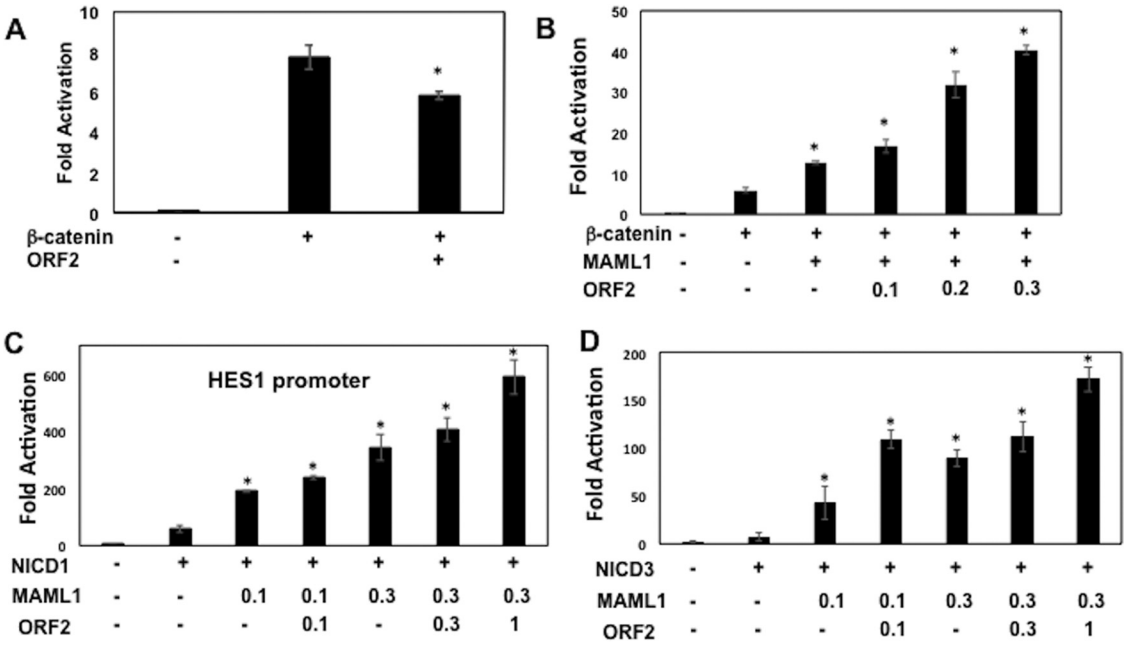

FIG 6 ORF2 stimulates transactivation potential of MAML1 for β-catenin and NICD-dependent transcription. (A) Neuro-2A cells were cotransfected with 0.1 μg of TOPFlash luciferase reporter, 0.1 μg of β-catenin (S33Y), and 0.1 μg of ORF2. (B) Neuro-2A cells were cotransfected with 0.1 μg of TOPFlash luciferase reporter, 0.1 μg of S33Y (β-catenin), 0.1 μg of MAML1, and increasing concentrations of ORF2 (0.1 to 0.3 μg). (C and D) Neuro-2A cells were cotransfected with 0.3 μg of an HES1 luciferase reporter, 0.3 μg of NICD1 or NICD3, different concentrations of the MAML1 plasmid (0.1 μg and 0.3 μg), and the indicated concentrations of ORF2 (0.1, 0.3, and 1 μg). For all studies, luciferase assays were performed at 48 h after transfection using a dual-luciferase assay. A plasmid expressing *Renilla* luciferase under the control of a minimal herpesvirus TK promoter was used as an internal control for all samples. The results are the average of three independent experiments, and error bars denote standard errors. An asterisk indicates significant differences ($P < 0.05$) in promoter activity as determined by one-way analysis of variance and Fisher's least significant difference multiple-means comparison tests.

promoter activity. A similar trend was observed with NICD3 (Fig. 6D). We have consistently observed that NICD1 yielded approximately 4-fold higher levels of transactivation than NICD3, which was expected because the transactivation domain of NICD1 is more active than that of NICD3 (57, 58).

MAML1, β-catenin, and ORF2 cooperate to stimulate cell survival. Canonical Wnt signaling, due to β-catenin activation, promotes axonal guidance, neuronal differentiation, neuronal connectivity, and cell survival (33, 59–61). Combining this knowledge with our previous observations that ORF2 expression stimulates neurite formation in cells expressing Notch (42, 43), we tested whether β-catenin cooperates with ORF2 to stimulate neurite formation in Neuro-2A cells. For neurite formation assays, Neuro-2A cells are seeded at low density and then serum starved for 3 days (42, 43). Many Neuro-2A cells undergo apoptosis following serum withdrawal for 72 h, whereas a minor population sprout neurites and display a differentiated neuronal morphology. Under these conditions, we did not observe an increased frequency of neurite formation when Neuro-2A cells were cotransfected with ORF2, S33Y, and MAML1 (data not shown).

During the course of examining neurite formation, it became obvious that the combination of ORF2, S33Y (referred to as β-catenin in Fig. 7), and MAML1 increased the number of surviving β-Gal$^+$ cells compared to amounts with other combinations (Fig. 7A). A procedure based on counting surviving β-Gal$^+$ cells to quantify cell survival following an apoptotic stimulus (62–64) has been used extensively in our previous studies (15, 19, 65–67). Although the number of β-Gal$^+$ cells could have been influenced by the presence of transcription factors that were overexpressed, this amount was negligible because serum withdrawal induced apoptosis, and only surviving cells that express β-Gal$^+$ would remain attached to the dish. These studies revealed that ORF2 alone, ORF2 plus β-catenin, ORF2 plus MAML1, or MAML1 plus β-catenin consistently enhanced cell survival 2- to 3-fold compared to levels in control cultures transfected with β-catenin alone (Fig. 7B). Interestingly, when Neuro-2A cells were cotransfected with the three expression plasmids, there was a statistically significant increase in cell survival relative to the level with other combinations examined ($P < 0.05$) (Fig. 7B). These studies also revealed that fewer floating cells were present in medium following serum withdrawal when ORF2 combined with both MAML1 and β-catenin was used to transfect Neuro-2A cells than when other transfection combinations were used, which was consistent with enhanced cell survival (data not shown).

DISCUSSION

A transcription factor (β-catenin) activated by the canonical Wnt signaling pathway was expressed in a subset of TG neurons from latently infected but not uninfected calves. We examined β-catenin expression versus a specific Wnt or Wnt receptor because β-catenin protein expression is stabilized when canonical Wnt is stimulated (reviewed in reference 33) (Fig. 1A). Furthermore, 19 known Wnt family members and 11 human Wnt receptors have been identified, making it difficult to examine expression of each isoform. ORF2$^+$ neurons frequently contained β-catenin but not the lytic cycle regulatory protein, bICP0. Our studies also suggested that MAML1 was expressed in a subset of latently infected neurons (data not shown). MAML1 is a crucial

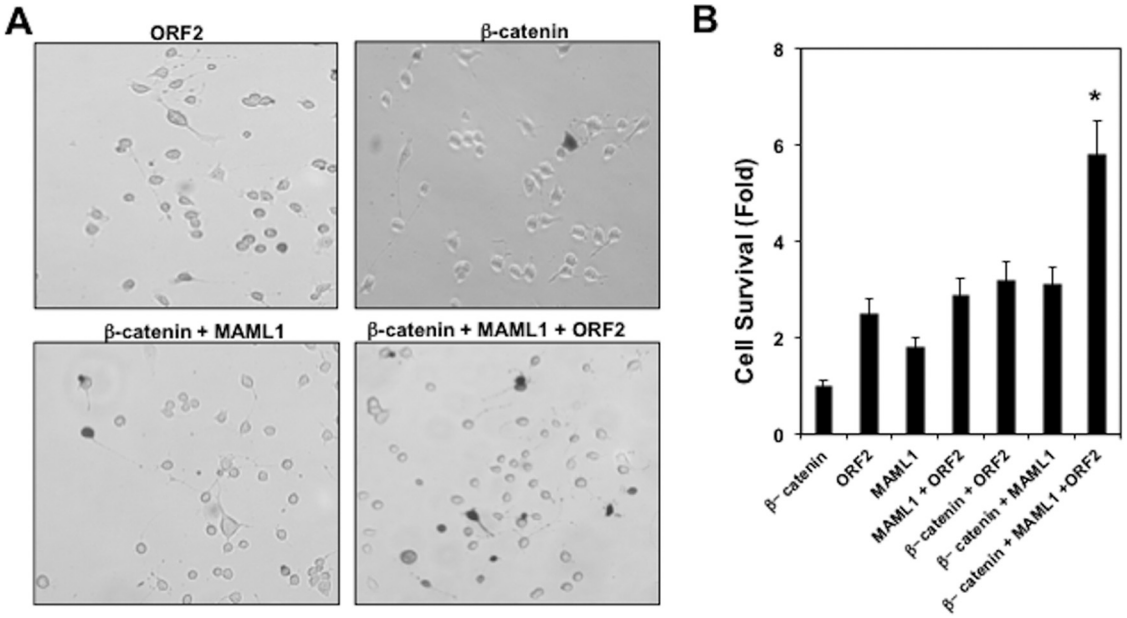

FIG 7 Regulation of cell survival by ORF2, β-catenin, and MAML1 following growth factor withdrawal. (A) Neuro-2A cells were cotransfected with a plasmid expressing β-catenin alone, or with MAML1, a plasmid expressing ORF2, and a plasmid expressing the LacZ gene (transfection control). One microgram of each plasmid was used for transfection, and an empty plasmid was used to maintain equal concentrations of plasmid DNA for the respective transfections. Twenty-four hours after transfection, cells were seeded into new plates at a low density (4,000 cells/cm^2) and then incubated with medium containing 0.5% serum for 3 days. Cells were fixed, and β-Gal$^+$ cells were counted as described in Materials and Methods. (B) The number of β-Gal$^+$ Neuro-2A cells transfected with the β-catenin expression plasmid (S33Y) was arbitrarily set at 1. The number of β-Gal$^+$ Neuro-2A cells in the respective studies was normalized to the β-catenin level to calculate the fold of cell survival. The average of four independent experiments is shown. An asterisk denotes significant differences ($P < 0.05$) in β-Gal$^+$ Neuro-2A cells containing β-catenin with MAML1 plus ORF2 relative to values with other plasmids or combinations, as determined by one-way analysis of variance and Fisher's least significant difference multiple-means comparison tests.

cofactor of Notch-mediated gene expression (30), and Notch1 stimulates BoHV-1 productive infection (22). Low levels of MAML1 expression in latently infected neurons may not have a major impact on Notch-dependent transcription because Notch1 or Notch3 expression is detected in bovine TG neurons only during DEX-induced reactivation from latency (22, 43).

Based on results presented in this study, we hypothesize that infection of sensory neurons causes significant damage; conversely, stabilization of β-catenin and MAML1 by ORF2 is predicted to enhance neuronal survival and maintain normal neuronal functions. This hypothesis is supported by studies demonstrating that the Wnt signaling pathway, via activation of β-catenin, regulates navigation of axons to their synaptic targets and stimulates axonal growth (reviewed in references 59, 60, and 68 to 70). Disruption of the Wnt signaling pathway also inhibits neural crest development and stimulates neurodegeneration (reviewed in references 35, 59, and 60). Finally, canonical Wnt signaling via β-catenin activation inhibits apoptosis in several cell types (71–73), including neurons (74). Although ORF2 interferes with apoptosis (15, 16), the cooperative effect of ORF2, S33Y, and MAML1 on survival of Neuro-2A cells following serum withdrawal was not expected. The ability of ORF2 and MAML1 to stabilize β-catenin levels in Neuro-2A cells correlates well with enhanced β-catenin-dependent transcription and cell survival.

Considering that MAML1 was not expressed in as many ORF2$^+$ TG neurons as β-catenin and that its expression level was low, it is fair to ask whether β-catenin and ORF2 can enhance neuronal survival if MAML1 is not expressed in the same neuron. Additional β-catenin coactivators, CBP (CREB-binding protein) (75), FLAP1 (leucine rich repeat-associated protein 1) (76), Pygopus (77), and the high-mobility group AT-hook 1 protein (HMGA1) have been identified previously (78). Interestingly, HMGA1 is induced by the Wnt/β-catenin signaling pathway (79, 80), suggesting that HMGA1 may be expressed in a subset of latently infected neurons. Additional studies are necessary to determine whether these coactivators are expressed in latently infected TG neurons and if they can cooperate with ORF2 and β-catenin to promote cell survival and transactivate β-catenin-dependent transcription.

During DEX-induced reactivation from latency, ORF2 expression was reduced (43), and expression of two Wnt antagonists (DKK-1 and SFRP2) was increased, which correlated with fewer neurons expressing β-catenin at 6 h after DEX treatment. Chronic stress or increased corticosteroids are known to induce DKK-1 expression and neuronal damage in the hippocampus (81), which is required for ischemic neuronal death (82). DKK-1 also mediates glucocorticoid-induced changes in human neuronal progenitor cell growth and differentiation (83), adding support to the finding that DEX stimulates DKK-1 gene expression in TG. SFRP2 may also regulate neuronal survival during reactivation because it induces cell death in the developing hindbrain (84); conversely, it promotes cell survival in mesenchymal cells (85). Finally, Notch3 and Notch signaling in general are stimulated during reactivation from latency (23, 43), which could influence neurite and axonal growth (24, 25, 86) and contribute to neuronal damage. Although DKK-1 and SFRP2 may have a paracrine-like effect on surrounding uninfected neurons, this may not be as dramatic as the effect in latently infected neurons because β-catenin was not detected

in neurons from uninfected calves. Currently, it is not known whether DKK-1 or SFRP2 directly influences reactivation from latency.

Wnt3a treatment or axin knockdown has been reported to reduce HSV-1 replication in L29 mouse cells (87). Axin is associated with β-catenin and negatively regulates its activity (reviewed in reference 33) (Fig. 1A), suggesting that activated β-catenin inhibits HSV-1 replication. Conversely, human cytomegalovirus replication is suppressed by Wnt antagonists (88). It is well established that gammaherpesviruses utilize the Wnt signaling pathways (89–91) to stimulate growth of latently infected cells. Considering that disregulated β-catenin expression enhances the growth of certain types of cancer cells (33), control of β-catenin expression by gammaherpesviruses is important for their oncogenic potential. In conclusion, these studies provide evidence that β-catenin stabilization during latency may be advantageous for maintaining BoHV-1 latency.

ACKNOWLEDGMENTS

This research was supported by a grant from the USDA-NIFA Competitive Grants Program (13-01041). A grant to the Nebraska Center for Virology (1P20RR15635) also supported certain aspects of these studies.

FUNDING INFORMATION

HHS | National Institutes of Health (NIH) provided funding to Clinton J. Jones under grant number 1P20RR15635. U.S. Department of Agriculture (USDA) provided funding to Clinton J. Jones under grant number 13-01041.

REFERENCES

1. **Turin L, Russo S, Poli G.** 1999. BHV-1: new molecular approaches to control a common and widespread infection. Mol Med **5:**261–284.
2. **Jones C.** 2009. Regulation of innate immune responses by bovine herpesvirus 1 and infected cell protein 0. Viruses **1:**255–275. http://dx.doi.org/10.3390/v1020255.
3. **Jones C, Chowdhury S.** 2007. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex, and development of improved vaccines. Adv Anim Health Res **8:**187–205. http://dx.doi.org/10.1017/S146625230700134X.
4. **Jones C, Geiser V, Henderson G, Jiang Y, Meyer F, Perez S, Zhang Y.** 2006. Functional analysis of bovine herpesvirus 1 (BHV-1) genes expressed during latency. Vet Microbiol **113:**199–210. http://dx.doi.org/10.1016/j.vetmic.2005.11.009.
5. **Perng G-C, Jones C.** 2010. Towards an understanding of the herpes simplex virus type 1 latency-reactivation cycle. Interdiscip Perspect Infect Dis **2010:**1–18.
6. **Jones C.** 1998. Alphaherpesvirus latency: its role in disease and survival of the virus in nature. Adv Virus Res **51:**81–133. http://dx.doi.org/10.1016/S0065-3527(08)60784-8.
7. **Jones C.** 2003. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. Clin Microbiol Rev **16:**79–95. http://dx.doi.org/10.1128/CMR.16.1.79-95.2003.
8. **Kutish G, Mainprize T, Rock D.** 1990. Characterization of the latency-related transcriptionally active region of the bovine herpesvirus 1 genome. J Virol **64:**5730–5737.
9. **Rock DL, Beam SL, Mayfield JE.** 1987. Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. J Virol **61:**3827–3831.
10. **Rock D, Lokensgard J, Lewis T, Kutish G.** 1992. Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. J Virol **66:**2484–2490.
11. **Devireddy LR, Jones C.** 1998. Alternative splicing of the latency-related transcript of bovine herpesvirus 1 yields RNAs containing unique open reading frames. J Virol **72:**7294–7301.
12. **Inman M, Lovato L, Doster A, Jones C.** 2001. A mutation in the latency-related gene of bovine herpesvirus 1 leads to impaired ocular shedding in acutely infected calves. J Virol **75:**8507–8515. http://dx.doi.org/10.1128/JVI.75.18.8507-8515.2001.
13. **Lovato L, Inman M, Henderson G, Doster A, Jones C.** 2003. Infection of cattle with a bovine herpesvirus 1 (BHV-1) strain that contains a mutation in the latency related gene leads to increased apoptosis in trigeminal ganglia during the transition from acute infection to latency. J Virol **77:**4848–4857. http://dx.doi.org/10.1128/JVI.77.8.4848-4857.2003.
14. **Inman M, Lovato L, Doster A, Jones C.** 2002. A mutation in the latency-related gene of bovine herpesvirus 1 disrupts the latency reactivation cycle in calves. J Virol **76:**6771–6779. http://dx.doi.org/10.1128/JVI.76.13.6771-6779.2002.
15. **Shen W, Jones C.** 2008. Open reading frame 2, encoded by the latency-related gene of bovine herpesvirus 1, has antiapoptotic activity in transiently transfected neuroblastoma cells. J Virol **82:**10940–10945. http://dx.doi.org/10.1128/JVI.01289-08.
16. **Sinani D, Jones C.** 2011. Localization of sequences in a protein encoded by the latency related gene of bovine herpesvirus 1 (ORF2) that inhibits apoptosis and interferes with Notch1 mediated *trans*-activation of the bICP0 promoter. J Virol **85:**12124–12133. http://dx.doi.org/10.1128/JVI.05478-11.
17. **Mott K, Osorio N, Jin L, Brick D, Naito J, Cooper J, Henderson G, Inman M, Jones C, Wechsler SL, Perng G-C.** 2003. The bovine herpesvirus 1 LR ORF2 is crucial for this gene's ability to restore the high reactivation phenotype to a herpes simplex virus-1 LAT null mutant. J Gen Virol **84:**2975–2985. http://dx.doi.org/10.1099/vir.0.19421-0.
18. **Perng G-C, Maguen B, Jin L, Mott KR, Osorio N, Slanina SM, Yukht A, Ghiasi H, Nesburn AB, Inman M, Henderson G, Jones C, Wechsler SL.** 2002. A gene capable of blocking apoptosis can substitute for the herpes simplex virus type 1 latency-associated transcript gene and restore wild-type reactivation levels. J Virol **76:**1224–1235. http://dx.doi.org/10.1128/JVI.76.3.1224-1235.2002.
19. **Perng G-C, Jones C, Ciacci-Zanella J, Stone M, Henderson G, Yukht A, Slanina SM, Hoffman FM, Ghiasi H, Nesburn AB, Wechsler SL.** 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript (LAT). Science **287:**1500–1503. http://dx.doi.org/10.1126/science.287.5457.1500.
20. **Meyer F, Perez S, Jiang Y, Zhou Y, Henderson G, Jones C.** 2007. Identification of a novel protein encoded by the latency-related gene of bovine herpesvirus 1. J Neurovirol **13:**569–578. http://dx.doi.org/10.1080/13550280701620754.
21. **Meyer F, Jones C.** 2009. The cellular transcription factor, CCAAT enhancer-binding protein alpha (C/EBP-alpha), has the potential to activate the bovine herpesvirus 1 immediate-early transcription unit 1 promoter. J Neurovirol **15:**123–130. http://dx.doi.org/10.1080/13550280802534771.
22. **Workman A, Sinani D, Pittayakhajonwut D, Jones C.** 2011. A protein (ORF2) encoded by the latency related gene of bovine herpesvirus 1 interacts with Notch1 and Notch3. J Virol **85:**2536–2546. http://dx.doi.org/10.1128/JVI.01937-10.
23. **Workman A, Eudy J, Smith L, Frizzo da Silva L, Sinani D, Bricker H, Cook E, Doster A, Jones C.** 2012. Cellular transcription factors induced in trigeminal ganglia during dexamethasone-induced reactivation from latency stimulate bovine herpesvirus 1 productive infection and certain viral promoters. J Virol **86:**2459–2473. http://dx.doi.org/10.1128/JVI.06143-11.
24. **Cornell R, Eisen JS.** 2005. Notch in the pathway: the roles of Notch signalling in neural crest development. Semin Cell Dev Biol **16:**663–672. http://dx.doi.org/10.1016/j.semcdb.2005.06.009.
25. **Berezovska O, McLean P, Knowles R, Frosh M, Lu FM, Lux SE, Hyman BT.** 1999. Notch1 inhibits neurite outgrowth in postmitotic primary neurons. Neuroscience **93:**433–439. http://dx.doi.org/10.1016/S0306-4522(99)00157-8.
26. **Justice NJ, Jan YN.** 2002. Variations on the Notch pathway in neural development. Curr Opin Neurobiol **12:**64–70. http://dx.doi.org/10.1016/S0959-4388(02)00291-X.
27. **Ehebauer M, Penelope P, Arias AM.** 2006. Notch, a universal arbiter of cell fate decisions. Science **314:**1414–1415. http://dx.doi.org/10.1126/science.1134042.
28. **Bray SJ.** 2006. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol **7:**678–689. http://dx.doi.org/10.1038/nrm2009.
29. **Wu L, Aster JC, Blacklow SC, Lake R, Artavanis-Taskonas S, Griffin JD.** 2000. MAML1, a human homolog of *Drosophila* mastermind, is a transcriptional coactivator for NOTCH receptors. Nat Genet **26:**484–489. http://dx.doi.org/10.1038/82644.

30. **Petcherski AG, Kimble J.** 2000. Mastermind is a putative activator for Notch. Curr Biol **10:**R471–R473. http://dx.doi.org/10.1016/S0960-9822(00)00577-7.
31. **Alves-Guerra MC, Ronchini C, Capobianco AJ.** 2007. Mastermind like 1 is a specific coactivator of beta-catenin transcription activation and is essential for colon carcinoma cell survival. Cancer Res **67:**8690–8698. http://dx.doi.org/10.1158/0008-5472.CAN-07-1720.
32. **Hayward P, Brennan K, Sanders P, Balayo T, DasGupta R, Perrimon N, Arlas AM.** 2005. Notch modulates Wnt signalling by associating with Armadillo/b-catenin and regulating its transcriptional activity. Development **132:**1819–1830. http://dx.doi.org/10.1242/dev.01724.
33. **Clevers H, Nusse R.** 2012. Wnt/B-catenin signaling and disease. Cell **149:**1192–1205. http://dx.doi.org/10.1016/j.cell.2012.05.012.
34. **Lu L, Gao Y, Zhang Z, Cao Q, Zhang X, Zou J, Cao Y.** 2015. Kdm2a/b lysine demethylases regulate canonical Wnt signaling by modulating the stability of nuclear beta-catenin. Dev Cell **33:**660–674. http://dx.doi.org/10.1016/j.devcel.2015.04.006.
35. **Grigoryan T, Ward P, Klaus A, Birchmeir W.** 2008. Deciphering the function of canonical Wnt signals in development and disease: conditional loss-and gain-of-function mutations of beta-catenin in mice. Gene Dev **22:**2308–2341. http://dx.doi.org/10.1101/gad.1686208.
36. **Brakenhoff RH.** 2011. Another NOTCH for cancer. Science **333:**1102–1103. http://dx.doi.org/10.1126/science.1210986.
37. **Hayward P, Kalmar T, Arlas AM.** 2008. Wnt/Notch signalling and information processing during development. Development **135:**411–424. http://dx.doi.org/10.1242/dev.000505.
38. **Perez S, Inman M, Doster A, Jones C.** 2005. Latency-related gene encoded by bovine herpesvirus 1 promotes virus growth and reactivation from latency in tonsils of infected calves. J Clin Microbiol **43:**393–401. http://dx.doi.org/10.1128/JCM.43.1.393-401.2005.
39. **Perez S, Lovato L, Zhou J, Doster A, Jones C.** 2006. Comparison of inflammatory infiltrates in trigeminal ganglia of cattle infected with wild type BHV-1 versus a virus strain containing a mutation in the LR (latency-related) gene. J Neurovirol **12:**392–397. http://dx.doi.org/10.1080/13550280600936459.
40. **Perez S, Meyer F, Saira K, Doster A, Jones C.** 2008. Premature expression of the latency-related RNA encoded by bovine herpesvirus 1 correlates with higher levels of beta interferon RNA expression in productively infected cells. J Gen Virol **89:**1338–1345. http://dx.doi.org/10.1099/vir.0.83481-0.
41. **Irizarry RA, Gautier L, Cope LM.** 2002. An R package for analyses of affymetrix oligonucleotide arrays, p 102–119. *In* Parmigiani G, Garrett ES, Irizarry RA, Zeger SL (ed), The analysis of gene expression of gene expression data: methods and software. Springer, New York, NY.
42. **Sinani D, Liu Y, Jones C.** 2014. Analysis of a bovine herpesvirus 1 protein encoded by an alternatively spliced latency related (LR) RNA that is abundantly expressed in latently infected neurons. Virology **464–465:**244–252. http://dx.doi.org/10.1016/j.virol.2014.06.038.
43. **Sinani D, Frizzo da Silva L, Jones C.** 2013. A bovine herpesvirus 1 protein expressed in latently infected neurons (ORF2) promotes neurite sprouting in the presence of activated Notch1 or Notch3. J Virol **87:**1183–1192. http://dx.doi.org/10.1128/JVI.02783-12.
44. **Morin PJ, Sparks AB, Korinek V, Barker N, Clever H, Vogelstein B, Kinzler KW.** 1997. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science **275:**1787–1790. http://dx.doi.org/10.1126/science.275.5307.1787.
45. **Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT.** 2003. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr Biol **13:**680–685. http://dx.doi.org/10.1016/S0960-9822(03)00240-9.
46. **Tan MJ, White EA, Sowa ME, Harper JW, Aster JC, Howley PM.** 2012. Cutaneous beta-human papillomavirus E6 proteins bind Mastermind-like coactivators and repress Notch signaling. Proc Natl Acad Sci U S A **109:**E1473–E1480. http://dx.doi.org/10.1073/pnas.1205991109.
47. **Wang T, Holt CM, Xu C, Ridley C, Jones R, Baron M, Trump D.** 2007. Notch 3 activation modulates growth behavior and cross talks to Wnt/TCF signalling pathway. Cell Signal **19:**2458–2467. http://dx.doi.org/10.1016/j.cellsig.2007.07.019.
48. **Fryer CJ, White JB, Jones KA.** 2004. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol Cell **16:**509–520. http://dx.doi.org/10.1016/j.molcel.2004.10.014.
49. **Kawanon Y, Kypta R.** 2003. Secreted antagonists of the Wnt signaling pathway. J Cell Sci **116:**2627–2634. http://dx.doi.org/10.1242/jcs.00623.
50. **Zorn AM.** 2001. Wnt signaling: antagonistic Dickkopfs. Curr Biol **11:**R592–R595. http://dx.doi.org/10.1016/S0960-9822(01)00360-8.
51. **Jiang Y, Inman M, Zhang Y, Posadas NA, Jones C.** 2004. A mutation in the latency related gene of bovine herpesvirus 1 (BHV-1) inhibits protein expression of a protein from open reading frame 2 (ORF-2) and an adjacent reading frame during productive infection. J Virol **78:**3184–3189. http://dx.doi.org/10.1128/JVI.78.6.3184-3189.2004.
52. **Frizzo da Silva L, Kook I, Doster A, Jones C.** 2013. Bovine herpesvirus 1 regulatory proteins, bICP0 and VP16, are readily detected in trigeminal ganglionic neurons expressing the glucocorticoid receptor during the early stages of reactivation from latency. J Virol **87:**11214–11222. http://dx.doi.org/10.1128/JVI.01737-13.
53. **Kook I, Doster A, Jones C.** 2015. Bovine herpesvirus 1 regulatory proteins are detected in trigeminal ganglionic neurons during the early stages of stress-induced escape from latency. J Neurovirol **21:**585–591. http://dx.doi.org/10.1007/s13365-015-0339-x.
54. **Jones C, da Silva LF, Sinani D.** 2011. Regulation of the latency-reactivation cycle by products encoded by the bovine herpesvirus 1 (BHV-1) latency-related gene. J Neurovirol **17:**535–545. http://dx.doi.org/10.1007/s13365-011-0060-3.
55. **Jones C.** 2013. Bovine herpes virus 1 (BHV-1) and herpes simplex virus type 1 (HSV-1) promote survival of latently infected sensory neurons, in part by inhibiting apoptosis. J Cell Death **6:**1–16. http://dx.doi.org/10.4137/JCD.S10803.
56. **Jones C, Inman M, Peng W, Henderson G, Doster A, Perng GC, Angeletti AK.** 2005. The herpes simplex virus type 1 locus that encodes the latency-associated transcript (LAT) enhances the frequency of encephalitis in male BALB/C mice. J Virol **79:**14465–14469. http://dx.doi.org/10.1128/JVI.79.22.14465-14469.2005.
57. **Borggrefe T, Oswald E.** 2009. The Notch signaling pathway: transcriptional regulation at Notch target genes. Cell Mol Life Sci **66:**1631–1646. http://dx.doi.org/10.1007/s00018-009-8668-7.
58. **Beatus P, Lundkvist J, Oberg C, Lendahl U.** 1999. The Notch 3 intracellular domain represses Notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters. Development **126:**3925–3935.
59. **Salinas PC.** 2012. Wnt signaling in the vertebrate central nervous system: from axon guidance to synaptic function. Cold Spring Harb Perspect Biol **4:**a008003. http://dx.doi.org/10.1101/cshperspect.a008003.
60. **Purro SA, Galli S, Salinas PC.** 2014. Dysfunction of Wnt signaling and synaptic disassembly in neurodegenerative diseases. J Mol Cell Biol **6:**75–80. http://dx.doi.org/10.1093/jmcb/mjt049.
61. **Kondo T, Matsuoka AJ, Shimomura A, Koehler KR, Chan RJ, Miller JM, Srour EF, Hashino E.** 2011. Wnt signaling promotes neuronal differentiation from mesenchymal stem cells through activation of Tlx3. Stem Cells **29:**836–846. http://dx.doi.org/10.1002/stem.624.
62. **Hsu H, Xiong J, Goeddel DV.** 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell **81:**495–504. http://dx.doi.org/10.1016/0092-8674(95)90070-5.
63. **Kumar S, Kinoshita M, Noda M, Copeland NG, Jenkins NA.** 1994. Induction of apoptosis by the mouse *Nedd2* gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1β-converting enzyme. Genes Dev **8:**1613–1626. http://dx.doi.org/10.1101/gad.8.14.1613.
64. **Liu X, Kim CN, Yang J, Jemmerson R, Wang X.** 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell **86:**147–157. http://dx.doi.org/10.1016/S0092-8674(00)80085-9.
65. **Inman M, Perng G-C, Henderson G, Ghiasi H, Nesburn AB, Wechsler SL, Jones C.** 2001. Region of herpes simplex virus type 1 latency-associated transcript sufficient for wild-type spontaneous reactivation promotes cell survival in tissue culture. J Virol **75:**3636–3646. http://dx.doi.org/10.1128/JVI.75.8.3636-3646.2001.
66. **Ciacci-Zanella J, Stone M, Henderson G, Jones C.** 1999. The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death. J Virol **73:**9734–9740.
67. **Shen W, Sa e Silva M, Jaber T, Vitvitskaia O, Li S, Henderson G, Jones C.** 2009. Two small RNAs encoded within the first 1.5 kb of the herpes simplex virus type 1 latency-associated transcript can inhibit productive infection and cooperate to inhibit apoptosis. J Virol **83:**9131–9139. http://dx.doi.org/10.1128/JVI.00871-09.
68. **Bhardwaji D, Nager M, Camats J, David M, Benguira A, Dopazo A, Canti C, Herreros J.** 2013. Chemokines induce axon outgrowth down-

stream of hepatocyte growth factor and TCF/β-catenin signaling. Front Cell Neurosci **7:**52. http://dx.doi.org/10.3389/fncel.2013.00052.
69. **Murase S, Mosser E, Schuman EM.** 2002. Depolarization drives beta-catenin into neuronal spines promoting changes in synaptic structure and function. Neuron **35:**91–105. http://dx.doi.org/10.1016/S0896-6273(02)00764-X.
70. **Bamji SX, Rico B, Kimes N, Reichardt LF.** 2006. BDNF mobilizes synaptic vesicles and enhances synaptic vesicles and enhances synapse formation by disrupting cadherin-beta-catenin interactions. J Cell Biol **174:**289–299. http://dx.doi.org/10.1083/jcb.200601087.
71. **Chen S, Guttridge DC, You Z, Zhang Z, Fribley A, Mayo MW, Kitajewski J, Wang CY.** 2001. Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription. J Cell Biol **152:**87–96. http://dx.doi.org/10.1083/jcb.152.1.87.
72. **Masckauchan TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J.** 2005. Wnt/beta-catenin signaling induces proliferation, survival, and Interleukin-8 in human endothelial cells. Angiogenesis **8:**43–51. http://dx.doi.org/10.1007/s10456-005-5612-9.
73. **Biechele TL, Kulikauskas RM, Toroni RA, Lucero OM, Swift RD, James RG, Robin NC, Dawson DW, Moon RT, Chien AJ.** 2012. Wnt/β-catenin signaling and AXIN1 regulate apoptosis mediated by inhibition of BRAFV600E kinase in human melanoma. Sci Signal **5:**ra3. http://dx.doi.org/10.1126/scisignal.2002274.
74. **Zhao S, Fu J, Liu X, Wang T, Zhang J, Zhao Y.** 2012. Activation of Akt/GSK-3β/β-catenin signaling pathway is invovlved in survival of neurons after traumatic brain injury in rats. Neurol Res **34:**400–407. http://dx.doi.org/10.1179/1743132812Y.0000000025.
75. **Takemaru K-T, Moon RT.** 2000. The transcriptional coactivator CBP interacts with β-catenin to activate gene expression. J Cell Biol **149:**249–254. http://dx.doi.org/10.1083/jcb.149.2.249.
76. **Lee Y-H, Stallup MR.** 2006. Interplay between Fli-1 and FLAP1 for regulation of β-catenin dependent transcription. Nucleic Acids Res **34:**5052–5059. http://dx.doi.org/10.1093/nar/gkl652.
77. **Mieszczanek J, de la Roche M, Bienz M.** 2008. A role of Pygopus as an anti-repressor in facilitating Wnt-dependent transcription. Proc Natl Acad Sci U S A **105:**19324–19320. http://dx.doi.org/10.1073/pnas.0806098105.
78. **Xing J, Cao G, Fu C.** 2014. HMGA1 interacts with β-catenin to positively regulate Wnt/β-catenin signaling in colorectal cancer cells. Pathol Oncol Res **20:**847–851. http://dx.doi.org/10.1007/s12253-014-9763-0.
79. **Bush BM, Brock AT, Deng JA, Nelson RA, Jr, Sumter TF.** 2013. The Wnt/β-catenin/TCF pathway up-regulates high-mobility group A1 expression in colon cancer. Cell Biochem Funct **31:**228–236. http://dx.doi.org/10.1002/cbf.2876.
80. **Akaboshi S-I, Watanabe S, Hino Y, Sekita Y, Xi Y, Araki K, Yamamura K-ichi, Oshima M, Ito T, Baba H, Nako M.** 2009. HMGA1 is induced by Wnt/β-catenin pathway and maintains cell proliferation in gastric cancer. Am J Pathol **175:**1675–1685. http://dx.doi.org/10.2353/ajpath.2009.090069.
81. **Matrisciano F, Buscetti CL, Bucci D, Orlando R, Caruso A, Molinaro G, Cappuccion I, Riozzi B, Gradini R, Motolese M, Caraci F, Copani A, Scaccianoce S, Melchiorri D, Bruno V, Battaglia G, Nicoletti F.** 2011. Induction of the Wnt antagonist Dickkopf-1 is involved in stress-induced hippocampal damage. PLoS One **6:**e16447. http://dx.doi.org/10.1371/journal.pone.0016447.
82. **Mastroiacovo F, Busceti CL, Biagioni F, Moyanova SG, Meisler MH, Battaglia G, Caricasole A, Bruno V, Nicoletti F.** 2009. Induction of the Wnt antagonist, Dickkopf-1, contributes to the development of neuronal death in models of brain focal ischemia. J Cereb Blood Flow Metab **29:**264–276. http://dx.doi.org/10.1038/jcbfm.2008.111.
83. **Moors M, Bose R, Johansson-Haque K, Edoff K, Okret S, Ceccatelli S.** 2012. Dickkopf mediates glucocorticoid-induced changes in human neural progenitor cell proliferation and differentiation. Toxicol Sci **125:**488–495. http://dx.doi.org/10.1093/toxsci/kfr304.
84. **Ellies DL, Church V, Francis-West P, Lumsden A.** 2000. The Wnt antagonist cSFRP2 modulates programmed cell death in the developing hindbrain. Development **127:**5285–5295.
85. **Mirotsou M, Zhang Z, Deb A, Zhang L, Gnecchi M, Noiseux N, Mu H, Pachori A, Dzau V.** 2007. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci U S A **104:**1643–1648. http://dx.doi.org/10.1073/pnas.0610024104.
86. **El Bejjani R, Hammerlund M.** 2012. Notch signaling inhibits axon regeneration. Neuron **73:**268–278. http://dx.doi.org/10.1016/j.neuron.2011.11.017.
87. **Choi E.-J, Kim S, Jho E, Song K-J, Kee S-H.** 2013. Axin expression enhances herpes simplex virus type 1 replication by inhibiting virus-mediated cell death in L929 cells. J Gen Virol **94:**1636–1646. http://dx.doi.org/10.1099/vir.0.051540-0.
88. **Kapoor A, He R, Venkatadri R, Forman M, Arav-Boger R.** 2013. Wnt modulating agents inhibit human cytomegalovirus replication. Antimicrob Agents Chemother **57:**2761–2767. http://dx.doi.org/10.1128/AAC.00029-13.
89. **Hayward SD, Liu J, Fujimuro M.** 2006. Notch and Wnt signaling: mimicry and manipulation by gamma herpesviruses. Sci STKE **2006:**re4.
90. **Angelova M, Ferris M, Swan KF, McFerrin HE, Pridjian G, Morris CA, Sullivan DE.** 2014. Kaposi's sarcoma-associated herpesvirus G-protein coupled receptor activates the canonical Wnt/β-catenin signaling pathway. Virol J **11:**218. http://dx.doi.org/10.1186/s12985-014-0218-8.
91. **Fujimuro M, Wu FY, ApRhys C, Kajumbula H, Young DY, Hayward GS, Hayward SD.** 2003. A novel viral mechanism for dysregulation of β-catenin in Kaposi's sarcoma-associated herpesvirus latency. Nat Med **9:**300–306. doi:http://dx.doi.org/10.1038/nm829.