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Regulation of Notch-mediated transcription by a bovine herpesvirus 1 encoded protein (ORF2) that is expressed in latently infected sensory neurons

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Abstract Bovine herpesvirus 1 (BoHV-1) is an *Alphaherpesvirinae* subfamily member that establishes lifelong latency in sensory neurons. The latency-related RNA (LR-RNA) is abundantly expressed during latency. An LR mutant virus containing stop codons at the amino-terminus of open reading frame (ORF)2 does not reactivate from latency and replicates less efficiently in tonsils and trigeminal ganglia. ORF2 inhibits apoptosis, interacts with Notch family members, and interferes with Notch-dependent transcription suggesting ORF2 expression enhances survival of infected neurons. The Notch signaling pathway is crucial for neuronal differentiation and survival suggesting that interactions between ORF2 and Notch family members regulate certain aspects of latency. Consequently, for this study, we compared whether ORF2 interfered with the four mammalian Notch family members. ORF2 consistently interfered with Notch1–3-mediated transactivation of three cellular promoters. Conversely, Notch4-mediated transcription was not consistently inhibited by ORF2. Electrophoretic shift mobility assays using four copies of a consensus-DNA binding site for Notch/CSL (core binding factor (CBF)-1, Suppressor of Hairless, Lag-2) as a probe revealed ORF2 interfered with Notch1 and 3 interactions with a CSL family member bound to DNA. Additional studies demonstrated ORF2 enhances neurite sprouting in mouse neuroblastoma cells that express Notch1–3, but not Notch4. Collectively, these studies indicate that ORF2 inhibits Notch-mediated transcription and signaling by interfering with Notch interacting with CSL bound to DNA.

Keywords BoHV-1 · ORF2 · Notch-mediated transcription · Neuronal differentiation · Latency in sensory neurons

Introduction

Bovine herpesvirus 1 (BoHV-1) is an important bovine pathogen that primarily infects cells within the upper respiratory tract as well as cells lining the ocular and nasal cavity (Jones 2009; Jones, and Chowdhury 2007; Turin et al. 1999). Although virus is cleared by a robust immune response, sensory neurons within trigeminal ganglia (TG) become latently infected (Jones et al. 2006; Jones and Chowdhury 2007). In contrast to other viral genes, the BoHV-1 latency-related (LR) RNA can be readily detected in latently infected neurons (Jones 1998, 2003; Jones, et al. 2006; Kutish et al. 1990; Rock et al. 1987, 1992). The LR gene contains two open reading frames (ORF1 and ORF2) and two reading frames without an initiating methionine, which are designated as RF-B and RF-C (Kutish et al. 1990). ORF2 isoforms, including the 15 days (15d) ORF, can be generated due to alternative splicing of the LR-RNA (Devireddy and Jones 1998) (Fig. 1a). The 15d ORF contains an additional 36 amino acids derived from RF-B (Fig. 1b), is more stable than ORF2 in transfected Neuro-2A cells, and has similar functions as ORF2 (Sinani et al. 2014). Regardless of how polyA+ LR-RNA is spliced, the transcript overlaps and is antisense relative to the infected protein 0 (bICP0) coding sequences, reviewed in (Jones et al. 2006, 2011) suggesting bICP0 expression is

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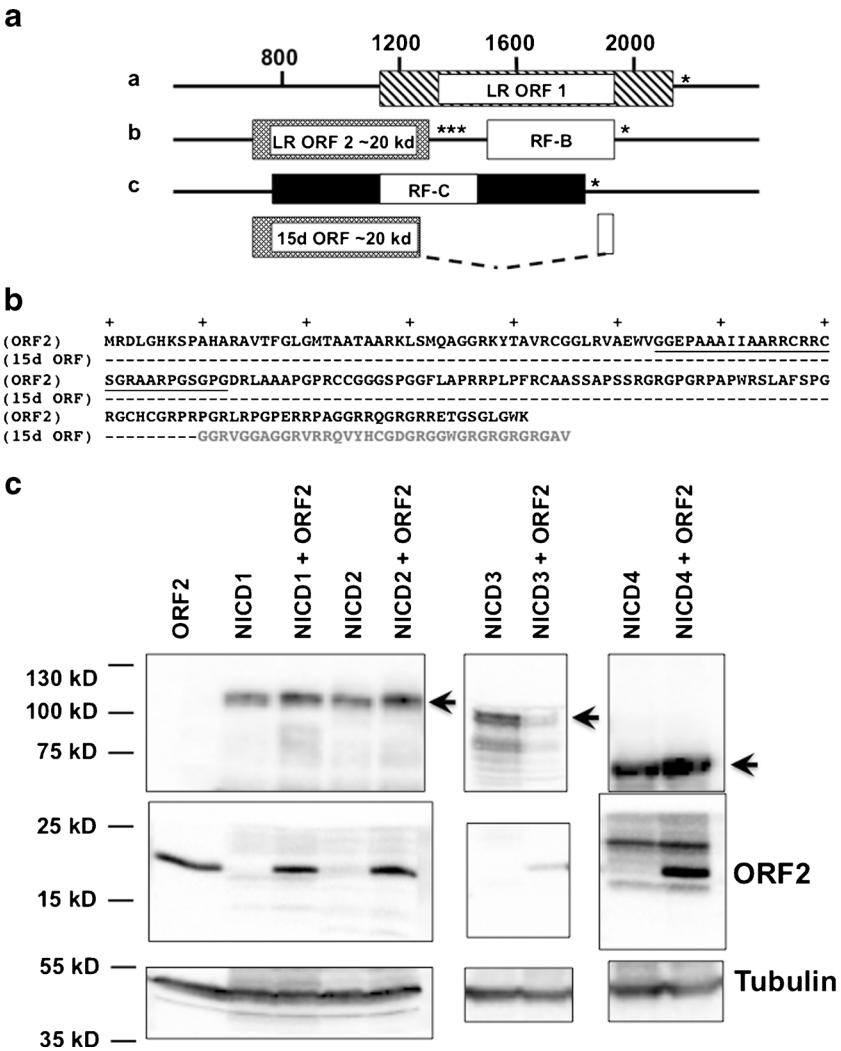

Fig. 1 ORF2 reduces NICD3 steady state levels, but not other NICD members in transfected Neuro-2A cells. **a** Location of ORFs within the LR gene. The numbering system of the LR gene and location of ORF2 is derived from a previous study (Kutish et al. 1990). Reading frames that lack an initiating methionine are designated RF-B and RF-C. LR ORF1, ORF2, and RF-C are located in reading frames *a–c*, respectively. RF-B, like ORF2, is in reading frame *b*. The 15d ORF is comprised of ORF2 sequences and contains 36 amino acids derived from RF-B due to alternative splicing (Devireddy and Jones 1998). **b** Comparison of ORF2 amino acid sequences and 15d ORF. Amino acid sequence of ORF2: *dashed lines* in 15d ORF reflect identical amino acids as ORF2. Underlined amino acids are the nuclear localization sequence. The 36 gray amino acids in the 15d ORF are derived from C-terminal sequences of RF-B, which was the result of alternative splicing. The plus signs denote every 10th amino acid. **c** Neuro-2A cells were transfected with 2 μg of the designated plasmid that express a Flag-tagged NICD expression plasmid alone or with 2 ug Flag-ORF2. Forty hours after transfection, total lysate was prepared and Western blot analysis performed using the Flag antibody (1:1000 dilution) or anti-Notch3 (1:300) (Santa Cruz). Twenty-five micrograms total cell lysate was used to detect NICD protein expression, while 100 μg total cell lysate was used for ORF2 detection. The images are representative of three independent experiments. Molecular weight markers are denoted to the *right*. Tubulin protein expression was used as a loading control for this study

influenced by LR-RNA. An LR mutant virus that does not express ORF2 due to insertion of three stop codons at the amino-terminus of ORF2 has reduced pathogenesis in calves, in part because virus shedding from certain tissue is reduced (Inman et al. 2001). The LR mutant virus does not establish latency as efficiently as wt BoHV-1 and does not reactivate from latency following treatment with the synthetic corticosteroid dexamethasone (DEX) (Inman et al. 2002).

ORF2 protein expression, not merely RNA expression, can inhibit apoptosis in mouse neuroblastoma cells (Ciacci-Zanella et al. 1999; Lovato et al. 2003; Shen and Jones 2008; Sinani and Jones 2011). Notch1, Notch3, or C/EBP-alpha interacts with ORF2 and these interactions interfere with Notch1- or 3-mediated transactivation (Meyer et al. 2007; Meyer and Jones 2008; Workman et al. 2011). The ability of ORF2 and ORF2 isoforms to interact with and influence Notch signaling is proposed to be important during the latency-reactivation cycle because Notch3 expression and genes stimulated by Notch are induced during reactivation from latency (Workman et al. 2011, 2012). Furthermore,

Notch1 protein expression increases following infection of cultured rabbit cells (Workman et al. 2011).

Mammals encode four Notch receptor family members (Notch 1–4) (Bray 2006; Ehebauer et al. 2006). Notch family membranes are membrane-tethered transcription factors that regulate neuronal maintenance, development, differentiation, as well as development of nearly all non-neuronal cell types (Berezovska et al. 1999; Cornell and Eisen 2005; Justice and Jan 2002). When the Notch receptor is specifically bound by a ligand (Jagged1, Jagged2, Delta-like1, Delta-like3, or Delta-like4, for example), the Notch intracellular domain (NICD) is cleaved, and then enters the nucleus. The NICD interacts with a member of the CSL family of transcriptional factors, core binding factor (CBF)1, Su(H), or Lag1 (also referred to as RBP*j* binding proteins) subsequently activating downstream genes. Mastermind (MAML) is also part of the Notch/CSL complex that is bound to DNA (Fryer et al. 2004). When CSL family members are not bound to Notch, transcription is repressed.

Notch1 (Naidr et al. 2003; Sade et al. 2004) and Notch3 (Wang et al. 2007) can inhibit apoptosis by activating the AKT protein kinase. Surprisingly, other reports reported that Notch induces apoptosis (Bray 2006; Ehebauer et al. 2006). This discrepancy could be related to the finding that low levels of constitutively active Notch1 intracellular domain (NICD1) inhibit apoptosis of neural progenitors in the absence of growth factors whereas higher NICD1 levels promotes apoptosis (Oishi et al. 2004). Deregulated Notch signaling and/or Notch mutations can also have an oncogenic effect in a growing number of malignancies (breast cancer, lung cancer, and T cell acute lymphoblastic leukemia, for example). Furthermore, Notch signaling can maintain the survival and growth of cancer stem cells, reviewed in (Koch and Radtke 2011; Sethi and Kang 2011). In summary, Notch family members play crucial roles during mammalian growth and differentiation.

For this study, we compared the ability of ORF2 and an ORF2 isoform (15d ORF) to regulate all four Notch family members. ORF2 and the 15d ORF consistently interfered with NICD1–3-mediated transactivation of three promoters that are regulated by Notch family members. ORF2 also interfered with the ability of NICD1 and 3 to engage a CSL family member bound to DNA. Conversely, ORF2 and the 15d ORF did not interfere with NICD4-mediated transactivation. Consistent with these studies, ORF2 overcame the ability of NICD1–3, but not NICD4, to interfere with neurite formation in mouse neuroblastoma cells.

Materials and methods

Cells

Murine neuroblastoma cells (Neuro-2A) were 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).

Plasmids and transient transfections

The LR-specific complementary DNA (cDNA) identified in TG at 15 days after infection was cloned into the vector pCMV-Tag-2B (Stratagene) and was previously shown to express this novel ORF2 isoform (Shen and Jones 2008; Sinani et al. 2013, 2014). A FLAG epitope is present at the N-terminus of the ORF and the human IE CMV promoter drives its expression.

Notch1, Notch2 and Notch4 ICD, and luciferase constructs hairy enhancer of split 1 (HES1) and four consensus CSL binding sites (4xCSL) were purchased from Addgene. Notch3 ICD constructs were kindly provided by U. Lendahl, Karolinska Institute, Stockholm, Sweden. A plasmid containing the firefly luciferase gene downstream of the HES5 promoter was a kind gift from (R. Kopan, Washington University, St. Louis, MO). All plasmids were transfected into mouse neuroblastoma (Neuro-2A) cells in 60-mm dishes by using TransIT Neural (MIR2145; Mirus) according to the manufacturer's instructions.

Western blot analysis

Western blotting was preformed as described previously (Shen and Jones 2008; Sinani et al. 2013, 2014). In brief, Neuro-2A cells in 60-mm dishes were transfected with the designated plasmids. Forty-eight hours after transfection, cells were scraped from the dish, washed once with PBS, lysed in RIPA buffer (50 mM Tris–HCl, pH 8150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM EDTA) 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 NICD1, 2, 3, and 4 were performed using a mouse anti-FLAG antibody (Sigma F1804) (1:1000) or rabbit anti-Notch3 antibody with a dilution of 1:300 (sc-5593; Santa Cruz Biotechnology).

Dual-luciferase reporter assay

Luciferase reporter assays were performed following transfection of Neuro-2A cells essentially as previously described (Shen and Jones 2008; Sinani et al. 2013, 2014). In brief, 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, the medium was replaced with fresh EMEM containing 0.5 % FCS to lower the basal levels of promoter activity. Cells were co-transfected with a plasmid containing the firefly luciferase gene downstream of the

HES1, HES5, or 4xCSL promoter (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 (1 μg DNA), and an ORF2 or 15d ORF expression plasmid (1 μg DNA). To maintain equal plasmid amounts in the transfection mixtures, the 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).

Electrophoretic mobility shift assay)

Neuro-2A cells were transfected with NICD1-4 with an empty expression vector or with the ORF2 expression plasmid. Forty-eight hours after transfection, whole-cell lysate was prepared. Cells were washed with phosphate-buffered saline (PBS) and suspended in NP-40 lysis buffer {50 mM Tris (pH 8.0), 150 mM NaCl, 1 % NP-40, 10 % glycerol, 1 mM EDTA, and 1x protease inhibitors (Thermo-Scientific)}. Cell lysate was incubated on ice for 30 min and then clarified by centrifugation at 12,000×g at 4 °C for 15 min.

Fifty micrograms of protein extract was incubated in 30 μl of binding buffer (10 mM HEPES, pH 8, 50 mM KCl, 8 mM $MgCL_2$, 0.5 mM EDTA, 150 ng/ul BSA, 1 mM DTT, 10 % glycerol) in the presence of 2 μg poly(dI-dC) (P4929; Sigma) and 0.5 pmol of double-stranded DNA probe labeled with 10 μCi of (γ-32P)-ATP. Protein concentrations were quantified by the Bradford assay. Incubation proceeded for 30 min at 4 ° C. For competition assays, 50 pmol cold 4xCSL consensus probe was incubated with cell lysate for 20 min prior to addition of radiolabeled probe. DNA-protein complexes were run in a 5 % polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) for 3 h at 100 V. The gel was dried and exposed to a phosphorimager plate and analyzed using a Bio-Rad Personal Molecular Imager™. The 4xCSL binding site oligonucleotide was synthesized by Integrated DNA Technology (Iowa) as single-stranded DNA and then allowed to anneal.

The 4xCSL plus strand is: 5′-CGTGGGAACGTGG GAACGTGGGAACGTGGGAA-3′.

The 4xCSL negative strand is: 5′-TTCCCACGTTC CCACGTTCCCACGTTCCCACG-3′.

Neurite formation assay

The neurite formation assay was performed as previously described (Sinani et al. 2013, 2014). In brief, Neuro-2A cells grown in 60-mm plates were co-transfected with a human cytomegalovirus promoter plasmid expressing the designated NICD (1 μg DNA), an ORF2 construct (1 μg DNA), and the pCMV-β-Gal plasmid (1 μg DNA). To induce neurite sprouting, 24 h after transfection, cells were seeded into new plates at a low density of 2000/cm^2 and starved in medium with 0.5 % serum for 3 days. Cells were then fixed and stained, and a β-galactosidase (β-Gal) assay was performed as previously described. The percentage of cells with β-Gal + neurites was calculated by dividing the number of β-Gal + cells with a neurite length at least twice the diameter of the cell by the total number of β-Gal + cells. The results are an average of three independent experiments.

Results

Expression of Notch family members in neuro-2A cells

Initial studies examined NICD expression in Neuro-2A cells and whether expression was influenced by ORF2. A yeast two-hybrid screen previously revealed that ORF2 interacted with Notch3 more frequently than Notch1; conversely Notch2 and Notch4 were not detected in the screen (Workman et al. 2011). We may not have detected Notch2 and Notch4 in the two-hybrid screen because the cDNA library did not contain these transcripts or ORF2 does not physically interact with these proteins. Neuro-2A cells were used for these studies because they can be readily transfected, ORF2 is consistently expressed in these cells whereas in other cells, it is not; these cells are models for studying neuro-biology, and ORF2 can promote neurite formation in these cells when they express NICD1 or NICD3 (Sinani et al. 2013, 2014; Sinani and Jones 2011). Furthermore, NICD family members are not readily detectable in Neuro-2A cells, which allows one to over-express an NICD member and characterize its functions in Neuro-2A cells without being concerned that other Notch members expressed endogenously will influence the effects of a single NICD on our studies. Plasmids that express NICD1–4 yielded the correct sized protein in transiently transfected Neuro-2A cells (Fig. 1, position of the respective NICD members is denoted by arrows). When ORF2 was co-transfected with the respective NICD family members, only NICD3 protein levels were reduced, which was consistent with a previous study (Sinani et al. 2013; Workman et al. 2011). In several studies, we have seen that NICD4 levels actually increased slightly in cell transfected with ORF2. Additional studies demonstrated that NICD3 co-localizes with ORF2 at the periphery of Neuro-2A cells but NICD1 disperses ORF2 to all areas of the nucleus, which is consistent with previous studies (Workman et al. 2011). NICD2 and NICD4, in the absence of ORF2, were localized to peripheral areas of the nucleus in transfected Neuro-2A cells, which made it difficult to discern whether ORF2 influenced their localization (data not shown).

Regulation of Notch-mediated transcription by ORF2 and the 15d ORF

Although previous studies demonstrated ORF2 interferes with NICD1- and 3-mediated transactivation (Sinani et al. 2014; Sinani and Jones 2011; Workman et al. 2011), we did not test whether ORF2 interferes with NICD2- or 4-mediated transcription. It was also of interest to examine the influence of ORF2 on three cellular promoters known to be transactivated by NICD family members because adjacent transcription factor binding sites and the number of CSL binding sites in a promoter can play a significant role with respect to transactivation (Borggrefe and Oswald 2009; Liu et al. 2010). Consequently, we examined three different Notch-regulated promoters, hairy enhancer of split 1 (HES1), HES5, and a simple promoter containing four consensus CSL binding sites (4xCSL).

NICD1 stimulated HES1 promoter activity more than 12-fold and ORF2 or the 15d ORF significantly reduced NICD1-mediated transactivation (Fig. 2a). NICD2 did not stimulate HES1 promoter activity as efficiently as NICD1; however, ORF2 and the 15d ORF interfered with transactivation. Although NICD3 only stimulated HES1 promoter activity threefold, ORF2 and the 15d ORF interfered with transactivation (Fig. 2b). In contrast, NICD4-mediated transactivation was not inhibited by ORF2 or the 15d ORF. In fact, ORF2, but not the 15d ORF, had a slight stimulatory effect on NICD4-mediated transactivation of HES1 promoter activity.

HES5 promoter activity was stimulated more than 16-fold by NICD1, and ORF2 as well as the 15d ORF reduced transactivation by approximately 2-fold (Fig. 3a). NICD2 stimulated HES5 promoter activity more than 10-fold. Although ORF2 and the 15d ORF significantly interfered with NICD2-mediated transactivation of HES5 promoter activity, inhibition was not as dramatic as that observed with NICD1. NICD3-mediated transactivation of HES5 promoter activity was less than NICD1 or NICD2 (Fig. 3b). ORF2 and the 15d ORF reduced NICD3-mediated transactivation to near basal promoter activity, which is consistent with previous studies (Sinani et al. 2013, 2014). Like the HES1 promoter, ORF2 and the 15d ORF had a modest, but significant, stimulatory effect on HES5 promoter activity when co-transfected with NICD4.

NICD1 stimulated 4xCSL promoter activity more than 150-fold and ORF2 or the 15d ORF significantly reduced NICD1-mediated transactivation (Fig. 4a). Although NICD2 and NICD3 (Fig. 5b) mediated transactivation of the 4xCSL promoter was less than NICD1, ORF2 and the 15d ORF inhibited transactivation. In contrast to the HES1 and HES5 promoters, NICD4-mediated transactivation of 4xCSL promoter activity was reduced by ORF2 and the 15d ORF (Fig. 4b). In summary, these studies demonstrated that ORF2 and the 15d ORF consistently interfered with NICD1–3 meditated transactivation of all three promoters examined.

ORF2 interferes with the ability of NICD1 and NICD3 to form a stable complex with CSL bound to DNA

To stimulate transcription, an NICD family member must form a stable complex with a CSL member specifically bound to DNA, MAML, and other transcriptional co-activators

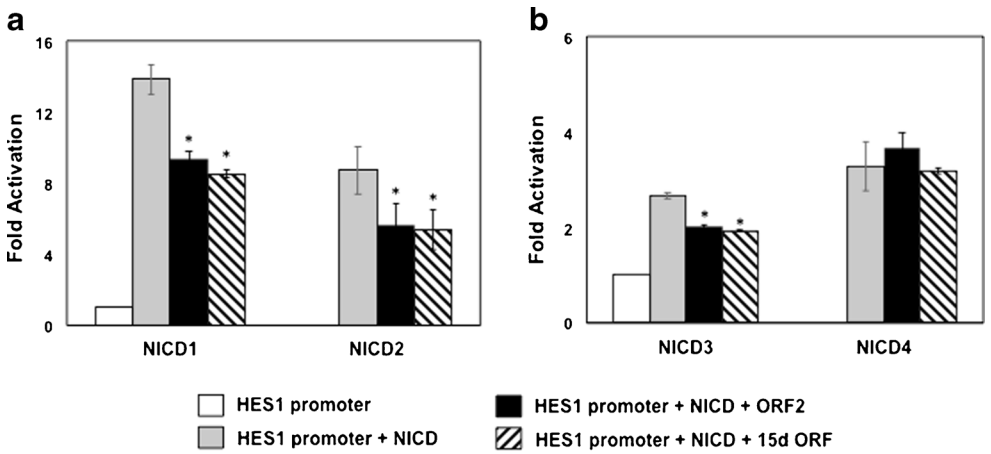

Fig. 2 Effects of ORF2 and the 15d ORF on NICD-mediated transactivation of HES1 promoter activity. Neuro-2A cells were co-transfected with a plasmid containing the *Firefly* luciferase gene downstream of the HES1 promoter, a plasmid expressing Notch1–4 ICD and a plasmid expressing ORF2 or 15d ORF. As a control, the HES1 promoter was co-transfected with an empty vector. Forty-eight hours after transfection, promoter activity was measured 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. The results are the average of three independent experiments and error bars denote standard deviation. An *asterisk* denotes significant differences ($P < 0.05$) in promoter activation by the respective NICD in the presence of ORF2 and 15d ORF, as determined by the one-way ANOVA and Fisher's LSD multiple means comparison tests

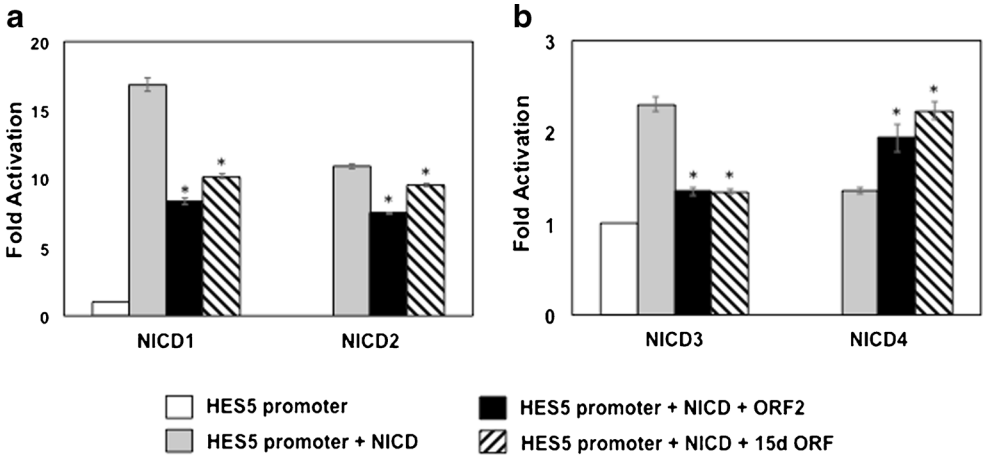

Fig. 3 Effects of ORF2 and the 15d ORF on NICD-mediated transactivation of HES5 promoter activity. Neuro-2A cells were co-transfected with a plasmid containing the *Firefly* luciferase gene downstream of the HES5 promoter, a plasmid expressing a NICD protein, and a plasmid expressing ORF2 or 15d ORF. As a control, the HES5 promoter was co-transfected with an empty vector. Forty-eight hours after transfection promoter activity was measured 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. The results are the average of three independent experiments and *error bars* denote standard deviation. An *asterisk* denotes significant differences ($P < 0.05$) in promoter activation by the respective NICD in the presence of ORF2 and 15d ORF, as determined by the one-way ANOVA and Fisher's LSD multiple means comparison tests

(Borggrefe and Oswald 2009; Bray 2006); see Fig. 5a, i for schematic of this complex. In the absence of Notch expression, CSL recruits co-repressors to DNA, which represses transcription (Fig. 5a, ii). We suggest ORF2 interferes with Notch-mediated transcription by one of two possible mechanisms: (1) ORF2 sequesters NICD and prevents it from interacting with CSL and/or MAML to form a stable DNA protein complex (Fig. 5a, iii); or (2) ORF2 + NICD stably interact with a CSL family member; however, this interaction does not activate transcription because important cofactors do not interact with this complex, MAML for example (Fig. 5a, iv).

To test which possible scenario occurs, electrophoretic mobility shift assays (EMSA) were performed by incubating a radioactive 4xCSL probe with extracts prepared from Neuro-2A cells that were transfected with the respective NICD. A prominent shifted band was detected when extracts prepared from Neuro-2A cells were incubated with the 4xCSL probe (Fig. 5b, lanes N2A, closed circle). This shifted band is likely a CSL protein bound to the 4xCSL probe because Neuro-2A cells do not express readily detectable amounts of Notch1 or Notch3 (Workman et al. 2011). When Neuro-2A cells were transfected with NICD1–3, additional high molecular weight bands were readily detected (Fig. 5b, arrows denote the

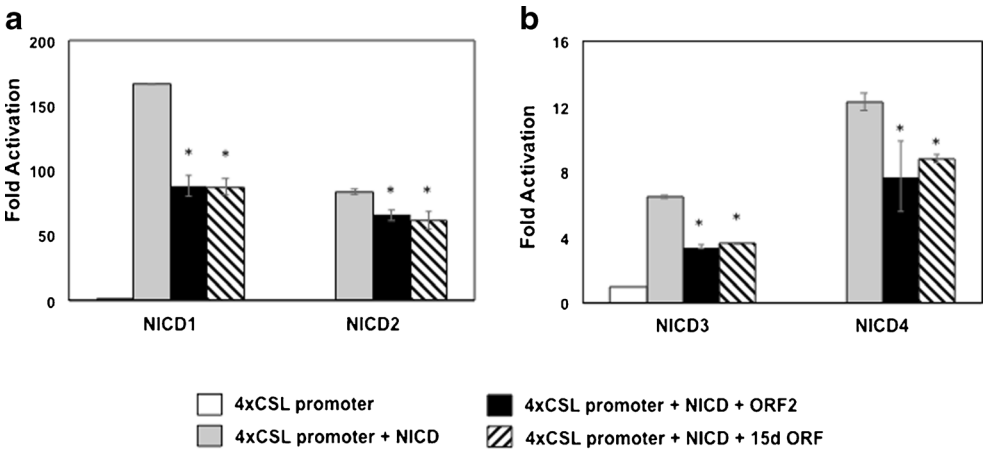

Fig. 4 Effects of ORF2 and 15d ORF on NICD-mediated transactivation of 4xCSL promoter activity. Neuro-2A cells were co-transfected with a plasmid containing the *Firefly* luciferase gene downstream of the 4xCSL promoter, a plasmid expressing the designated NICD protein and a plasmid expressing ORF2 or 15d ORF. As a control, the 4xCSL promoter construct was co-transfected with an empty vector. Forty-eight hours after transfection, promoter activity was measured 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. The results are the average of three independent experiments and *error bars* denote standard deviation. An *asterisk* denotes significant differences ($P < 0.05$) in promoter activation by the respective NICD in the presence of ORF and 15d ORF, as determined by the one-way ANOVA and Fisher's LSD multiple means comparison tests

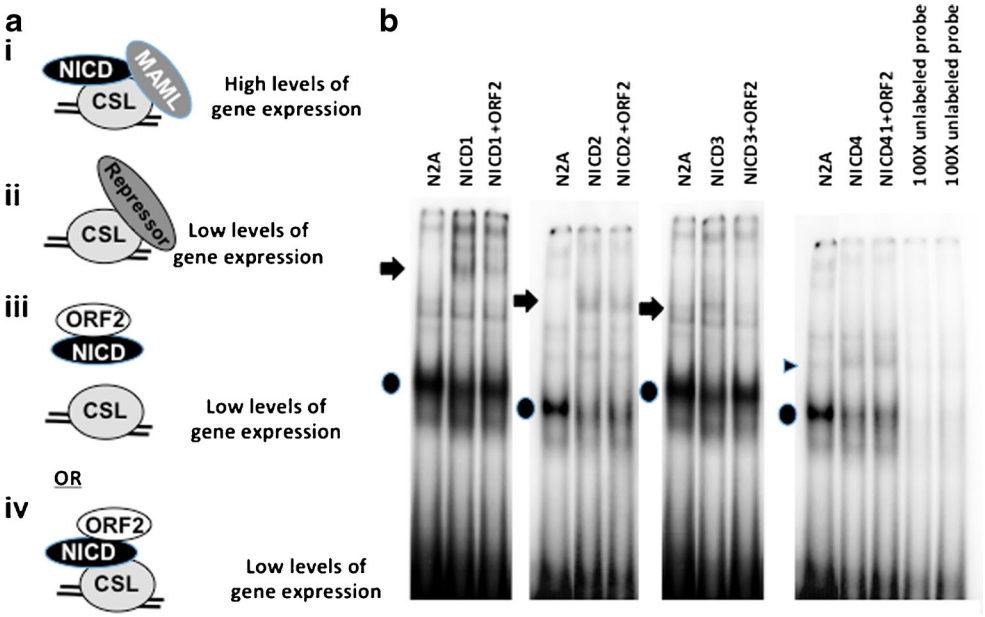

Fig. 5 ORF2 prevent the formation of stable NICD1–3/CSL complex. EMSA was performed using a probe that contains four consensuses CSL binding site that was described in "Materials and Methods." The ^{32}P-radiolabeled probe was incubated with 50 μg of Neuro-2A cell lysate from cells transfected with the NICD1-4 with or without ORF2 expression construct. For competition assays, 50 pmol of the cold 4xCSL consensus probe was incubated with NICD1 and NICD 2 transfected cell extracts. N2A denotes cell lysate prepared from Neuro-2A cells that was not transfected. Closed circles denote shift bands induced by a CSL protein bound to the 4xCSL probe. *Arrows* denote high molecular weight shifted bands, which could be NICD interacting with CSL and other transcriptional cofactors. This high molecular weight band was quantified using a Bio-Rad PMI system and Quantity One software and the levels of the band in cells transfected with an NICD compared to that in cells transfected with the same NICD and ORF2. The *closed triangle* denotes minor shifted bands in Neuro-2A cells transfected with NICD4

position of high molecular weight shifted bands), which is consistent with NICD interacting with CSL and other transcriptional cofactors, including MAML. Transfection of Neuro-2A cells with NICD4 did not lead to additional high molecular weight shifted bands; however, there was a slight increase in a high molecular weight band (denoted by a closed triangle). When the ORF2 expression plasmid was co-transfected with an NICD1 or NICD3 expression plasmid, we consistently saw reduced levels of the high molecular weight band (approximately 40 % reduction; lane NICD + ORF2, shifted band denoted by closed arrow). There was only a slight reduction in this high molecular weight band when cells were co-transfected with ORF2 and NICD2 (approximately 20 % decrease) and there was no apparent differences in cells transfected with NICD4. When excess amounts of the cold 4xCSL oligonucleotide were added to the reaction, all shifted radioactive bands were not detected. Collectively, these studies provide evidence that ORF2 sequesters NICD1 and NICD3; consequently, these NICD members do not appear to form stable complexes with the CSL-DNA complex.

ORF2 overcomes NICD1–3-mediated inhibition of neurite formation in neuro-2A cells

We previously demonstrated that NICD1 and NICD3 expression interfere with neurite formation in Neuro-2A cells and that ORF2 induces neurite formation in Neuro-2A cells that express NICD1 or NICD3 (Sinani et al. 2013, 2014). This function correlates with ORF2 binding Notch1 or Notch3 and interfering with Notch-mediated transactivation of certain viral promoters (Workman et al. 2011). Conversely, ORF2 does not have a dramatic effect on neurite formation unless NICD1 or NICD3 is expressed. It is well established that Notch signaling interferes with neuronal differentiation in the ophthalmic branch of TG (Lassiter et al. 2010), inhibits neurite sprouting (Berezovska et al. 1999; Franklin et al. 1999; Levy et al. 2002a; c; Sestan et al. 1999), and axon repair (El Bejjani and Hammerlund 2012), and maintains neuronal progenitors in an undifferentiated state (Hitoshi et al. 2002) due to activation of HES1 and HES5 gene expression (Ohtsuka et al. 1999). Consequently, neuronal cell death can occur (Raff et al. 2002).

When Neuro-2A cells were seeded at low density and then serum starved for 3 days, neurite positive cells and cells lacking neurites were detected (Fig. 6a). Numerous cells also detach from the plate indicating they do not survive growth factor withdrawal. Co-transfection of Neuro-2A cells with an NICD member and a plasmid that expresses the Lac Z gene to "mark" transfected cells, β-Gal + cells that were rounded up and possessed long neurites (two times the size of the cell body) were not readily detected after serum withdrawal (Fig. 6b). When Neuro-2A cells were co-transfected with

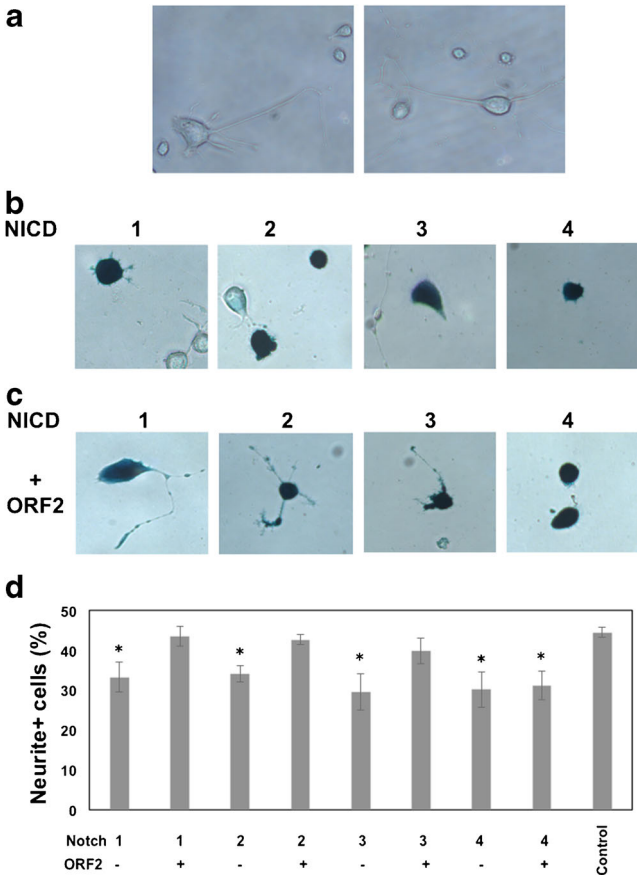

Fig. 6 ORF2 stimulates neurite formation in Neuro-2A cells expressing NICD1-3. **a** To induce neurite sprouting, Neuro-2A cells were seeded into new plates at a low density (2000 cells/cm^2) and then incubated in media containing 0.5 % serum for 3 days. **b** Neuro-2A cells were transfected with 1 ug plasmid expressing the designated NICD and 1 ug plasmid expressing the Lac Z gene (transfection control). Twenty-four hours after transfection, Neuro-2A cells were starved as described earlier. Cells were then fixed and β-gal + cells identified as described in the "Materials and Methods." **c** Neuro-2A cells were co-transfected with 1-ug plasmid expressing the designated NICD protein, 1-ug plasmid expressing ORF2, and 1-ug plasmid expressing the Lac Z gene (transfection control). Neurite formation was performed as described (**a**, **b**). **d** The percent of β-Gal + cells containing neurites was calculated by dividing the number of β-gal + cells with a neurite length at least twice the diameter of the cell by the total number of β-gal + cells. The average of three independent experiments is shown with the respective standard deviation. An *asterisk* denotes significant differences ($P < 0.05$) in β-Gal + Neuro-2A cells containing neurites following co-transfection with the ORF2 reporter and the designated NICD family member relative to β-Gal + Neuro-2A cells with neurites following transfection with a plasmid expressing just an NICD plus empty vector, as determined by the one-way ANOVA and Fisher's LSD multiple means comparison tests

NICD1, 2, or 3 and ORF2 and β-Gal + cells that had long neurites were readily detected (Fig. 6c). In contrast, ORF2 did not readily induce neurite formation when Neuro-2A cells were transfected with NICD4. Quantification of the data revealed ORF2 significantly stimulated neurite formation in Neuro-2A cells co-transfected with NICD1, 2, or 3, but not NICD4 (Fig. 6d).

Discussion

ORF2 is a multi-functional protein that can inhibit apoptosis (Shen and Jones 2008; Sinani et al. 2014; Sinani and Jones 2011). ORF2 does not resemble other proteins, which has made it difficult to predict functional domains. Although the LR gene and HSV-1 latency-associated transcript (LAT) both inhibit apoptosis (Ciacci-Zanella et al. 1999; Jones 2015; Perng et al. 2000; Perng and Jones 2010), there does not appear to be an ORF2-like protein encoded by LAT. Conversely, LAT encodes two small non-coding RNAs (sRNA1 and sRNA2) that can interfere with apoptosis in transiently transfected cells (Shen et al. 2009) and are expressed in TG of latently infected mice (Peng et al. 2008). Recent studies demonstrated that sRNA1 and sRNA2 promote expression of an HSV-1 entry mediator (HVEM), which also interferes with immune responses in latently infected TG and increases survival of latently infected neurons (Allen et al. 2014). Interestingly, LR gene expression, in particular ORF2, restores the ability of an HSV-1 LAT null mutant to reactivate from latency in small animal models of latency (Mott et al. 2003; Perng et al. 2002). Consequently, we suggest that the LR gene and LAT are functional homologues, even though the mechanism by which they promote the latency-reactivation cycle may not be exactly the same.

Although ORF2 directly interacts with Notch3 and to a lesser degree, Notch1 (Workman et al. 2011), it does not appear to directly interact with Notch4. Notch4 is the smallest and most divergent notch family member, and lacks a transcriptional activation domain (Borggrefe and Oswald 2009; Bray 2006; Ehebauer et al. 2006). Unlike Notch1, which is expressed in nearly all tissues, Notch4 is primarily expressed in vascular endothelial cells (James et al. 2014). Notch1 and 2 null mice are embryonic lethal whereas Notch3 (Krebs et al. 2003) and 4 are not required (Swiatek et al. 1994). However, Notch3-deficient mice exhibit uncontrolled muscle growth inducing hyperplasia or marked arterial structural defects (Kitamoto and Hanaoka 2010; Valerie et al. 2004). Notch4 null mice are viable and fertile but exhibit a delayed growth in retinal angiogenesis suggesting a role for Notch4 in embryonic development (James et al. 2014). Notch4 has also been reported to promote carcinogenesis and metastasis in certain tumors, breast cancer, and melanoma (for example, see Hardy et al. 2010; Nagamatsu et al. 2014).

Previously published studies (Sinani et al. 2013, 2014; Sinani and Jones 2011) and those in this report demonstrated that ORF2 interferes with NICD1–3-mediated transactivation of model promoters. Notch-mediated transcription requires an interaction between a CSL family member and MAML, reviewed in (Borggrefe and Oswald 2009; Bray 2006) and as depicted in Fig. 5a, i. Our studies provided evidence that ORF2 sequesters NICD1 and 3 and interferes with the formation of a stable complex between a CSL family member and

DNA. This conclusion is based on comparing EMSA results in cell lysate prepared from Neuro-2A cells co-transfected with plasmids that express ORF2 and NICD1–3 versus cell lysate prepared from Neuro-2A cells transfected with an empty vector and NICD1 and 3. With respect to NICD2, we also saw a slight reduction in the high molecular weight shifted band detected in Neuro-2A cells transfected with NICD2, which implies ORF2 associates with NICD2 but the association is not as stable as NICD1 or NICD3. The effect ORF2 had on NICD4 with respect to interactions with CSL bound to four copies of a CSL binding site was difficult to assess because NICD4, unlike NICD1–3, did not induce high molecular weight complexes when over-expressed in Neuro-2A cells. Although our studies suggest ORF2 sequesters NICD1 and 3, ORF2 may also interfere with interactions between Notch and other transcriptional co-activators required for Notch-mediated transcription.

Our studies imply that ORF2 promotes the establishment and maintenance of latency, in part due to interactions with Notch3 and Notch1 (Fig. 7a). Neurons containing damaged neurites or axons can die (Coleman and Freeman 2010; Raff et al. 2002): conversely, neurite sprouting repairs damaged axons (El Bejjani and Hammerlund 2012). Activated Notch signaling in neurons inhibits neurite sprouting (Berezovska et al. 1999; Levy and Darnell 2002; Levy et al. 2002b; Sestan et al. 1999) and the repair of axons (El Bejjani and Hammerlund 2012), in part by stimulating HES1 and HES5 expression (Ohtsuka et al. 1999). During BoHV-1 latency, cattle are frequently exposed to "low levels" of stress, which does not result in successful reactivation of latency. However,

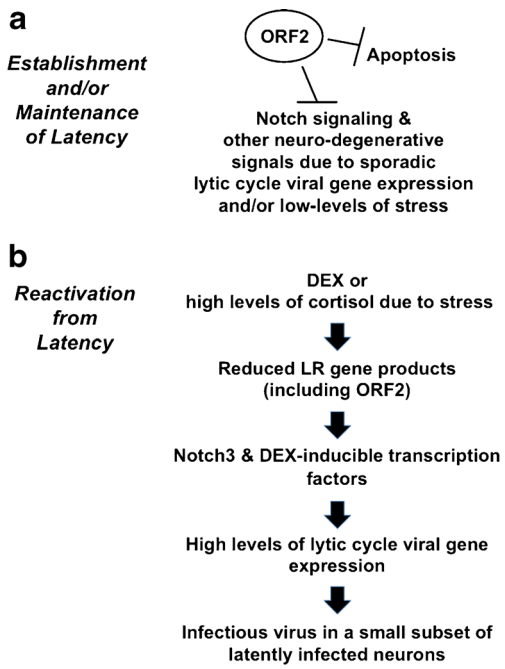

Fig. 7 Schematic of putative roles ORF2 and stress play during the BoHV-1 latency-reactivation cycle. For details, see text

these episodes of low levels of stress may induce Notch3 and/or lytic cycle viral gene expression in a subset of latently infected neurons. These studies suggest that the ability of ORF2 to stimulate neurite sprouting (Sinani et al. 2013, 2014) maintains neuronal health of latently infected neurons, in part by interfering with Notch-mediated transactivation of HES1 and HES5 promoters. In particular, the ability of ORF2 to sequester Notch3 may maintain normal differentiated properties in latently infected sensory neurons during the establishment and maintenance of latency or following low levels of stress. Considering the importance of preventing neurodegeneration during life-long latent infections, it would not be surprising to find that ORF2 or other LR gene products influence additional signaling pathways that promote neuronal survival.

During DEX-induced reactivation from latency, several events occur that are proposed to induce lytic cycle viral gene expression and shedding of infectious virus in certain latently infected neurons (summarized in Fig. 7b). For example, Notch3 RNA levels (Workman et al. 2011), protein levels (Sinani et al. 2013), and the Notch signaling pathway (Workman et al. 2012) are induced during DEX-induced reactivation from latency. Conversely, ORF2 protein expression (Sinani et al. 2013) and LR-encoded micro-RNA levels (Jaber et al. 2010) decrease during DEX-induced reactivation. Consequently, we hypothesize that activation of the Notch pathway stimulates viral replication and perhaps reactivation from latency when ORF2 is not abundantly expressed in a latently infected neuron. Support for this prediction comes from the finding that Notch1, but not Notch3, enhances BoHV-1 productive infection and ORF2 interferes with this function (Workman et al. 2011). Notch1 also stimulates the BoHV-1 immediate-early transcription unit 1 (IEtu1) and bICP0 early promoters (Workman et al. 2011). Finally, Notch1 and Notch3 transactivate the glycoprotein C (gC) promoter (Workman et al. 2011). In addition to activation of Notch-dependent transcription during reactivation, DEX stimulates expression of additional cellular transcription factors that transactivate certain BoHV-1 promoters and productive infection (Workman et al. 2012). In summary, the ability of ORF2 to interfere with Notch-dependent transcription may influence the switch from maintenance of latency to reactivation from latency.

Dysregulation of the Notch signaling pathway or gain of function mutations in Notch family members is required for development of T cell acute lymphoblastic leukemia and a growing number of solid tumors (reviewed in Brakenhoff 2011; Koch and Radtke 2011; Sethi and Kang 2011). Although there is a small molecule that inhibits gamma-secretase mediated cleavage of Notch after ligand binding (Shih and Wang 2007), this class of inhibitors can be toxic and have serious side effects; furthermore, they do not interfere with nuclear functions of NICD. Consequently, there is a

need for novel therapeutic strategies designed to inhibit the nuclear functions of Notch, which would complement and perhaps replace gamma-secretase inhibitors. Based on our studies, we suggest ORF2 is a potential candidate for interfering with NICD1–3 mediated growth of tumor cells. Future studies designed to identify small domains of ORF2 that can sequester NICD1–3 are being pursued.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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