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Zhangfei, a novel regulator of the human nerve growth factor receptor, trkA

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The replication of herpes simplex virus (HSV) in epithelial cells, and during reactivation from latency in sensory neurons, depends on a ubiquitous cellular protein called host cell factor (HCF). The HSV transactivator, VP16, which initiates the viral replicative cycle, binds HCF as do some other cellular proteins. Of these, the neuronal transcription factor Zhangfei suppresses the ability of VP16 to initiate the replicative cycle. It also suppresses Luman, another cellular transcription factor that binds HCF. Interactions of nerve growth factor (NGF) and its receptor tropomyosin-related kinase (trkA) appear to be critical for maintaining HSV latency. Because the neuronal transcription factor Brn3a, which regulates trkA expression, has a motif for binding HCF, we investigated if Zhangfei had an effect on its activity. We found that Brn3a required HCF for activating the trkA promoter and Zhangfei suppressed its activity in non-neuronal cells. However, in neuron-like NGF-differentiated PC12 cells, both Brn3a and Zhangfei activated the trkA promoter and induced the expression of endogenous trkA. In addition, capsaicin, a stressor, which activates HSV in *in vitro* models of latency, decreased levels of Zhangfei and trkA transcripts in NGF-differentiated PC12 cells. *Journal of NeuroVirology* (2008) **14,** 425–436.

Keywords: Brn3a; host cell factor; herpes simplex virus; HCF; nerve growth factor; PC12 cells; trkA; Zhangfei

Introduction

Herpes simplex virus type 1 (HSV-1) causes recurrent lesions on epithelial surfaces. The virus uses two complimentary strategies to avoid immune surveillance while maintaining itself in its primary host and continuing the chain of infection to other hosts. Initial infection of epithelial cells by HSV-1 leads to active replication of the virus and lysis of the host cells. The virus also infects sensory neurons innervating the site of viral replication. The viral genome is delivered by retrograde axonal transport to the neuronal cell body in sensory ganglia where it establishes a latent infection. Periodic reactivation from latency in response to a variety of stressors leads to replication of the latent virus in the neuron and transport of virus to epithelial surfaces where viral replication causes recurrent lesions and potential dissemination to other hosts (reviewed in Roizman, 2001).

The expression of viral genes during lytic infection in epithelial cells is temporally regulated so that the approximately 80 viral genes involved can be categorized as immediate-early (IE or α), early (E or β), or late (L or γ), depending on when in the replicative cycle they are expressed. Because the efficient expression of E and L genes depends on IE gene products, these regulatory proteins are crucial for the progression of the replicative cycle (reviewed in Roizman, 2001).

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Initiation of the transcription of the IE genes is induced by the assembly of a multiprotein complex made up of the HSV-1 virion protein 16 (VP16) and two cellular proteins: Oct-1 and host cell factor (HCF) (reviewed in Wysocka and Herr, 2003). VP16 recognizes HCF through its HCF-binding motif (HBM), a four–amino acid domain, EHAY, which, as the consensus sequence D/EHxY, is conserved in several cellular and viral HCF-binding proteins (Luciano and Wilson, 2003). The VP16-HCF heterodimer then recognizes the POU domain of Oct1 bound to TAATGARAT (R is a purine) motifs present in multiple copies in the promoters of all HSV IE genes. The activation domain of VP16 interacts with components of the transcription machinery, leading to the expression of IE genes. Immediate-early proteins subsequently regulate the expression of the E and L genes (Jones, 2003). Recent reports (Narayanan *et al*, 2007; Tyagi *et al*, 2007) suggest that HCF acts as a transcriptional coactivator or corepressor of viral and cellular genes by recruiting histone-modifying enzymes to promoters.

Following infection of sensory neurons innervating the epithelial site of viral replication, the HSV-1 virion is transported along axonal microtubules to the neuronal cell body. This process requires proteins present in the viral tegument (Bearer and Satpute-Krishnan, 2002; Luxton *et al*, 2005), a structure that surrounds the viral nucleocapsid. The tegument comprises, among other viral proteins, VP16. Although the molecular mechanisms that suppress the expression of viral replicative cycle genes as a prelude to the establishment of a latent infection in the neuron have not been defined, they most likely involve the blocking of IE gene expression (reviewed in Efstathiou and Preston, 2005; Preston, 2000).

During latency no viral proteins can be detected consistently in the neuron and the viral genome is silent with the exception of the latency-associated transcripts, which have an antiapoptotic role (Gupta *et al*, 2006; Jin *et al*, 2003; Thompson and Sawtell, 2001). A variety of stimuli have been shown to result in the reactivation of the latent viral genome. Among these is the disruption of nerve growth factor (NGF)-induced signaling in the neuron (Block *et al*, 1994; Hill *et al*, 1997; Jordan *et al*, 1998; Laycock *et al*, 1994; Wilcox and Johnson, 1988; Wilcox *et al*, 1990). NGF is a neurotropin that plays an important role in the differentiation and survival of sensory neurons (Bibel and Barde, 2000; Huang and Reichardt, 2003). Its high-affinity receptor in neurons is the tropomyosin-related kinase (trkA) (Huang and Reichardt, 2003; Parada *et al*, 1992).

Because the latently infected neuron contains no detectable VP16 during viral reactivation, the induction of HSV IE gene expression, and subsequently the viral replicative cycle, must be mediated by cellular proteins. In addition, HCF appears to be critical for the induction of HSV-1 IE gene expression and, consequently, the induction of the replicative cycle in epithelial cells (Khurana and Kristie, 2004; Narayanan *et al*, 2005). Also, the nuclear localization of HCF in neurons correlates with reactivation of HSV-1 from latency (Kristie *et al*, 1999). It is therefore conceivable that viral IE gene–inducing cellular proteins require and bind HCF. To examine this possibility, our laboratory and others have identified two neuronal proteins, Luman (Freiman and Herr, 1997; Lu *et al*, 1997) and Zhangfei (Lu and Misra, 2000b), that bind HCF and require it for their activity. Luman is an endoplasmic reticulum (ER)-anchored basic leucine zipper (b-Zip) protein. When released from the ER by regulated proteolysis, Luman is a potent transcription activator of promoters that contain cyclic AMP and unfolded protein response elements (Liang *et al*, 2006; Lu *et al*, 1997; Raggo *et al*, 2002). These elements include those in the promoters of the HSV-1 IE gene ICP0 and the latency-associated transcripts (Lu *et al*, 1997). Zhangfei is also a b-Zip protein. In contrast to Luman, it acts as a suppressor and reduces gene activation by both Luman (Misra *et al*, 2005) and VP16 (Akhova *et al*, 2005). Its effect on VP16 leads to a profound decrease in viral replication in HSV-1–infected cells that express Zhangfei (Akhova *et al*, 2005).

Among the several cellular proteins that possess an HBM is another neuronal transcription factor called Brn3a (although its ability to bind HCF has not been established). This factor has been shown to be important in regulating the expression of trkA during differentiation of sensory neurons in the developing mouse embryo (Ma *et al*, 2000, 2003). We recently identified *cis*-acting domains in the proximal trkA promoter that bind Brn3a and showed that exogenous Brn3a induces the expression of trkA in NGF-differentiated PC12 cells and in medulloblastoma cells that do not normally express trkA (Valderrama and Misra, 2008). In this article we examine Brn3a-Zhangfei interactions to determine if Zhangfei can suppress the ability of Brn3a to activate the trkA promoter. We discovered that Brn3a required HCF for activity and that Zhangfei suppressed the activity of Brn3a in non-neuronal cells. However, in neuron-like NGF-differentiated PC12 cells, Zhangfei not only did not suppress Brn3a, it was also capable of activating the expression of trkA in the absence of Brn3a.

Results

Brn3a requires nuclear HCF for efficient activation of the trkA promoter

Luciano and Wilson (2003) identified almost 50 viral and cellular proteins that posses the D/EHxY HBM. The proteins with HBM include Brn3a, which has the sequence EHKY (a comprehensive list of

proteins with HBMs is in Luciano and Wilson, 2003). To determine if Brn3a requires HCF for activity, we exploited the natural ability of ER-anchored full-length Luman to sequester HCF in the cytoplasm (Misra *et al*, 2005) as a means of depleting cells of nuclear HCF. Briefly, we used a mutant of full-length Luman (Lu N160G), which does not bind Luman response elements in promoters and is therefore transcriptionally inactive. This mutant, however, retains HCF in the ER and has the ability to severely inhibit the activity of transcription factors that require HCF while having no effect on factors that are HCF independent (Misra *et al*, 2005). We compared the activity of Brn3a in the presence of another mutant, Luman DHTY78AGTA. This mutant has an altered HBM and does not sequester HCF (Lu *et al*, 1998; Misra *et al*, 2005). In cells expressing this mutant, most of the HCF is nuclear. We cotransfected cells with a plasmid expressing Brn3a and ptrkA1043, a CAT reporter plasmid with 1043 bp of sequence that lies upstream from the start of transcription of the human trkA gene, as well as plasmids specifying Luman DHTY78AGTA or Luman N160G. We found that whereas Luman N160G reduced levels of activation by more than 50%, Luman DHTY78AGTA had no effect (Figure 1A, B). This suggested that like other HCF-binding proteins, Brn3a requires HCF for efficient activation of the trkA promoter.

We then determined if Brn3a was able to bind directly to HCF. We constructed and purified glutathione *S*-transferase (GST)-linked Brn3a and synthesized biotinylated Brn3a and examined the ability of these proteins to bind radiolabeled HCF. As a positive control we used Luman, which binds HCF, and as a negative control we included a mutant of Luman (DHTY78AGTA), which does not bind HCF. HCF bound to the proteins was precipitated with glutathione beads (for GST fusion proteins) or avidin-coated magnetic beads (biotinylated proteins). In both binding assays Brn3a did not appear to bind HCF (Figure 1C). This suggests that although Brn3a requires HCF for activating the trkA promoter, the *in vitro* synthesized proteins do not form dimers that are stable enough to be recovered in ''pull-down'' assays.

Zhangfei suppresses the ability of Brn3a to activate the trkA promoter in non-neuronal cells

Because Zhangfei can suppress the activity of at least two other HCF-binding transcription factors, Luman and VP16 (Akhova *et al*, 2005; Misra *et al*, 2005), we examined the ability of Brn3a to activate the 190-bp trkA minimal promoter (ptrkA+190) in the presence of increasing amounts of a plasmid specifying Zhangfei. To examine the interactions of Brn3a and Zhangfei without the confounding effects of other neuronal factors, we performed these experiments in non-neuronal Vero cells that do not express Brn3a or other neuronal factors. Figure 2A shows that Zhangfei suppressed the activity of Brn3a in a dose-dependent manner.

To determine if Zhangfei was directly responsible for the suppression, we examined the ability of Zhangfei to suppress Brn3a in the presence or absence of a plasmid specifying siRNA against Zhangfei. This plasmid completely abrogates the ability of Zhangfei to suppress Luman, whereas a plasmid expressing nonspecific siRNA has no effect (Misra *et al*, 2005). Zhangfei-specific siRNA also reduced ZF protein levels in Vero cells transfected to express the protein by 35% (Figure 2B). The siRNA inhibited the ability of Zhangfei to suppress the activity of Brn3a (Figure 2C). In the presence of siRNA Zhangfei, not only was the suppressive effect of Zhangfei on Brn3a eliminated, but also a combination of Brn3a and Zhangfei activated the promoter to a greater extent than Brn3a alone. These results are consistent with our observations on the effects of Zhangfei on the other HCF-binding proteins, VP16 and Luman (Akhova *et al*, 2005; Misra *et al*, 2005).

To determine if HCF binding by Zhangfei was required for Brn3a suppression, we compared the ability of Zhangfei or its HBM domain mutant (ZF Y224A), which does not bind HCF (Lu and Misra, 2000b), to suppress Brn3a activation. In contrast to Zhangfei, its HBM mutant did not suppress Brn3a activation of either ptrkA1043 (Figure 2D) or the shorter promoter construct (ptrkA+190; data not shown).

The effect of Zhangfei is cell type and promoter specific

We next examined the effect of Zhangfei on Brn3a in rat pheochromocytoma cells (PC12) treated with nerve growth factor (NGF). When PC12 cells are treated with NGF, they differentiate into neuron-like cells, which have been used extensively as surrogates for neurons in various studies on neuronal function and HSV-1 latency (Colgin *et al*, 2001; Smith *et al*, 1992; Wilcox and Johnson, 1987, 1988; Wilcox *et al*, 1990). NGF-treated cells were transfected with ptrkA+190 and plasmids specifying either Brn3a or Zhangfei, or a combination of the plasmids. Figure 3A shows that in contrast to Vero cells (Figure 3B; also see Figure 2A), in NGF-differentiated PC12 cells Zhangfei did not suppress the ability of Brn3a to activate the minimal trkA promoter. In NGF-differentiated PC12 cells, again in contrast to its effects in Vero cells, Zhangfei activated the trkA promoter even in the absence of Brn3a.

Our results suggested that the effect of Zhangfei on the trkA promoter was cell type specific. To determine if the effect of Zhangfei was promoter and target transcription factor specific as well, we examined the effect of Zhangfei on the ability of Luman to activate a promoter with unfolded protein response elements (pCAT3BATF6). For this experiment we used Luman S221Op, a truncated mutant of Luman that is not anchored in the ER and is constitutively

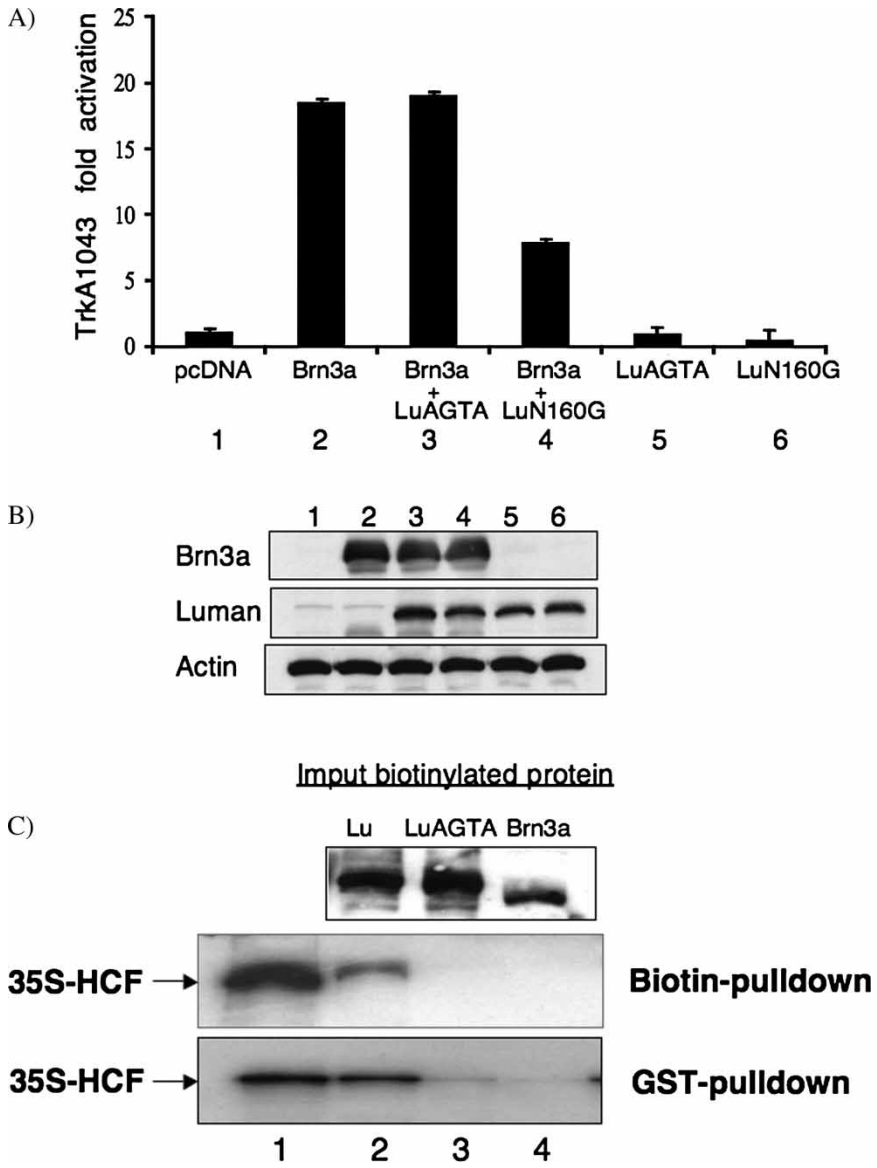

Figure 1 Brn3a requires HCF for efficient activation of the trkA promoter. (**A**) Brn3a requirement for HCF was assessed by coexpressing Brn3a in Vero cells with an ER-associated mutant of Luman (Lu N160G) that possesses no transcriptional activity but retains HCF in the cytoplasm. As a control, Brn3a was either expressed alone or with an ER-associated mutant of Luman with an altered HBM (Lu DHTY:AGTA), which does not retain HCF in the cytoplasm. Neither Lu N160G nor Lu DHTY:AGTA activates the trkA promoter. All transfections contained the reporter plasmid ptrkA1043. (**B**) Immunoblot of cell lysates in **A** screened for Brn3a, Luman, or β-actin. (**C**) *In vitro* binding of radioactive [^{35}S]HCF to Brn3a as assessed by GST or biotin-avidin precipitation. Lane 1, input HCF alone; lane 2, HCF + GST or biotinylated Luman; lane 3, HCF + GST or biotinylated Luman (DHTY78AGTA, mutated HBM); lane 4, HCF + GST or biotinylated Brn3a.

active (Raggo *et al*, 2002). As reported earlier, in Vero cells Luman S221Op activated the unfolded protein response element–containing promoter several hundred fold and Zhangfei completed abrogated this activity (Figure 3D). Although the activity of Luman S221Op was not as profound in NGF-differentiated PC12 cells (Figure 3C) as in Vero cells (Figure 3D), Zhangfei had a suppressive effect (Figure 3C).

The ability of Zhangfei to activate the trkA promoter (Figure 3A) in NGF-differentiated PC12 was surprising because it does not do so in Vero cells. To determine if HCF is required for Zhangfei to activate trkA, we examined Zhangfei Y224A (Figure 3E) and discovered that the mutant was as effective in activating the minimal trkA promoter as the wild-type protein. This suggests that although Zhangfei can activate the trkA promoter in NGF-differentiated PC12 cells, it does not require HCF to do so.

Zhangfei activates endogenous expression of trkA in PC12 cells

We have previously shown (Valderrama and Misra, 2008) that Brn3a activates the trkA promoter *in vitro* and the expression of exogenous Brn3a in

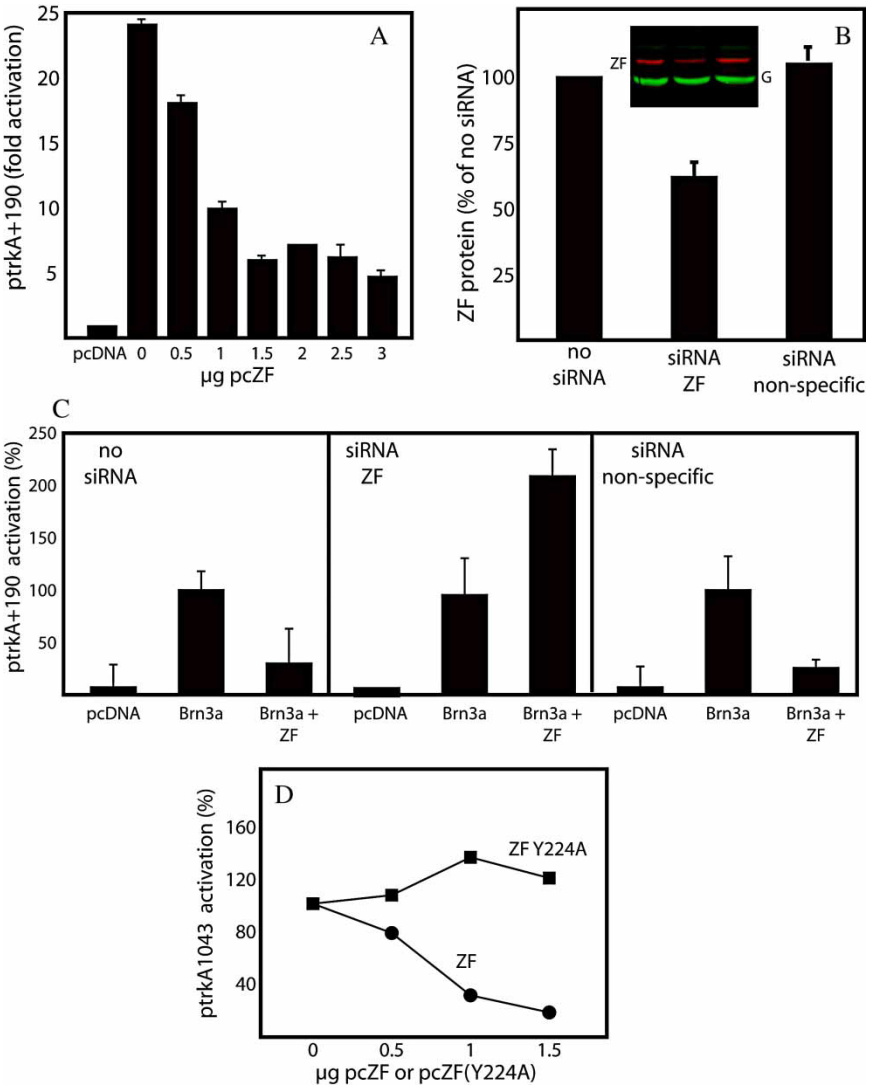

Figure 2 Zhangfei suppresses the ability of Brn3a to activate the trkA promoter in non-neuronal cells. (**A**) Vero cells were transfected with the trkA promoter reporter ptrkA+190, a plasmid that specifies Brn3a and varying amounts (0 to 3 µg) of a plasmid that expresses Zhangfei. The first bar (pcDNA) represents CAT activity of the reporter without Brn3a and is regarded as 1. Total DNA in each reaction was adjusted to 6 µg with the empty vector pcDNA3. (**B**) siRNA against Zhangfei reduces expression of the protein in Vero cells transfected to express Zhangfei. Vero cells were transfected with a plasmid expressing Zhangfei and no additional plasmid, plasmid expressing siRNA Zhangfei, or a nonspecific siRNA (siRNA Ubc13). Cell lysates were examined by immunoblotting for Zhangfei (ZF; red in the inset) or GAPDH (G; green). The intensity of the bands was measured and calculated as a ratio of Zhangfei to GAPDH. The ratio for cells expressing no siRNA is expressed as a 100%. The graph combines the results of three independent experiments. (**C**) siRNA against Zhangfei inhibits its suppressive effect on Brn3a. Vero cells were transfected with the trkA reporter and pcDNA, a plasmid specifying Brn3a, and a Zhangfei expressing plasmid. One batch of cells also received a plasmid expressing siRNA ZF or another plasmid expressing a nonspecific siRNA. (**D**) Zhangfei must bind HCF to suppress Brn3a activity. Vero cells were transfected with a trkA promoter reporter (pTrkA1043), a plasmid expressing Brn3a and varying amounts of a plasmid expressing either Zhangfei or a mutant with a HBM that does not bind HCF (ZFY224A). CAT activity was measured 48 h after transfection.

NGF-treated PC12 cells and in medulloblastoma cells leads to an increase in endogenous trkA transcripts. To determine if Zhangfei, like Brn3a, could enhance endogenous trkA expression in NGF-differentiated PC12 cells, we transfected these cells with plasmids expressing Brn3a and Zhangfei, either alone or in combination. When compared to trkA transcript levels in untransfected, NGF-differentiated PC12 cells, both Brn3a and Zhangfei enhanced trkA RNA levels between 20- and 30-fold (Figure 4). Although Brn3a and Zhangfei did not appear to act synergistically, cells expressing both proteins had significantly higher levels of transcripts than either of the two proteins on their own.

Stress decreases Zhangfei and trkA transcripts in NGF-differentiated PC12 cells

Zhangfei, which is present in sensory neurons, suppresses the ability of VP16 to activate HSV IE genes, leading us to hypothesize that it might play a

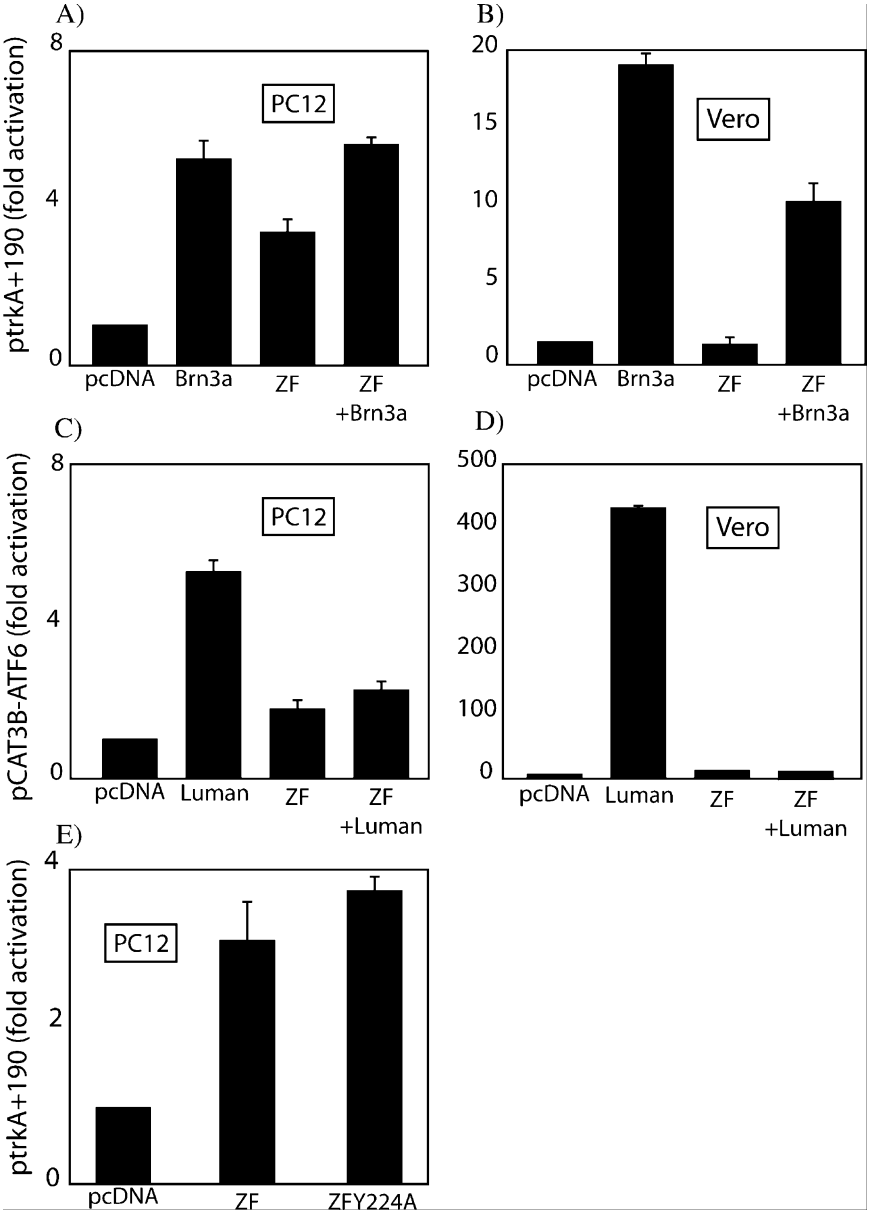

Figure 3 The effect of Zhangfei is dependent on the promoter and cell type. (**A**) PC12 cells were transfected with the trkA reporter, pTrkA + 190, and pcDNA3, or plasmids expressing Brn3a alone, Zhangfei alone, or both Brn3a and Zhangfei. After overnight incubation, cells were treated with NGF and assayed for CAT 48 h later. (**B**) Vero cells were transfected with the trkA reporter and pcDNA, a plasmid specifying Brn3a, and a Zhangfei expressing plasmid. (**C**) PC12 cells were transfected with a reporter with Luman-responsive UPRE binding sites (pCATB3-ATF6 and pcDNA3), or plasmids expressing Luman alone, Zhangfei alone, or both Luman and Zhangfei. After overnight incubation, cells were treated with NGF and assayed for CAT 48 h later. (**D**) Vero cells were transfected with pCATB3-ATF6 and pcDNA, a plasmid specifying Luman, and a Zhangfei-expressing plasmid. (**E**) Cells were transfected with ptrkA + 190 and pcDNA3, or plasmids expressing Zhangfei and/or mutant ZhangfeiY224A (mutated HBM).

role in initiating HSV latency (Akhova *et al*, 2005). Because the NGF-trkA pathway appears to be critical for maintaining HSV latency, we examined the possibility that stressors that lead to reactivation from latency suppress the expression of Zhangfei and, consequently, trkA. Because capsaicin, a stressor, leads to HSV-1 reactivation (Hunsperger and Wilcox, 2003) and PC12 cells express VR1, the capsaicin receptor (Ganju *et al*, 1998; Qiao *et al*, 2004; Someya *et al*, 2004), we quantitated Zhangfei and trkA transcripts by quantitative polymerase chain reaction (QPCR) in capsaicin-treated, NGF-differentiated PC12 cells. Figure 5A shows that capsaicin decreased steady-state levels of both Zhangfei (*left panel*) and trkA (*right panel*) transcripts. The decrease in the levels of RNA, though modest, was consistent. To rule out the possibility that the effects of capsaicin were due to toxicity, we

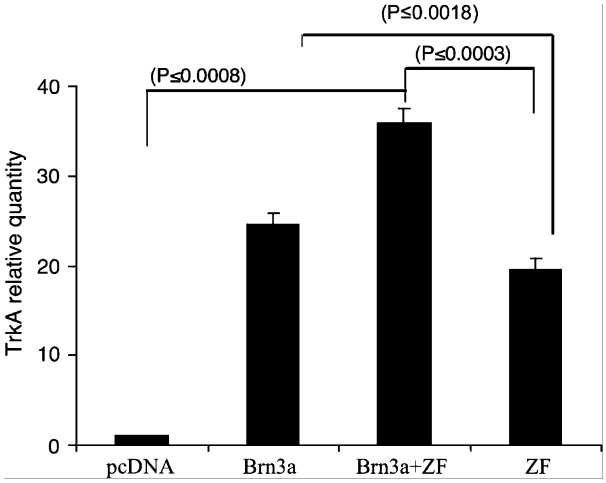

Figure 4 Brn3a and Zhangfei activate endogenous trkA in NGF-differentiated PC12 cells. Cells were transfected with plasmids expressing pcDNA, Brn3a, Brn3a + Zhangfei, or Zhangfei. Forty-eight hours after NGF treatment, RNA from the cells was assayed for trkA transcripts using quantitative real-time PCR. Values are normalized to GAPDH transcripts in each sample and the value for the sample with no Brn3a or Zhangfei was designated as 1. Bars are standard errors of means.

compared the viability of capsaicin-treated and control cells after 24 h of treatment. We found no differences (450 nm absorbance of 1.55 ± 0.417 for control versus 1.58 ± 0.07 for capsaicin-treated cells), showing that for the duration of the experiment capsaicin was not toxic to NGF-differentiated PC12 cells.

Discussion

Evidence from cultured neuronal cells as well as experimental animal models for HSV-1 latency and reactivation suggest an important role for NGF-trkA signaling in the establishment and maintenance of viral latency (Hill *et al*, 1997; Kriesel, 1999; Laycock *et al*, 1994; Wilcox and Johnson, 1988). Recent data showing that neuronal cell-death caused by pseudorabies virus, which is closely related to HSV-1, is modulated by Brn3a, a transcription factor known to activate trkA expression, support these observations (Geenen *et al*, 2007). In addition, HCF is critical for the initiation of the replicative cycle of HSV-1 and other alpha-herpesviruses, such as varicella-zoster virus and pseudorabies virus (Khurana and Kristie, 2004; Kristie *et al*, 1999; Narayanan *et al*, 2005). The recent observation that Brn3a possesses a HBM and could potentially require HCF for activity, prompted us to investigate a possible link between Brn3a, trkA, NGF signaling, HCF, and HSV-1 latency and reactivation. We had previously observed that Zhangfei has a suppressive effect on Luman (Misra *et al*, 2005) and VP16 (Akhova *et al*, 2005), two other HCF-binding proteins that influence HSV-1 gene expression. Based on these observations, we hypothesized that Zhangfei would also suppress the ability of Brn3a to activate the expression of trkA, that this would have an impact on NGF-trkA signaling and, consequently, on HSV-1 reactivation from latency. Our results showed that Zhangfei did not suppress the ability of Brn3a to activate the trkA promoter and, contrary to our expectations, induced the expression of trkA in the absence of Brn3a.

We studied whether Brn3a needed HCF for its transcription activation function and, if so, if direct interaction between the proteins was required. We found (Figure 1A) that Brn3a required HCF for transcription activation of the trkA promoter. However, we could not demonstrate HCF binding to Brn3a *in vitro* (Figure 1C). These results suggested that either Brn3a requires conformational changes to bind to HCF or requires the presence of other proteins found in the cellular environment. Brn3a's dependency on HCF for the transactivation of trkA in non-neuronal cells suggested that it was possible that Zhangfei could regulate Brn3a's function. As with Luman and VP16, Zhangfei suppressed Brn3a transactivation of trkA in an HCF dependent manner in Vero cells. In contrast, in NGF-differentiated PC12 cells, a model of sympathetic neurons, Zhangfei had no repressive effects on Brn3a *trans*-activation function. Instead, Zhangfei activated the trkA minimal promoter (ptrkA + 190) and increased trkA endogenous transcription without the requirement to bind to HCF.

Previous studies (Akhova *et al*, 2005; Misra *et al*, 2005) and the results described here suggest that Zhangfei, which does not appear to bind known response elements for b-Zip transcription factors on its own, can either suppress or activate other factors. Zhangfei is one of four viral and human b-Zip factors that lack an asparagine residue in their basic domain. In other b-Zip factors this residue has been shown to be critical for binding to DNA response elements. The other factors that lack the asparagine residue are CCAAT/enhancer-binding protein homologous protein (CHOP) (Ubeda *et al*, 1999; Ubeda *et al*, 1996), the human T-cell leukemia virus b-Zip factor (HBZ) (Gaudray *et al*, 2002), and the Kaposi sarcoma herpesvirus b-Zip protein (K-Bzip) (Izumiya *et al*, 2003a,b; Liao *et al*, 2003). CHOP has gene-activating as well as -suppressing roles depending on its dimerization partner and the nature of the cell expressing the proteins. As a dimer of the b-Zip factors C/EBPα and C/EBPβ, CHOP blocks conversion of 3T3L1 cells to adipocytes (Batchvarova *et al*, 1995) and by suppressing osteocalcin gene transcription in osteoblasts prevents osteoblast differentiation (Shirakawa *et al*, 2006). In contrast, as a partner of C/EBPβ in some cells, CHOP activates the expression of carbonic anhydrase (Sok *et al*, 1999). CHOP also acts as a positive regulator of gene expression in association with non-C/EBP proteins such as ATF3 (Chen *et al*,

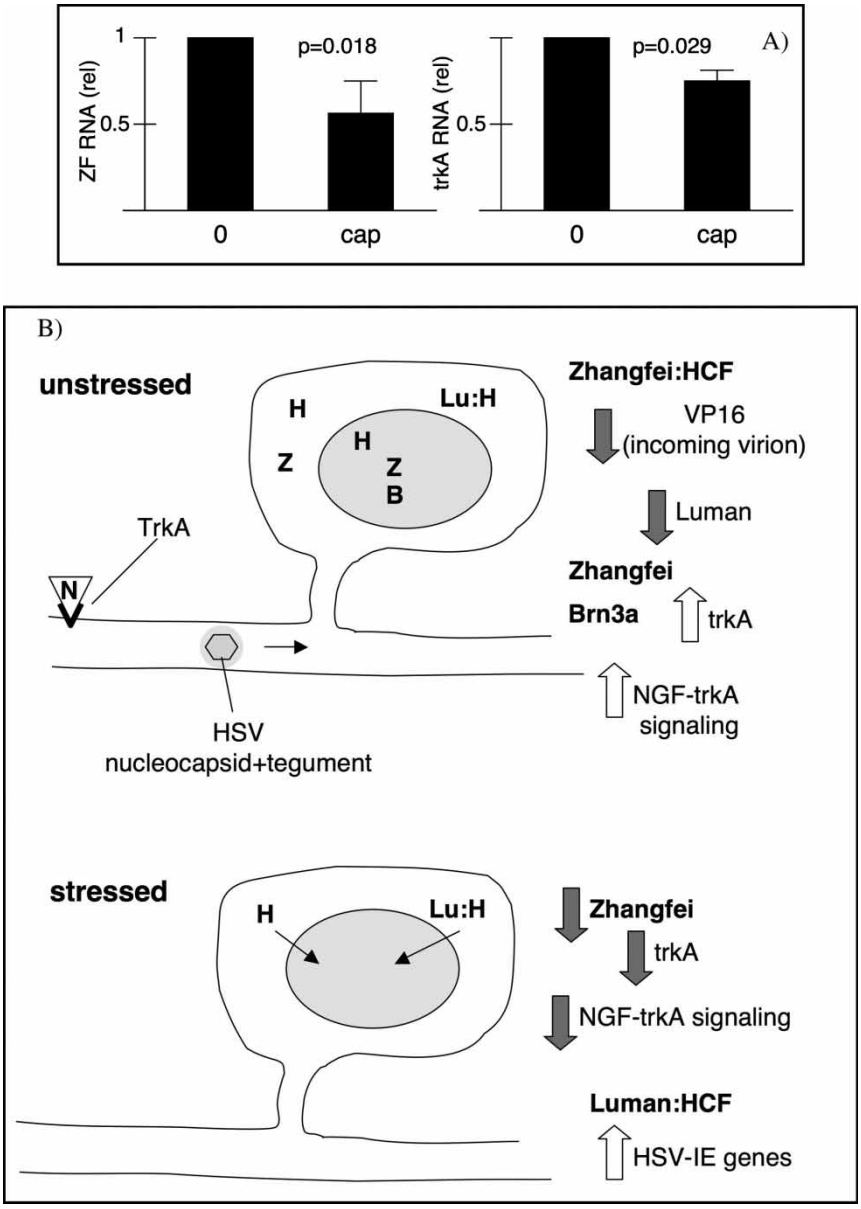

Figure 5 (**A**) Capsaicin-induced stress reduces ZF expression in NGF-differentiated PC12 cells. NGF-differentiated PC12 cells were incubated overnight in medium containing either 0.1 μM capsaicin or an equivalent amount of solvent (no treatment). Cells were harvested and Zhangfei, trkA, and GAPDH transcripts quantitated by QPCR. Amounts of RNA [RNA (rel)] are relative to samples with no capsacin. The values are a mean of $2\Delta C_T$ from five experiments. A student *t* test was used to calculate the *P* value (.018 and .029) (**B**) Model for the role of Zhangfei in unstressed and stressed neurons. N = NGF; trkA = NGF receptor tyrosine kinase; H = HCF; Lu = Luman; B = Brn3a; L = Luman. In unstressed neurons Luman and associated HCF is anchored in the ER. Zhangfei, in an HCF-independent manner, along with Brn3a activates the expression of trkA. trkA enables NGF-mediated signaling, which suppresses HSV-1 reactivation in latently infected neurons. Zhangfei also suppresses HSV-1 lytic cycle by suppressing VP16 associated with incoming viral nucleocapsids with associated VP16 (tegument). Zhangfei also suppresses any Luman that might be released from the ER, thereby further blocking the HSV-1 lytic cycle. In response to stress, the expression of Zhangfei is suppressed, thereby decreasing trkA expression and, consequently, suppressing NGF signaling and triggering HSV-1 reactivation. A reduction in Zhangfei also allows proteolytically activated Luman to induce the expression of IE genes and hence the HSV-1 lytic cycle.

1996), JunD, c-Jun, and c-Fos (Ubeda and Habener, 2003; Ubeda *et al*, 1999).

Some of the functions of Zhangfei, such as the suppressive effects on Luman and VP16 and the effect on Brn3a in non-neuronal cells, are dependent on binding HCF. A mutant of Zhangfei that does not bind HCF is inefficient in this role. In contrast, the effect of Zhangfei on the activation of trkA in neuronal cells was independent of HCF.

Based on these observations we propose a hypothetical role for Zhangfei in the establishment of HSV-1 latency as well as its reactivation from this

state (Figure 5B): In unstressed neurons the normal function of Zhangfei is to activate trkA expression. Drawing parallels with CHOP, Zhangfei probably accomplishes this as a partner of another b-Zip protein such as ATF4 (Hogan *et al*, 2006). trkA expression in these cells promotes NGF-trkA signaling. In unstressed neurons HCF is held in the cytoplasm (Kristie *et al*, 1999), possibly in association with ER-anchored Luman (Lu and Misra, 2000a). Because some Luman is constitutively released from the ER and untimely gene activation by Luman might be detrimental to the cell (Raggo *et al*, 2002), the role of Zhangfei is to suppress Luman's activity in an HCF-dependent manner. Zhangfei also, again in an HCF-dependent manner, suppresses the VP16-induced expression of viral IE genes in herpes simplex virions delivered to the neuronal cell body, thereby promoting the establishment of latency. The suppression of Luman, which can also activate IE genes, aids in this.

In response to stress, we hypothesize that two simultaneous events occur. The expression of Zhangfei ceases, leading to decrease in trkA and, consequently, NGF-trkA signaling. This primes the neuron for HSV-1 reactivation (Block *et al*, 1994; Hill *et al*, 1997; Jordan *et al*, 1998; Laycock *et al*, 1994; Wilcox and Johnson, 1988; Wilcox *et al*, 1990). Stress also increases the release of Luman and HCF from the ER and their translocation to the nucleus where they further facilitate reactivation by activating the expression of IE genes.

Although at this stage we have very little direct evidence to support this model, our preliminary results indicating that capsaicin-induced stress in NGF-differentiated PC12 cells reduces the levels of Zhangfei and trkA transcript (Figure 5A) lend some credence to it. Experiments to test the model are in progress.

Materials and methods

Cell culture

Vero cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), with 10% newborn calf serum and 1% penicillin-streptomycin. Rat pheochromocytoma (PC12) cells (provided by D. D. Mousseau, University of Saskatchewan, Saskatoon, SK, Canada) were maintained in complete medium (CM) containing RPMI 1640, 10% horse serum (heat treated to inactivate complement), 5% fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin. To differentiate PC12 cells, they were ''fasted'' in low-serum medium, RPMI 1640 supplemented with 1% of fetal bovine serum and 1% penicillin-streptomycin, overnight before treatment with 100 ng/ml of nerve growth factor (NGF; Cederlane Laboratories, Hornby, ON, Canada). Every second day, half the culture medium was replaced with fresh NGF-containing medium. After 6 days of treatment, cells showed extensive neurite outgrowth and were considered to be differentiated. NGF was reconstituted in 0.02% acetic acid in fetal bovine serum before dilution. A 1 mM stock solution of capsaicin (Alexis Biochemicals, Lausen, Switzerland) was prepared in methanol or ethanol. Capsaicin was added to differentiated PC12 cells at a concentration of 0.1 μM. Control cells received an equivalent amount of alcohol. Cells were harvested for RNA quantitation 24 h later. Cell proliferation reagent WST-1 (Roche Applied Science, Indianapolis, IN, USA) was used to assess the toxicity of capsaicin for NGF-differentiated PC12 cells. Cells in collagen-coated 96-well plates were incubated with either 0.1 μM capsaicin or an equivalent amount of ethanol for 24 h. WST-1 (10 μl or 1/10 volume) was added and cells incubated for 1 h and absorbance measured at 450 nm (reference 655 nm).

Plasmids

The plasmid pRK-55, coding for human Brn3a, was a gift from Mengqing Xiang (Robert Wood Johnson Medical School, Piscataway, NJ, USA). Using polymerase chain reaction (PCR) from HeLa cell genomic DNA, a 1043-bp portion of the trkA promoter region was amplified (-1073 to -30 from the trkA initiator codon) and cloned into the chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT3basic (Clontech, Mountain View, CA, USA). The resulting plasmid was named ptrkA1043.

To construct ptrkA1043, 1043 bp of DNA sequences that lie upstream from the initiation codon for human trkA were amplified by using the primers CGCTGGTACCTTGAGACGGATGGGTTAGTGC and ATTACTCGAGGCAGCCGTCTGTGCGCTCC (underlined nucleotides represent the target sequences) and the product cloned between the KpnI and XhoI sites of the chloramphenicol acetyl transferase (CAT) reporter plasmid pCAT3Basic. The veracity of the inserted DNA was confirmed by sequencing. The plasmid ptrkA + 190 contains the distal 109 bp of the trkA upstream sequences in pCAT3Basic. Details of the construction of the plasmids have been previously described (Valderrama and Misra, 2008). Brn3a activates the reporter CAT in both these plasmids (Valderrama and Misra, 2008). The reporter pCAT3BATF6 has five copies of the unfolded protein response element linked to coding sequences for CAT. Plasmids expressing Zhangfei (Lu and Misra, 2000b) and Luman (Misra *et al*, 2005; Raggo *et al*, 2002) have also been described previously.

Transfections

Vero and PC12 cells were transfected using the calcium phosphate method as described previously (Chen and Okayama, 1988). Vero cells were plated at a density of 1×10^6 cells, transfected the next day, and harvested 48 h later. PC12 cells were seeded at a

density of 2×10^6 per well (collagen coated) in CM and incubated overnight at 37°C in a 10% CO_2 incubator. The next day, medium was replaced with RPMI 1640 + 10% normal calf serum + 1% penicillin-streptomycin + 2 mM l-glutamine, and incubated for at least 1 hour at 37°C in 5% CO_2 incubator. DNA concentrations for transfection were 2 μg of reporter plasmid, 1.5 μg pCMVBGal and 1.5 μg of pRK-Brn3a or Luman (pcLuS221Op, which expresses the truncated constitutively active form of Luman), and 2.5 μg of Zhangfei or Zhangfei mutant with an altered HBM (pcZFY224A, which cannot bind HCF) DNA/well. After 5 h in 5% CO_2, cells were shocked with glycerol as follows: cells were rinsed with RPMI and then incubated for 45 s with 0.5 ml of prewarmed 25% glycerol in 2 × N,N-bis[2-Hydroxyethyl]-2 aminoethanesulfonic acid (BES) was added. Cells were rinsed with RPMI 1640 and incubated in 10% CO_2 with 2 ml of PC12 CM or low-serum medium (1% fetal bovine serum) if cells were treated with NGF. Cells were harvested 48 h later for analysis.

Chloramphenicol acetyl transferase (CAT) assays
For chloramphenicol acetyl transferase (CAT) assays 250 ng or 1 μg of pCMVBGal, a plasmid specifying β-galactosidase, was added to transfection mixtures in Vero and PC12 cells, respectively. Lysates were assayed for β-galactosidase and for CAT using an enzyme-linked immunosorbent assay kit (Roche Applied Science). CAT values were adjusted for transfection efficiency using β-galactosidase values. In figures expressing data as CAT results, each data point is the average of replicate transfections, with the bar representing the standard deviation from the mean. The data are representative of three or more independent experiments.

Antibodies and immunoblot and immunoprecipitation
Antibodies against Brn3a, Luman, and Zhangfei were produced at the University of Saskatchewan Animal Resources Centre by immunizing rabbits with about 150 μg of protein in Freund's complete adjuvant as described previously (Misra *et al*, 1995). The anti-Brn3a serum specifically detects Brn3a in immunoblots of *in vitro* synthesized Brn3a (TnT; Promega, Madison, WI, USA) and lysates of mammalian cells transfected to express Brn3a (data not shown). Procedures for immunoblotting and *in vitro* translation of control plasmids and HCF (pSL7) plasmid were as previously described (LaBoissiere *et al*, 1999). Immunoprecipitation and glutathione *S*-transferase (GST) pull-down assays were performed with either ^{35}S-labeled proteins (Lu and Misra, 2000a) or biotinylation proteins (Suzuki *et al*, 2004). In brief, ^{35}S-labeled HCF fusion protein comprising amino and carboxy termini of host cell factor (N-C) and biotin-labeled Luman, Luman (DHTY78AGTA), or Brn3a were synthesized using a TnT system (Promega). ^{35}S-HCF was precipitated with GST-Luman, GST-Luman GST-Luman (DHTY78AGTA), or GST-Brn3a produced in *Escherichia coli*, or with biotinylated proteins linked to avidin beads. The precipitates were washed extensively and analyzed by sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) and autoradiography.

Quantitative real-time PCR (QPCR)
Total RNA was extracted with Trizol (Invitrogen) as suggested by the manufacturer. First-strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) was used for QPCR. Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) was used to quantitate transcripts of interest. The concentration of transcripts in each sample was normalized to a relative amount of GAPDH transcripts expressed as cycle threshold (C_T). Samples were amplified in a Mx3005XP QPCR thermocycler (Stratagene) using the following thermocycle conditions: 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Data were analyzed using the thermocycler-associated software. The primer sets for transcript amplification were used at a final concentration of 40 nM. The sequences of the primers were as follows trkAcd-F (forward): GAGGGCAAAGGCTCTGGACTCCA, trk Acd-R (reverse): AGACTCCGAAGCGCACGATG; Gapdh-F: GCCTCCTGCACCACCAACTG, Gapdh-R: GGGCCATCCACAGTCTTCTGG.

Suppression of Zhangfei expression using siRNA
Several portions of the Zhangfei-coding regions were assessed for their ability, as siRNA, to suppress Zhangfei protein synthesis. Double-stranded oligonucleotides representing a 5' overhanging BspI site, 22 Zhangfei-coding nucleotides in the sense orientation, a nine-nucleotide loop, 22 complementary nucleotides, five T residues as a polymerase III terminator, and an overhanging XbaI site were substituted for green fluorescent protein–coding sequences in plasmid mU6pro (Yu *et al*, 2002). The construct mU6ZF14 was the most effective in suppressing Zhangfei protein synthesis. This was designed to target the Zhangfei-coding sequence GGAGCTGCGGGCCGAGAAT. It suppressed Zhangfei protein expression, as determined in immunoblots, by 25% to 80% and completely suppressed the ability of Zhangfei to inhibit the ability of Luman to activate a unfolded protein response element (UPRE)-containing promoter. Control siRNA, mutant human Ubc13-coding sequences AATCCAGATGATCCAATAGCA, cloned in pmU6pro (Andersen *et al*, 2005) had no effect on Zhangfei expression.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Akhova O, Bainbridge M, Misra V (2005). The neuronal host cell factor-binding protein Zhangfei inhibits herpes simplex virus replication. *J Virol* **79:** 14708–14718.

Andersen PL, Zhou H, Pastushok L, Moraes T, McKenna S, Ziola B, Ellison MJ, Dixit VM, Xiao W (2005). Distinct regulation of Ubc13 functions by the two ubiquitin-conjugating enzyme variants Mms2 and Uev1A. *J Cell Biol* **170:** 745–755.

Batchvarova N, Wang XZ, Ron D (1995). Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153). *EMBO J* **14:** 4654–4661.

Bearer EL, Satpute-Krishnan P (2002). The role of the cytoskeleton in the life cycle of viruses and intracellular bacteria: tracks, motors, and polymerization machines. *Curr Drug Targets Infect Disord* **2:** 247–264.

Bibel M, Barde YA (2000). Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev* **14:** 2919–2937.

Block T, Barney S, Masonis J, Maggioncalda J, Valyi-Nagy T, Fraser NW (1994). Long term herpes simplex virus type 1 infection of nerve growth factor-treated PC12 cells. *J Gen Virol* **75(Pt 9):** 2481–2487.

Chen BP, Wolfgang CD, Hai T (1996). Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10. *Mol Cell Biol* **16:** 1157–1168.

Chen CA, Okayama H (1988). Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **6:** 632–638.

Colgin MA, Smith RL, Wilcox CL (2001). Inducible cyclic AMP early repressor produces reactivation of latent herpes simplex virus type 1 in neurons in vitro. *J Virol* **75:** 2912–2920.

Efstathiou S, Preston CM (2005). Towards an understanding of the molecular basis of herpes simplex virus latency. *Virus Res* **111:** 108–119.

Freiman RN, Herr W (1997). Viral mimicry: common mode of association with HCF by VP16 and the cellular protein LZIP. *Genes Dev* **11:** 3122–3127.

Ganju P, O'Bryan JP, Der C, Winter J, James IF (1998). Differential regulation of SHC proteins by nerve growth factor in sensory neurons and PC12 cells. *Eur J Neurosci* **10:** 1995–2008.

Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM (2002). The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. *J Virol* **76:** 12813–12822.

Geenen K, Nauwynck HJ, De Regge N, Braeckmans K, Favoreel HW (2007). Brn-3a suppresses pseudorabies virus-induced cell death in sensory neurons. *J Gen Virol* **88:** 743–747.

Gupta A, Gartner JJ, Sethupathy P, Hatzigeorgiou AG, Fraser NW (2006). Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. *Nature* **442:** 82–85.

Hill JM, Garza HHJ, Helmy MF, Cook SD, Osborne PA, Johnson EMJ, Thompson HW, Green LC, O'Callaghan RJ, Gebhardt BM (1997). Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits. *J NeuroVirol* **3:** 206–211.

Hogan MR, Cockram GP, Lu R (2006). Cooperative interaction of Zhangfei and ATF4 in transactivation of the cyclic AMP response element. *FEBS Lett* **580:** 58–62.

Huang EJ, Reichardt LF (2003). Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* **72:** 609–642.

Hunsperger EA, Wilcox CL (2003). Capsaicin-induced reactivation of latent herpes simplex virus type 1 in sensory neurons in culture. *J Gen Virol* **84:** 1071–1078.

Izumiya Y, Lin SF, Ellison T, Chen LY, Izumiya C, Luciw P, Kung HJ (2003a). Kaposi's sarcoma-associated herpesvirus K-bZIP is a coregulator of K-Rta: physical association and promoter-dependent transcriptional repression. *J Virol* **77:** 1441–1451.

Izumiya Y, Lin SF, Ellison TJ, Levy AM, Mayeur GL, Izumiya C, Kung HJ (2003b). Cell cycle regulation by Kaposi's sarcoma-associated herpesvirus K-bZIP: direct interaction with cyclin-CDK2 and induction of G1 growth arrest. *J Virol* **77:** 9652–9661.

Jin L, Peng W, Perng GC, Brick DJ, Nesburn AB, Jones C, Wechsler SL (2003). Identification of herpes simplex virus type 1 latency-associated transcript sequences that both inhibit apoptosis and enhance the spontaneous reactivation phenotype. *J Virol* **77:** 6556–6561.

Jones C (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16:** 79–95.

Jordan R, Pepe J, Schaffer PA (1998). Characterization of a nerve growth factor-inducible cellular activity that enhances herpes simplex virus type 1 gene expression and replication of an ICP0 null mutant in cells of neural lineage. *J Virol* **72:** 5373–5382.

Khurana B, Kristie TM (2004). A Protein Sequestering System Reveals Control of Cellular Programs by the Transcriptional Coactivator HCF-1. *J Biol Chem* **279:** 33673–33683.

Kriesel JD (1999). Reactivation of herpes simplex virus: the role of cytokines and intracellular factors. *Curr Opin Infect Dis* **12:** 235–238.

Kristie TM, Vogel JL, Sears AE (1999). Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency. *Proc Natl Acad Sci U S A* **96:** 1229–1233.

LaBoissiere S, Hughes T, O'Hare P (1999). HCF-dependent nuclear import of VP16. *EMBO J* **18:** 480–489.

Laycock KA, Brady RH, Lee SF, Osborne PA, Johnson EM, Pepose JS (1994). The role of nerve growth factor in modulating herpes simplex virus reactivation in vivo. *Graefes Arch Clin Exp Ophthalmol* **232:** 421–425.

Liang G, Audas TE, Li Y, Cockram GP, Dean JD, Martyn AC, Kokame K, Lu R (2006). Luman/CREB3 induces transcription of the endoplasmic reticulum stress response protein, Herp, through an ERSE-II element. *Mol Cell Biol* **26:** 7999–8010.

Liao W, Tang Y, Lin SF, Kung HJ, Giam CZ (2003). K-bZIP of Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) binds KSHV/HHV-8 Rta and represses Rta-mediated transactivation. *J Virol* **77:** 3809–3815.

Lu R, Misra V (2000a). Potential role for Luman, the cellular homologue of herpes simplex virus VP16 (alpha gene trans-inducing factor), in herpesvirus latency. *J Virol* **74:** 934–943.

Lu R, Misra V (2000b). Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16. *Nucleic Acids Res* **28:** 2446–2454.

Lu R, Yang P, O'Hare P, Misra V (1997). Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor. *Mol Cell Biol* **17:** 5117–5126.

Lu R, Yang P, Padmakumar S, Misra V (1998). The herpesvirus transactivator VP16 mimics a human basic domain leucine zipper protein, Luman, in its interaction with HCF. *J Virol* **72:** 6291–6297.

Luciano RL, Wilson AC (2003). HCF-1 functions as a coactivator for the zinc finger protein Krox20. *J Biol Chem* **278:** 51116–51124.

Luxton GW, Haverlock S, Coller KE, Antinone SE, Pincetic A, Smith GA (2005). Targeting of herpesvirus capsid transport in axons is coupled to association with specific sets of tegument proteins. *Proc Natl Acad Sci U S A* **102:** 5832–5837.

Ma L, Lei L, Eng SR, Turner E, Parada LF (2003). Brn3a regulation of TrkA/NGF receptor expression in developing sensory neurons. *Development* **130:** 3525–3534.

Ma L, Merenmies J, Parada LF (2000). Molecular characterization of the TrkA/NGF receptor minimal enhancer reveals regulation by multiple *cis* elements to drive embryonic neuron expression. *Development* **127:** 3777–3788.

Misra V, Rapin N, Akhova O, Bainbridge M, Korchinski P (2005). Zhangfei is a potent and specific inhibitor of the host cell factor-binding transcription factor Luman. *J Biol Chem* **280:** 15257–15266.

Misra V, Walker S, Hayes S, O'Hare P (1995). The bovine herpesvirus alpha gene *trans*-inducing factor activates transcription by mechanisms different from those of its herpes simplex virus type 1 counterpart VP16. *JVirol* **69:** 5209–5216.

Narayanan A, Nogueira ML, Ruyechan WT, Kristie TM (2005). Combinatorial transcription of herpes simplex virus and varicella zoster virus immediate early genes is strictly determined by the cellular coactivator HCF-1. *J Biol Chem* **280:** 1369–1375.

Narayanan A, Ruyechan WT, Kristie TM (2007). The coactivator host cell factor-1 mediates Set1 and MLL1 H3K4 trimethylation at herpesvirus immediate early promoters for initiation of infection. *Proc Natl Acad Sci U S A* **104:** 10835–10840.

Parada LF, Tsoulfas P, Tessarollo L, Blair J, Reid SW, Soppet D (1992). The Trk family of tyrosine kinases: receptors for NGF-related neurotrophins. *Cold Spring Harbor Symp Quant Biol* **57:** 43–51.

Preston CM (2000). Repression of viral transcription during herpes simplex virus latency. *J Gen Virol* **81(Pt 1):** 1–19.

Qiao S, Li W, Tsubouchi R, Murakami K, Yoshino M (2004). Role of vanilloid receptors in the capsaicin-mediated induction of iNOS in PC12 cells. *Neurochem Res* **29:** 687–693.

Raggo C, Rapin N, Stirling J, Gobeil P, Smith-Windsor E, O'Hare P, Misra V (2002). Luman, the cellular counterpart of herpes simplex virus VP16, is processed by regulated intramembrane proteolysis. *Mol Cell Biol* **22:** 5639–5649.

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Roizman B, Knipe DM (2001). Herpes simplex viruses and their replication. In Field's Virology. Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE (ed). Lippincott Williams & Wilkins, Philadelphia. Pp 2399–2460

Shirakawa K, Maeda S, Gotoh T, Hayashi M, Shinomiya K, Ehata S, Nishimura R, Mori M, Onozaki K, Hayashi H, Uematsu S, Akira S, Ogata E, Miyazono K, Imamura T (2006). CCAAT/enhancer-binding protein homologous protein (CHOP) regulates osteoblast differentiation. *Mol Cell Biol* **26:** 6105–6116.

Smith RL, Pizer LI, Johnson EMJ, Wilcox CL (1992). Activation of second-messenger pathways reactivates latent herpes simplex virus in neuronal cultures. *Virology* **188:** 311–318.

Sok J, Wang XZ, Batchvarova N, Kuroda M, Harding H, Ron D (1999). CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI. *Mol Cell Biol* **19:** 495–504.

Someya A, Kunieda K, Akiyama N, Hirabayashi T, Horie S, Murayama T (2004). Expression of vanilloid VR1 receptor in PC12 cells. *Neurochem Int* **45:** 1005–1010.

Suzuki H, Ogawa C, Usui K, Hayashizaki Y (2004). In vitro pull-down assay without expression constructs. *Biotechniques* **37:** 918, 920.

Thompson RL, Sawtell NM (2001). Herpes simplex virus type 1 latency-associated transcript gene promotes neuronal survival. *J Virol* **75:** 6660–6675.

Tyagi S, Chabes AL, Wysocka J, Herr W (2007). E2F activation of S phase promoters via association with HCF-1 and the MLL family of histone H3K4 methyltransferases. *Mol Cell* **27:** 107–119.

Ubeda M, Habener JF (2003). CHOP transcription factor phosphorylation by casein kinase 2 inhibits transcriptional activation. *J Biol Chem* **278:** 40514–40520.

Ubeda M, Vallejo M, Habener JF (1999). CHOP enhancement of gene transcription by interactions with Jun/Fos AP-1 complex proteins. *Mol Cell Biol* **19:** 7589–7599.

Ubeda M, Wang XZ, Zinszner H, Wu I, Habener JF, Ron D (1996). Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. *Mol Cell Biol* **16:** 1479–1489.

Valderrama X, Misra V (2008). Novel Brn3a *cis*-acting sequences mediate transcription of human trkA in neurons. *J Neurochem* **105:** 425–435.

Wilcox C, Johnson E (1987). Nerve growth factor deprivation results in the reactivation of latent herpes simplex virus in vitro. *J Virol* **61:** 2311–2315.

Wilcox C, Johnson E (1988). Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro. *J Virol* **62:** 393–399.

Wilcox CL, Smith RL, Freed CR, Johnson EMJ (1990). Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. *J Neurosci* **10:** 1268–1275.

Wysocka J, Herr W (2003). The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. *Trends Biochem Sci* **28:** 294–304.

Yu JY, DeRuiter SL, Turner DL (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* **99:** 6047–6052.