Axin expression enhances herpes simplex virus type 1 replication by inhibiting virus-mediated cell death in L929 cells

Eun-Jin Choi,¹ Sewoon Kim,² Eek-hoon Jho,² Ki-Joon Song¹ and Sun-Ho Kee¹

¹Laboratory of Cell Biology, Department of Microbiology and Bank for Pathogenic Virus, College of Medicine, Korea University, Seoul, 136-705, Korea
²Department of Life Science, University of Seoul, Seoul 130-743, Korea

Herpes simplex virus type 1 (HSV-1) replicates in various cell types and induces early cell death, which limits viral replication in certain cell types. Axin is a scaffolding protein that regulates Wnt signalling and participates in various cellular events, including cellular proliferation and cell death. The effects of axin expression on HSV-1 infection were investigated based on our initial observation that Wnt3a treatment or axin knockdown reduced HSV-1 replication. L929 cells expressed the axin protein in a doxycycline-inducible manner (L-axin) and enhanced HSV-1 replication in comparison to control cells (L-EV). HSV-1 infection induced cell death as early as 6 h after infection through the necrotic pathway and required de novo protein synthesis in L929 cells. Subsequent analysis of viral protein expression suggested that axin expression led to suppression of HSV-1-induced premature cell death, resulting in increased late gene expression. In analysis of axin deletion mutants, the regulators of the G-protein signalling (RGS) domain were involved in the axin-mediated enhancement of viral replication and reduction in cell death. These results suggest that viral replication enhancement might be mediated by the axin RGS domain.

INTRODUCTION
Herpes simplex virus type 1 (HSV-1) is a large (~152 kb) fast-replicating DNA virus that infects many cell types (Whitley & Roizman, 2001). HSV-1 infection causes various human diseases such as orofacial lesions, and ocular, brain and disseminated viral diseases (Whitley & Roizman, 2001). At the cellular level, HSV-1-infected cells undergo structural and biochemical alterations termed cytopathic effects, which ultimately lead to cell lysis and death (Nguyen & Blaho, 2006). In addition to necrotic cell death, HSV-1 infection triggers the apoptotic cell death pathway. Apoptosis is first triggered and later blocked in cells infected with HSV-1 (Aubert et al., 1999; Koyama & Adachi, 1997; Nguyen & Blaho, 2006), suggesting that HSV-1 expresses both pro-apoptosis and anti-apoptosis genes. The triggering of apoptosis seems to occur in the absence of protein synthesis within 3 h post-infection in HEp-2 cells (Aubert et al., 1999), but Vero cells require de novo protein synthesis for HSV-1-dependent apoptosis (Nguyen et al., 2005). Many lines of evidence have shown the involvement of mitochondrial membrane perturbances and caspase-3 activation during HSV-1-mediated apoptosis (Kraft et al., 2006; Nguyen & Blaho, 2006), whereas HSV-1 mutations such as ICP4 (infected-cell polypeptide 4) deletion (d120) induce caspase-independent apoptosis (Galvan et al., 1999). In contrast, anti-apoptosis genes appear to be expressed 6 h post-infection (Aubert et al., 1999), and numerous viral gene products such as ICP4, ICP27, ICP34.5 and US5 kinase have anti-apoptosis effects (Nguyen & Blaho, 2006). Cellular proteins such as Bcl-2 and NF-κB seem to be involved during HSV-1-mediated anti-apoptosis. In addition, some viral proteins interact with specific cellular proteins to attenuate apoptosis. For example, the interaction of HSV-1 U3 kinase with programmed cell death protein 4 participates in the attenuation of apoptosis in cells infected with the HSV-1 d120 mutant (Wang et al., 2011). The balance between pro- apoptotic and anti-apoptotic effects of HSV-1 gene expression may determine the rate of infected-cell death. If the balance in cell death regulation shifts to apoptosis, HSV-1 infection may trigger premature death of host cells, which produces an unfavourable environment for viral replication. In accordance with this hypothesis, inhibiting apoptosis using the caspase inhibitor zVAD increases viral persistence and replication (Wood & Shillitoe, 2011). Among other cell death-related mechanisms, autophagy seems to inhibit HSV-1 replication (Pei et al., 2011), and the anti-autophagic effect of HSV-1 ICP34.5 has been well described (Alexander et al., 2007; Orvedahl et al., 2007). Additionally, HSV-1 infection induces autophagy in some cells including fibroblasts and macrophages (English et al., 2009; McFarlane et al., 2011). As identifying the HSV-1-induced...
cell death mechanisms appears to be dependent on differences in cell type (Aubert & Blaho, 2003; English et al., 2009; Galvan & Roizman, 1998; McFarlane et al., 2011; Nguyen et al., 2005), cellular factors may be important for HSV-1-induced cell death and viral replication. Thus, in this study, the effects of axin expression were investigated in terms of HSV-1 replication and virus-mediated cell death to analyse the influence of host factors during HSV-1 infection.

The most well-known function of axin is negative regulation of canonical Wnt signalling through formation of the β-catenin destruction complex. Upon Wnt interaction with its receptor [e.g. frizzled (Fz) or LRP], β-catenin dissociates from axin and is transported into the nucleus to transcriptionally upregulate proliferation-related genes such as myc and cyclin D. In addition, axin appears to participate in the induction of cell death in Chinese hamster ovary (CHO) cells (Neo et al., 2000). In several systems, axin appears to be involved in regulation rather than direct induction of cell death. Axin overexpression enhances mitotic defect-induced cell death in L929 cells (Choi et al., 2011). Additionally, axin functions in UV-induced cell death by phosphorylating p53 at Ser 46, which is catalysed by HIPK2 (Li et al., 2007). Subsequent observations showed that Pirh2 competes with HIPK2 to bind axin and abrogates HIPK2-mediated p53 phosphorylation, which allows cells to survive under low-dose UV irradiation (Li et al., 2009). Therefore, axin determines cell fate by controlling p53 activation status. In contrast, some lines of evidence suggest that axin plays a cytoprotective role under specific conditions. Reduction of β-catenin levels showed a neuroprotective effect in neurons and in a Drosophila model of Huntington’s disease (HD) (Godin et al., 2010), and axin overexpression increases lifespan in HD Drosophila (Dupont et al., 2012). Moreover, axin expression appears to alter mitochondrial function, which attenuates staurosporine (STS)-induced mitochondria-mediated cell death in HeLa cells (Shin et al., 2012). These observations suggest that axin may play a protective role in cells under some harmful conditions, although antiproliferative or cell death effects are more frequently observed.

We observed that HSV-1 infected and replicated more efficiently in axin-expressing L929 cells in comparison to control cells. This enhanced HSV-1 replication effect was accompanied by reduced cell death.

RESULTS

Axin expression facilitates HSV-1 replication and reduces HSV-1-induced cell death

HSV-1-infected cells were treated with Wnt3a-conditioned medium (Wnt3a-CM) to determine the effects of Wnt signalling activity on viral replication. Treatment with Wnt3a-CM induced an increase in β-catenin levels in L929 cells (Fig. 1a), suggesting the activation of β-catenin-dependent Wnt signalling. HSV-1 infection reduced β-catenin levels, which was surpassed by Wnt3a-CM treatment (Fig. 1b). Viral replication, which was monitored by viral capsid protein ICP5 expression in Wnt3a-CM-treated L929 cells, appeared to be reduced in comparison to that of control untreated cells (Fig. 1b). In the case of the β-catenin knockdown experiment, only a slight increase of viral replication was observed in L929 cells (Fig. 1c, d). To evaluate further, knockdown of endogenous axin, which is a negative regulator of β-catenin-dependent Wnt signalling, was performed and a reduction of viral replication was observed in L929 cells (Fig. 1e, f). These results suggest that HSV-1 replication may be influenced by β-catenin-dependent Wnt signalling activity.

Next, we analysed the effects of axin expression on HSV-1 replication, because axin is an important regulatory protein in β-catenin-dependent Wnt signalling (Zeng et al., 1997). For this, axin-expressing L929 cells (L-axin), which express axin and GFP using a dual promoter system in a doxycycline-inducible manner, were established. Axin expression was monitored using the tagged Myc peptide in L-axin cells (Fig. 2a). Wnt3a-CM treatment increased the β-catenin level in L-axin cells, but this effect was less apparent compared to that in L929 cells (Fig. 1b). Additionally, the efficiency of HSV-1 replication appeared to be higher in L-axin than in L929 cells regardless of Wnt3a treatment (Fig. 1b). The increase of HSV-1 replication appeared to be axin-expression-specific since the increase was more apparent in the presence of doxycycline (Fig. 2b). A detailed time-dependent analysis showed that a higher expression of ICP5 was observed from 16 h post-infection in L-axin cells compared to that in control L-EV cells (Fig. 2c). The increase in HSV-1 replication in L-axin cells was confirmed by immunofluorescence analysis (Fig. 2d). A quantitative plaque-forming analysis using infected-cell culture supernatant showed about a fivefold increase in virus release from L-axin cells compared to that from L-EV cells (Fig. 2e, f). In conclusion, our results suggest that axin expression may enhance HSV-1 replication, providing supportive evidence of an inverse relationship between HSV-1 replication and β-catenin-dependent Wnt signalling.

HSV-1-mediated cell death was analysed. In phase-contrast microscopic observations, HSV-1 infection-induced cell death was less clear in L-axin cells than in L-EV cells (Fig. 3a). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that HSV-1-infected L-EV cells showed significant cell death in dose- and time-dependent manners, but these effects decreased significantly in L-axin cells (Fig. 3b). Similarly, HSV type 2 (HSV-2) produced less apparent cell death in L-axin cells than in L-EV cells, although this effect was obvious only 48 h after infection (Fig. 3c). A mixed culture of L-axin and parental L929 cells was infected with HSV-1 and analysed using time-lapse imaging. Because L929 cells did not express GFP, the GFP-expressing cells were L-axin cells. L929 cells underwent the cell death process, whereas many GFP-expressing L-axin cells remained viable throughout the imaging period (Fig. 3d). These cell images were used to directly count viable and
dead cells (Fig. 3e). Cell death in L929 cells was observed from 6 h post-infection, whereas L-axin cells began to die at 15 h post-infection. Taken together, our results suggest that axin expression reduces or delays HSV-1-induced early cell death and facilitates viral replication.

The detailed mechanisms of HSV-1-mediated cell death were elucidated. In live and dead cell staining at 16 h post-infection (Fig. 4a), a greater abundance of dead cells was observed for L-EV cells in comparison with L-axin cells. This cell death appeared to occur in a caspase-3-independent manner because HSV-1 infection failed to induce caspase-3 activation or poly(ADP-ribose) polymerase cleavage in both L-EV and L-axin cells (Fig. 4b). For a detailed analysis of HSV-1-induced cell death, L-EV and L-axin cells were infected with HSV-1, and subjected to flow cytometric analysis (Fig. 4c). At 16 h post-infection, the Annexin V-PE and 7-amino-actinomycin D (7-AAD) positive population was increased from 2.06 to 11.44% in L-EV cells, whereas there was no significant increase in L-axin cells. Annexin V-PE positive and 7-AAD negative cells were not increased upon HSV-1 infection in both cells, suggesting that HSV-1 induced non-apoptotic, necrotic cell death. The proportion of dead cells in both virus-infected L-EV and L-axin cells appeared to be less abundant in comparison to the results of the MTT assay (Fig. 3b). This discrepancy might result from the loss of shrunken dead cells during stringent washing steps. Furthermore, treatment with necrostatin-1, which is known as necroptosis inhibitor (Degterev et al., 2005), facilitated HSV-1 replication and decreased virus-induced cell death in L929 cells (Fig. 4d, e). These results suggest that HSV-1-mediated cell death was induced through the necrotic pathway and reduction of necrotic cell death might enhance HSV-1 replication in L-axin cells.

**HSV-1-mediated cell death requires viral replication and de novo protein synthesis**

L-EV and L-axin cells were infected with UV-irradiated HSV-1 to determine whether the axin-mediated reduction in cell death was caused by engagement of the virus–receptor

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**Fig. 1.** HSV-1 replication is inversely related to Wnt signalling. (a) L929 cells were incubated in media with the indicated dilutions of Wnt3a-CM or without Wnt3a-CM for 24 h. Activation of Wnt signalling was identified by increased expression of β-catenin (β-CTN). (b) L929 or L-axin cells treated with Wnt3a-CM were infected with HSV-1 (m.o.i.=6) for 16 h. Immunoblot analysis showed that treatment with Wnt3a decreased viral replication in L929 and L-axin cells. (c) L929 cells were transfected with two kinds of β-catenin siRNA (B1, siCatenin-B1; B2, siCatenin-B2) and scrambled control siRNA (S). At 24 and 48 h after siRNA transfection, a reduction in β-catenin expression was identified by immunoblot analysis. (d) At 24 h post-transfection of siRNA, L929 cells were infected with HSV-1 for 16 h and subjected to immunoblot analysis. Knockdown of β-catenin slightly increased HSV-1 replication in L929 cells. (e) Immunoblot analysis revealed that knockdown of axin decreased HSV-1 replication at 16 h post-infection in L929 cells. (f) A reduction in axin expression by siAxin transfection was confirmed by immunoprecipitation using the E5 mAb and immunoblot analysis using mAb G4, as previously described (Kim et al., 2009). Results are representative of three independent experiments. HC, heavy chain of IgG.
complex. Irradiated HSV-1 failed to express the late gene product ICP5 after infection (Fig. 5a). Upon infection with irradiated HSV-1, cell death of both L-EV and L-axin cells decreased significantly in comparison to cell death upon infection with non-irradiated virus (Fig. 5b). Furthermore, the protein translation requirement for HSV-1-mediated cytotoxic effects was analysed using the translation inhibitor cycloheximide (CHX). The effect of CHX was confirmed by failure of capsid protein synthesis after HSV-1 infection (Fig. 5c). CHX treatment also reduced HSV-1-mediated cell death in both L-EV and L-axin cells (Fig. 5d). Because the reduction in cell death was greater in L-EV cells, cell viability became similar in both L-EV and L-axin cells, suggesting that HSV-1-mediated cell death may require newly synthesized viral proteins after viral entry into L929 cells and that axin expression may suppress this HSV-1-mediated cell death. Next, expression of various viral proteins was investigated to identify which replication steps were influenced by cell death (Fig. 5e). For this, immunoblot analysis was performed using antibodies detecting an immediate-early (ICP4), early (ICP8) or late (ICP5) protein (Bryant et al., 2012; Grondin & DeLuca, 2000). Whereas the expression patterns of ICP4 and ICP8 were similar between L-EV and L-axin cells after HSV-1 infection, ICP5 expression level was higher in L-axin cells than in L-EV cells. These results suggest that HSV-1 induces cell death in an early phase of infection, which may reduce late gene expression. Therefore, axin-mediated inhibition of cell death may influence late gene expression more significantly than immediate-early or early gene expression.

Regulators of G-protein signalling (RGS) domain of the axin protein participate in facilitating HSV-1 replication

To analyse the axin protein domain responsible for enhancing HSV-1 replication, various axin deletion mutants were constructed, and L929 cells expressing these mutant proteins were infected with HSV-1. The results showed that the RGS domain of axin was required for HSV-1 replication, indicating that axin facilitates HSV-1 replication through its RGS domain.
axin genes were established (Fig. 6a). All cells expressed mutant axin and GFP simultaneously in a doxycycline-inducible manner. An initial analysis of infected-cell cytotoxicity showed that all deletion mutant-expressing cells, except those with the RGS domain deletion (L-D_R) mutant, showed cytoprotective effects upon HSV-1 infection (Fig. 6b). Subsequent immunoblot analysis of ICP5 expression from infected cells revealed that infected L-D_R cells showed an apparent reduction in ICP5 expression (Fig. 6c, d), suggesting suppression of viral replication. Although deletion of the glycogen synthase kinase 3 (GSK3β)-binding domain (L-DG) also produced some cell death and reduced viral replication compared to those of L-axin cells, these effects appeared less clear than those in L-EV and L-ΔR cells (Fig. 6b–d). These results raised the possibility that the axin RGS domain may influence HSV-1 infection through a distinct mechanism in addition to regulating β-catenin stability through destruction of the β-catenin complex.

Together with the DIX domain, the RGS domain is well conserved between axin and its homologue axin2 (Behrens et al., 1998). Because the RGS domain appears to be related to axin-mediated enhancement of viral replication and suppression of cytotoxicity, we speculated that axin2 may have a similar effect to axin. To evaluate the effect of axin2 on...
HSV-1 replication, L929 cells expressing axin2 were established (L-axin2). L-axin2 cells infected with HSV-1 showed increased ICP5 expression compared to that in L-EV cells (Fig. 7a), although this increase in ICP5 expression appeared to be less efficient than that in L-axin cells. Subsequent phase-contrast microscopic observations revealed that L-axin2 cells were resistant to the cytotoxic effects of HSV-1 compared to those of L-EV cells (Fig. 7b). This result was confirmed through the quantification of cell viability, which was performed by direct counting of viable and dead cells using the phase-contrast images from Fig. 7b (Fig. 7c). Taken together, the effect of axin on HSV-1 replication might be mediated, at least in part, by a function related to the axin RGS domain.

**DISCUSSION**

Axin is a concentration-limiting factor regulating β-catenin-dependent Wnt signalling activity (Salahshor & Woodgett, 2005), and the increase in axin level results in antiproliferative effects in various cells (Huang et al., 2009; Salahshor & Woodgett, 2005). In this study, we showed that Wnt3a treatment and axin knockdown reduced HSV-1 replication (Fig. 1), suggesting an inverse relationship between Wnt signalling activity and viral replication. In the case of β-catenin knockdown, only a slight increase of HSV-1 replication was observed (Fig. 1d). This result led us to speculate that there are additional mechanisms in addition to the regulation of β-catenin-dependent Wnt signalling. Given that the axin mutant with deletion of the GSK3β-binding domain (L-DG) showed relatively higher HSV-1 replication than L-EV and L-DG (Fig. 6), axin affects HSV-1 replication through RGS domain-related functions, as well as by regulation of β-catenin-dependent Wnt signalling. Therefore, the effect of β-catenin knockdown on HSV-1 replication may be less efficient in comparison to that of axin expression. Although axin
mediated cell death in HeLa cells (Shin et al. 2006). Axin overexpression confers protection against mutant huntingtin toxicity in the Drosophila model (Dupont et al., 2012). In addition, axin expression attenuates STS-induced mitochondrial-mediated cell death in HeLa cells (Shin et al., 2012). Axin also shows a protective effect against Salmonella invasiveness in intestinal epithelial cells (Zhang et al., 2012). In that report, the DIX domain but not the RGS domain was required for axin to inhibit Salmonella invasion. In our results, enhancement of HSV-1 replication required the RGS domain, suggesting that different intrinsic properties of axin may be involved in these two events. Axin interacts with numerous cellular proteins as a scaffolding protein (Salahshor & Woodgett, 2005), and a small amount of axin is sufficient in normal cells (Lee et al., 2003).

HSV-1 infection leads to undergo structural and biochemical alterations, which result in necrotic cell death involving cell lysis (Nguyen & Blaho, 2006; Roizman, 1974). Additionally, HSV-1 can trigger apoptosis at multiple steps of the infection through expression of pro-apoptotic and anti-apoptotic molecules (Nguyen & Blaho, 2006). During the early stage of infection, HSV-1 infection induces apoptotic early death, whereas apoptosis is blocked by viral proteins in the late stage, and necrotic cell death ensues. Our results show HSV-1 infection-induced cell death occurred as early as 6 h post-infection in L929 cells, but this early cell death was apparently reduced in L-axin cells (Fig. 3d, e). However, apoptotic signs related to HSV-1-induced cell death in control L-EV cells were difficult to detect. Instead, HSV-1 might induce necrotic cell death because infected L-EV cells showed perturbation of the cell membrane, which was assessed by 7-AAD staining (Fig. 4c). Moreover HSV-1-induced cell death was inhibited by treatment with necrostatin-1 in L929 cells (Fig. 4e). These results suggest that HSV-1 infection-induced necrotic cell death in L929 cells. Previous observations showed that L929 cells are vulnerable to necrotic cell death under specific conditions. For example, zVAD, which is a caspase-3 inhibitor that generally shows low cytotoxicity in most cell lines (Van Noorden, 2001), produces robust necrotic cell death and autophagy in L929 cells (Chen et al., 2011; Wu et al., 2011). Different from other cells, treatment of L929 cells with TNF causes necrotic cell death and autophagy (Vercammen et al., 1998a; Ye et al., 2011), and this cell death was exaggerated by treating the L929 cells with zDEVD or zVAD (Vercammen et al., 1998b). Considering this unique property of L929 cells in response to cell death, HSV-1 infection preferentially induced premature necrotic cell death in L929 cells, which might cause limited viral replication. In addition, HSV-1-induced cell death seemed to require de novo protein synthesis (Fig. 5c, d), and axin expression did not affect ICP4 and ICP8.

![Fig. 5. HSV-1-mediated cell death requires viral replication and de novo protein synthesis. (a, b) L-EV and L-axin cells were infected with UV-irradiated HSV-1 for 16 h and subjected to immunoblot analysis (a) or MTT assay (b). UVC irradiation blocked viral replication and UV-irradiated HSV-1 did not efficiently induce cell death. The error bars indicate the sds. (c, d) L-EV and L-axin cells were infected with HSV-1 in the presence of CHX for 16 h and subjected to immunoblot analysis (c) or MTT assay (d). Inhibition of viral protein synthesis by CHX did not efficiently induce HSV-1-mediated cell death. The error bars indicate the sds. (e) L-EV and L-axin cells were infected with HSV-1 for the indicated times and then subjected to immunoblot analysis using anti-ICP4, anti-ICP8 and anti-ICP5 antibodies. In both cell types, the expression pattern of ICP4 and ICP8 was similar, whereas ICP5 expression was higher in L-axin cells than in L-EV cells. Results are representative of three independent experiments.](image-url)
expression (Fig. 5e). These results suggest that immediately or early proteins might induce premature necrotic cell death in L929 cells, which might cause restriction of late gene product expression and viral replication. In L-axin cells, suppressing HSV-1-induced cell death may produce a more favourable situation for viral replication.

In analysis of deletion mutants of axin, both the RGS and GSK3β-binding domain of axin may participate in axin-mediated modulation of HSV-1 replication and cell death (Fig. 6). Both domains were involved in β-catenin-independent Wnt signalling through binding to adenomatosis polyposis coli (APC) and GSK3β (Itoh et al., 1998; Kishida et al., 1998). Quantification of the results suggests that the RGS domain may have additional functions to axin-mediated regulation of β-catenin-dependent Wnt signalling activity because deletion of the GSK3β-binding domain showed only partial reversion of axin effects on viral replication and cell death (Fig. 6). This speculation may be supported by the results that β-catenin knockdown affects viral replication only slightly (Fig. 1d). In the literature, APC and the alpha subunit of the trimeric G-protein (Gzo) have been well described as RGS-binding proteins. In addition to regulation of β-catenin-dependent Wnt signalling, APC participates in numerous cellular events, such as cell migration, polarization, microtubule stability and cell death (Hanson & Miller, 2005; McCartney & Näthke, 2008). In terms of cell death, APC plays complicated roles. The expression of full-length APC in a colon carcinoma cell line induces apoptosis (Morin et al., 1996), and conversely loss of Drosophila APC induces apoptosis of retinal neurons (Ahmed et al., 1998). In some cases, APC-mediated regulation of cell cycle progress appears to be independent of β-catenin-mediated signals (Ishidate et al., 2000). More recently, it was described that Gzo directly acts on the axin RGS domain, which neutralizes the axin inhibitory function for Wnt signalling (Egger-Adam & Katanaev, 2012). Gzo may participate in overactivation of Wnt/Fz signalling, and the interaction of Gzo with Rab4 and Rab5 may be involved in Gzo-mediated Wnt signalling activation (Egger-Adam & Katanaev, 2008; Koval et al., 2011). In addition to Gzo binding ability, both the Rab5 and axin proteins show neuroprotective effects in the HD Drosophila model (Dupont et al., 2012; Ravikumar et al., 2008). These
observations lead to the speculation that axin, APC, Gzo and Rab proteins may interrelate to regulate some common cellular events including cell death.

Our results indicate that axin expression suppresses HSV-1-induced premature cell death, which results in facilitated viral replication. These axin effects may be related to suppression of Wnt signalling activity but the intrinsic functions of axin mediated by the RGS domain are also involved in these axin effects on HSV-1 infection.

**METHODS**

**Cells, chemicals and antibodies.** L929 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % (v/v) FBS (Cambrex). L929 cells expressed ectopic axin (L-axin), axin2 (L-axin2) or axin deletion mutants together with GFP simultaneously in a dual doxycycline-inducible manner (Jeon et al., 2007; Kim et al., 2009). Control cells were established by transfection with an empty vector with GFP (L-EV). Each mutant axin gene, which had a deletion of a specific domain, was transfected into L929 cells and cultured in DMEM containing G418. Final clones expressing the appropriate axin mutant were selected. Each mutant axin was expressed by the doxycycline-inducible system. Each axin deletion mutant contained an RGS domain deletion (amino acid numbers 127–226; ΔR), a mitogen-activated protein kinase 1 deletion (228–354; ΔM), a GSK3β deletion (377–406; ΔG), or a protein phosphatase 2A deletion (508–711; ΔP). STS, necrostatin-1 and CHX were purchased from Sigma. The following antibodies were used: β-actin, ICP5, Myc (Santa Cruz Biotechnology), ICP4, ICP8 (Abcam), β-catenin (BD Pharmingen), active caspase-3 (Cell Signaling Technology).

**Virus and drug treatment.** HSV-1 strain HF (ATCC VR-260) and HSV-2 strain G (ATCC VR-734) were propagated in Vero cells using Eagle’s minimum essential medium (EMEM) containing 2 % FBS. Inactivated HSV-1 was prepared by UVC irradiation (12 000 μJ) using an XL-1500 UV cross-linker (Spectronics). L929 cells were seeded and treated with doxycycline (1 μg ml⁻¹) for 24 h and then infected with virus. Cells were inoculated with virus stock (m.o.i. = 6) in serum-free media, and culture media were exchanged with fresh EMEM containing 2 % FBS and doxycycline after 2 h for an additional 16 h, unless indicated. Cells were pretreated with CHX (10 μg ml⁻¹) or necrostatin-1 for 1 h and were maintained in these drugs during the infection. The Wnt3a-CM was produced from L929 cells, as described previously (Shin et al., 2012). Briefly, control and Wnt3a-expressing L929 cells were grown for 24 h, and the culture medium was exchanged with fresh medium. After 24 h incubation, the medium was collected and used as control-conditioned medium (control-CM) or Wnt3a-CM. These conditioned media were used for activating the β-catenin-dependent Wnt signalling cascade in L-EV and L-axin cells.

**Plaque assay.** The supernatant obtained from HSV-1-infected L-EV or L-axin cells was diluted in tenfold steps and inoculated into confluent Vero cells. After 2 h incubation, the viruses were removed and the first overlay medium (EMEM, 1 % agarose) was added to each well. After 4 days, a second overlay medium (EMEM, 1 % agarose, 5 % neutral red) was added and incubated for 1 day. The plaques were counted after fixation in 4 % formaldehyde and detachment of the agarose.

**Transfection.** Cells were grown to 40–60% confluency and transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The sequences of two siRNAs specific to mouse axin and to mouse β-catenin were 5′-GGCAGAGAGCAGGUAUG-3′ (siAxin-A1), 5′-TGCCAAAGAAGCAGTGCG-3′ (siAxin-A2), 5′-AAGGCUUUUC-CAGGCUUCA-3′ (siCatenin-B1) and 5′-AAAGAUGUUGUG-GCCAAGUG-3′ (siCatenin-B2). siAxin or siCatenin was transfected into L929 cells and cultivated for 24 h. Then, the transfected cells were infected with HSV and subjected to immunoblot analysis at 16 h post-infection. The reduction in axin expression was monitored by immunoprecipitation using the E5 and G4 axin-specific monoclonal antibodies (Kim et al., 2009).

**MTT assay.** The MTT assay was performed using the CellTiter 96 non-radioactive cell proliferation assay kit (Promega) according to the manufacturer’s instructions. Coloured products were measured at an absorbance of 570 nm using a microplate reader (Spectramax; Molecular Devices). All experiments were performed in triplicate.

**Live imaging.** L929 and L-axin cells were mixed and plated on 35 mm dishes. At 4 h post-infection with HSV-1, live images were taken at 6 min intervals for 20 h using an Observer D1 phase-contrast microscope equipped with a charge coupled device camera (Carl Zeiss Jena).
**Immunofluorescence assay.** The immunofluorescence assay was performed as described previously (Jeon et al., 2007; Kim et al., 2009). Briefly, cells were grown on coverslips and infected with HSV-1. After washing with PBS (pH 7.4), the cells were pretreated with 0.4% Triton X-100 and 0.4% paraformaldehyde in PBS to remove soluble proteins, and then fixed. Following permeabilization, the cells were incubated with primary and secondary antibodies. Stained cells were analysed by fluorescence microscopy (Axioscope; Carl Zeiss). The live and dead assay was performed using LIVE/DEAD viability/cytotoxicity kit (Molecular Probes) according to the manufacturer’s instructions.

**Immunoblot analysis.** The immunoblot analysis was performed as described previously (Jeon et al., 2007; Kim et al., 2009). Briefly, cells were cultured in 100 mm dishes and infected with HSV-1. Following preparation of the cell lysates, SDS-PAGE was performed, and the proteins were transferred to PVDF membranes. After reacting the membranes with primary and secondary antibodies, target proteins were visualized using enhanced chemiluminescence (PerkinElmer).

**Flow cytometry.** FACS analysis was conducted as previously described (Choi et al., 2011) and the Annexin V-PE apoptosis detection kit (BD Pharmingen) was used according to the manufacturer’s instructions. Briefly, cells infected with HSV-1 were collected and resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂). After addition of Annexin V-PE or 7-AAD, cells were incubated in the dark for 15 min at room temperature. Stained cells were subjected to flow cytometry using a FACScantor II flow cytometer (BD Biosciences) with WinMDI 2.9 (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA) software.

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