

1 **Early phosphatidylinositol 3-kinase/Akt pathway activation limits**  
2 **poliovirus-induced JNK-mediated cell death**

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21 **ABSTRACT**

22

23 PV-induced apoptosis seems to play a major role in tissue injury in the central nervous system  
24 (CNS). We have previously shown that this process involves PV-induced Bax-dependent  
25 mitochondrial dysfunction mediated by early JNK activation in IMR5 neuroblastoma cells.  
26 We show here that PV simultaneously activates the phosphatidylinositol 3-kinase (PI3K)/Akt  
27 survival signaling pathway in these cells, limiting the extent of JNK activation, and thereby  
28 cell death. JNK inhibition is associated with PI3K-dependent negative regulation of the  
29 apoptosis signal-regulating kinase 1 (ASK1), which acts upstream from JNK in PV-infected  
30 IMR5 cells. In poliomyelitis, this survival pathway may limit the spread of PV-induced  
31 damage in the CNS.

32 Poliovirus (PV), from the *Picornaviridae* family, causes paralytic poliomyelitis — a  
33 disease in which the motor neurons are destroyed in association with PV replication. PV  
34 consists of a single-stranded positive RNA genome surrounded by a nonenveloped  
35 icosahedral protein capsid. The human PV receptor, CD155, and its simian counterparts  
36 belong to the immunoglobulin superfamily (24, 25, 31) and are related to the nectin family of  
37 adhesion molecules (28, 38).

38 PV is mostly transmitted via the fecal-oral route. It first infects the oropharynx and the  
39 digestive tract, and then spreads to the central nervous system (CNS) in which it mostly  
40 targets motor neurons. Studies in mouse models have shown that PV-infected motor neurons  
41 in the spinal cord die by apoptosis (10, 19). PV-induced apoptosis therefore seems to play a  
42 major role in the tissue injury occurring in the CNS.

43 PV triggers apoptosis *in vitro* in tissue cultures of human colon carcinoma cells  
44 (CaCo-2) (4), promonocytic cells (U937) (29), dendritic cells (41), murine L cells expressing  
45 CD155 (21, 36), HeLa cells (8, 39) and cultures of mixed mouse primary nerve cells (12)  
46 from the cerebral cortex of mice transgenic for CD155. Analyses of the apoptotic pathways  
47 induced following PV infection in several cell lines have demonstrated that mitochondria are  
48 key actors of PV-induced apoptosis. In particular, mitochondrial outer membrane  
49 permeabilization (MOMP) following PV infection leads to a loss of mitochondrial  
50 transmembrane potential and the release of proapoptotic molecules, including cytochrome *c*,  
51 from the mitochondria to the cytosol (8, 21). We recently demonstrated that MOMP in PV-  
52 infected neuronal IMR5 cells was dependent on Bax, a proapoptotic member of the Bcl-2  
53 family. Bax activation was mediated by c-Jun NH<sub>2</sub>-terminal kinase (JNK) phosphorylation  
54 after PV infection (6). JNK activation occurred early after PV infection whereas apoptotic  
55 features were observed later in PV-infected cells. These events may involve a balance  
56 between pro- and antiapoptotic signals following PV infection. Pro- and antiapoptotic events

57 potentially acting in synergy or competing with each other during the reproduction cycle of  
58 PV have been described by Agol's group (1, 39). However, the mechanisms involved in  
59 maintaining this delicate balance remain unclear.

60 Cells become committed to undergoing apoptosis in response to a collection of  
61 multiple survival and death signals. The phosphatidylinositol 3-kinase (PI3K) signaling  
62 pathway plays a crucial role in the transmission of survival signals in various cell types (14,  
63 26), including neurons (16). PI3K activates its downstream effector, the serine-threonine  
64 kinase Akt (also known as protein kinase B, PKB) by promoting its phosphorylation at the  
65 residues Thr308 and Ser473. Activated Akt then phosphorylates various substrates, activating  
66 antiapoptotic factors and inactivating proapoptotic factors. The role of PI3K/Akt in the  
67 regulation of cell survival and apoptosis in a number of viral infection models (11, 13, 17, 27,  
68 30), including infection with coxsackievirus B3 (18), rhinovirus (32), foot-and-mouth disease  
69 virus (35) and enterovirus 71 (40, 43) — all members of the *Picornaviridae* family — has  
70 recently been investigated.

71

## 72 **PV activates the PI3K/Akt survival signaling pathway in IMR5 cells**

73 We began by determining whether PV infection of IMR5 neuroblastoma cells resulted  
74 in Akt activation. IMR5 cells were infected with PV as previously described (6). Briefly, the  
75 growth medium (DMEM supplemented with 10% FBS) was discarded. The virus was then  
76 added to monolayers at a multiplicity of infection (MOI) of ten 50% tissue culture infective  
77 dose units (TCID<sub>50</sub>) per cell (this MOI was used for all assays performed in this study).  
78 Adsorption was allowed to proceed for 30 min at 37°C in humidified air containing 5% CO<sub>2</sub>.  
79 Cells were then washed twice with serum-free medium to remove unbound particles and  
80 incubated with fresh DMEM supplemented with 10% FBS at 37°C. The virus was allowed to  
81 grow for the indicated times. Time zero postinfection (p.i) corresponds to the inoculation time

82 point. Mock-infected cells were used as a negative control. As previously described (6), both  
83 adherent and detached cells were taken into account in all experiments. Kinetics of Akt  
84 phosphorylation at serine 473 (Ser473), which is required for full Akt activation (3), was  
85 investigated in mock- and PV-infected cells. Whole-cell lysates were analyzed at the indicated  
86 times p.i. by Western blotting with a specific anti-phospho (Ser473)-Akt antibody (Fig. 1A).  
87 We checked for equal protein loading on the total Akt Western blot. The amount of  
88 phosphorylated Akt increased until 30 min p.i., and then decreased; at 4 h p.i., the amount of  
89 phosphorylated Akt present was similar to that in mock-infected cells analyzed at the same  
90 time point. To check that the virus stock used in this study did not contain host-derived  
91 components that may activate Akt signaling pathway, we depleted the virus suspension of PV  
92 using an anti-PV antibody and infected cells with either the depleted or non-depleted  
93 suspension. In contrast to cells infected with the non-depleted stock, no Akt activation (30  
94 min p.i.) was detected in cells treated with the depleted suspension (Fig. 1A, bottom, left). We  
95 also checked that poliovirus, purified by isopycnic CsCl gradient centrifugation (9), could  
96 promote Akt activation (30 min p.i.), at an efficiency similar to that obtained with the virus  
97 preparations used in this study (Fig. 1A, bottom, right). We then investigated whether Akt  
98 activation in response to PV infection occurred through the PI3K pathway, by treating IMR5  
99 cells with a specific PI3K inhibitor, wortmannin (5), at a concentration of 100 nM and 500  
100 nM, 2 h before mock or virus infection. The concentration of the inhibitor was maintained  
101 during the adsorption period and PV infection. Cell lysates were collected 30 min after  
102 infection and subjected to Western blot analysis for the detection of Akt phosphorylation (Fig.  
103 1B, top). Wortmannin inhibited Akt phosphorylation at both concentrations without altering  
104 total Akt levels. The activation of Akt in response to PV infection was illustrated by  
105 immunofluorescence staining, 30 min p.i., with the same anti-phospho (Ser473)-Akt antibody.  
106 Representative staining patterns for mock-infected and PV-infected IMR5 cells treated with

107 wortmannin or left untreated are presented (Fig. 1B, bottom). As expected,  
108 immunofluorescence staining was detected only in infected cells in the absence of  
109 wortmannin. Thus, the rapid PV-induced phosphorylation of Akt involves a PI3K-dependent  
110 mechanism.

111 We investigated whether PV adsorption onto IMR5 cells induced Akt activation in the  
112 absence of PV replication by assessing Akt phosphorylation after the addition of UV-  
113 inactivated PV (UV cross-linked at 6,000  $\mu\text{J}/\text{cm}^2$ ) to IMR5 cells at a dilution corresponding to  
114 an MOI of 10 TCID<sub>50</sub> per cell (6). The complete abolition of viral infectivity by UV light  
115 treatment was confirmed by titration assay with undiluted viral suspension. We also checked  
116 that UV inactivation did not modify virus adsorption on cells, by comparing the binding  
117 efficiency of infectious and UV light-treated PV labeled with [<sup>35</sup>S]methionine (data not  
118 shown). Akt phosphorylation was induced in IMR5 cells 30 minutes after the addition of UV-  
119 inactivated PV, with an efficiency similar to that observed with infectious PV (Fig. 2). Thus,  
120 PV-cell receptor interaction alone is sufficient to induce Akt phosphorylation in the absence  
121 of viral replication.

122

### 123 **PI3K/Akt signaling pathway limits the amplitude of Bax activation, cytochrome *c*** 124 **release and apoptosis in PV-infected IMR5 cells**

125 We assessed the role of the PI3K/Akt signaling pathway in regulating the  
126 mitochondrial pathway of apoptosis in PV-infected cells, by blocking PI3K activation with  
127 wortmannin. The mitochondrial pathway is regulated by members of the Bcl-2 family,  
128 including the proapoptotic protein Bax, which promotes the release of cytochrome *c*. Bax-  
129 mediated cell death involves several well-controlled steps, including a conformational change  
130 resulting in exposure of the NH<sub>2</sub>-terminus. Mock- and PV-infected IMR5 cells were left  
131 untreated or were treated with 100 nM wortmannin for 2 h before PV infection. The

132 concentration of the inhibitor was maintained throughout both PV adsorption and replication.  
133 At 8 h p.i., a time point at which Bax activation is known to occur in PV-infected cells (6),  
134 whole-cell lysates were prepared in a lysis buffer containing 1% of the zwitterionic detergent  
135 CHAPS, which has no effect on Bax conformation (22). Bax was then immunoprecipitated  
136 with an anti-Bax antibody (6A7) that specifically recognizes Bax protein with an exposed  
137 NH2 terminus. The Bax protein immunoprecipitated from mock- and PV-infected cells was  
138 visualized by Western blotting (Fig. 3A, top). No activated Bax was detected in the  
139 immunoprecipitates from mock-infected cells. Consistent with our previous report (6), Bax  
140 was immunoprecipitated with the 6A7 antibody at 8 h p.i., indicating that PV infection was  
141 responsible for inducing the change in Bax conformation. Wortmannin enhanced Bax  
142 activation in IMR5-infected cells, without affecting the total amount of Bax (Fig. 3A,  
143 bottom). The effect of wortmannin on cytochrome *c* efflux from the mitochondria of PV-  
144 infected cells was also investigated. Whole-cell extracts from mock- or PV-infected cells were  
145 fractionated at 8 h p.i., to separate the cytosolic fraction from the heavy membrane fraction,  
146 including mitochondria, as previously described (6). Cytochrome *c* release was analyzed by  
147 Western blotting the cytosolic fraction. Much more cytochrome *c* was released in response to  
148 PV infection in cells treated with wortmannin than in untreated infected cells (Fig. 3B). These  
149 results suggest that PI3K may inhibit Bax-dependent MOMP during the PV infection of  
150 IMR5 cells.

151 We investigated the possible involvement of PV-mediated PI3K activation in the  
152 inhibition of apoptosis, by analyzing the kinetics of apoptosis in mock infected and infected  
153 cells treated or not treated with the specific PI3K inhibitor, wortmannin (Fig. 3C). Adherent  
154 and detached cells were harvested at the indicated times p.i. and apoptosis was analyzed by  
155 assessing chromatin condensation and fragmentation by flow cytometry after acridine orange  
156 (AO) nuclear dye staining, as previously described (6). We found that levels of PV-induced

157 apoptosis were higher in infected cells treated with wortmannin than in untreated infected  
158 cells. To confirm the role of PI3K/Akt signaling pathway in limiting PV-induced apoptosis,  
159 we down-regulated Akt expression with a specific siRNA. Western blot analysis with a  
160 specific antibody showed that Akt expression in IMR5 cells transfected with Akt siRNA was  
161 significantly weaker than in cells transfected with a nontargeted control siRNA (Fig. 3D, left).  
162 As expected, following PV infection (8 h p.i.), apoptosis levels were higher in Akt  
163 knockdown cells than in nontargeted control siRNA-transfected cells (Fig. 3D, right). These  
164 results suggest that PI3K/Akt pathway plays a role in inhibiting the mitochondrial apoptotic  
165 pathway in PV-infected IMR5 cells.

166

#### 167 **The PI3K/Akt signaling pathway does not affect PV growth, but delays PV release**

168 We evaluated the effects of PI3K/Akt signaling on the amount of total virus produced  
169 in IMR5 cells, by determining the kinetics of total virus yield by TCID<sub>50</sub> assays in the  
170 presence or absence of wortmannin. PI3K/Akt pathway inhibition had no effect on the total  
171 amount of virus produced (Fig. 4). As PV-induced apoptosis levels were higher in infected  
172 cells treated with wortmannin than in untreated infected cells, we assessed the possible effects  
173 of the increase in apoptosis levels on externalization of the virus. Viruses were released earlier  
174 in the presence of wortmannin (Fig. 4). Thus, PI3K/Akt seems to delay viral release without  
175 affecting virus production.

176

#### 177 **The PI3K/Akt signaling pathway limits JNK activation in PV-infected cells**

178 We have shown that Bax-dependent activation of the mitochondrial pathway of  
179 apoptosis is mediated by early JNK activation (6). JNK activation peaks 30 min p.i and then  
180 decreases in IMR5 neuroblastoma cells. It is possible that the PI3K/Akt pathway down  
181 regulates the JNK pathway, as recently reported in nonviral models (2, 23).



182 We assessed the effects of PI3K/Akt on JNK activation in PV-infected cells, by  
183 treating cells with wortmannin. JNK activation was investigated 30 min p.i., by Western  
184 blotting whole-cell lysates with an antibody against phosphorylated forms of JNK (Fig. 5A).  
185 As expected, phosphorylated JNK was detected 30 min p.i.. Larger amounts of  
186 phosphorylated JNK were found in infected cells treated with wortmannin than in untreated  
187 cells. Thus, activation of the PI3K/Akt pathway limits JNK activation in PV-infected IMR5  
188 cells.

189

### 190 **JNK activation is limited by the Akt-mediated phosphorylation of ASK1 in PV-infected** 191 **cells**

192 We then examined the possibility that a kinase, upstream of JNK, was inhibited by  
193 Akt, causing the observed limited JNK phosphorylation in PV-infected cells. Apoptosis  
194 signal-regulating kinase 1 (ASK1) has been shown to be a key regulator of the JNK pathway  
195 amenable to inhibition by Akt-mediated phosphorylation at Ser83 in nonviral systems (2, 23).  
196 We assessed the possible involvement of ASK1 in JNK activation in PV-infected IMR5 cells,  
197 by down-regulating ASK1 expression using specific siRNA (37). Western blot analysis with a  
198 specific antibody showed that ASK1 levels were significantly lower in IMR5 cells transfected  
199 with ASK1 siRNA than in cells transfected with a nontargeted control siRNA (Fig. 5B, left).  
200 Moreover, following PV infection, JNK activation in ASK1 knockdown cells was weaker  
201 than in cells transfected with the nontargeted control siRNA (Fig. 5B, right). Thus, ASK1  
202 plays an important role in JNK activation following PV infection in IMR5 cells.

203 We then investigated the possible limitation of ASK1 activity by PI3K/Akt-mediated  
204 phosphorylation at Ser83 in PV-infected cells. The kinetics of ASK1 phosphorylation at  
205 Ser83 in PV-infected cells was analyzed by Western blotting with a specific antibody against  
206 phosphorylated ASK1 (Fig. 5C). A transient increase in the level of ASK1 phosphorylation

207 was evident 30 minutes after infection, consistent with the pattern of Akt activation.  
208 Furthermore, treatment of the cells with the PI3K inhibitor wortmannin abolished the increase  
209 in ASK1 phosphorylation in PV-infected cells (Fig. 5D). Altogether, these results indicate  
210 that the PI3K/Akt pathway negatively regulates JNK activation by phosphorylating and  
211 inactivating ASK1 in PV-infected IMR5 cells.

212 This study provides evidence that the early PI3K/Akt survival pathway limits the  
213 magnitude of PV-induced JNK activation and cell death in IMR5 cells. We previously  
214 showed that PV-cell receptor interaction alone is sufficient to induce JNK phosphorylation, as  
215 for Akt activation. However, we also showed that JNK phosphorylation is necessary, but not  
216 sufficient, to trigger apoptosis that seems to require the active replication of PV. As  
217 previously reported by Agol's group (1, 39), several different courses of events may influence  
218 apoptosis in PV-infected cells between 30 min and 6-8 h p.i. These events may involve the  
219 interplay between cellular and viral proteins (7, 15, 20, 33, 34, 42). Thus, the early PI3K/Akt  
220 survival pathway seems to act upstream of this unidentified interplay. The PI3K/Akt pathway  
221 has been shown to play an antiapoptotic role in several viral infections (11). However, this is  
222 the first report, to our knowledge, of the limitation of JNK activation by PI3K/Akt mediating  
223 a survival pathway during a viral infection. We have also shown that the cross-talk between  
224 the PI3K/Akt and JNK pathways involved ASK1 inhibition. In poliomyelitis, this survival  
225 pathway may limit the spread of PV-induced damage in the CNS.

226

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365

## 366 **FIGURE LEGENDS**

367

368 **Fig. 1. PV induces early Akt phosphorylation in a PI3K-dependent manner in IMR5**  
369 **neuroblastoma cells**

370 **(A)** Kinetics of Akt activation in PV-infected neuronal cells. (Top) Akt activation was  
371 analyzed in whole-cell lysates at the indicated times p.i., by Western blotting with a specific  
372 anti-phospho (Ser473)-Akt antibody (Cell Signaling). Whole-cell lysates from mock-infected  
373 cells were analyzed at 30 min (first lane) and 240 min (last lane) post mock-infection,  
374 respectively. Blots were then stripped and reprobed with an antibody recognizing all forms of  
375 Akt (Cell Signaling), to confirm equal protein loading. (Bottom) Western blot analyses of Akt  
376 activation 30 min p.i.. (Left) Cells were infected with viral stock (PV) or viral stock depleted  
377 of PV (PV<sup>depleted</sup>) with anti-PV antibody. (Right) Cells were infected with viral stock (PV) or  
378 CsCl-purified PV (PV<sup>purified</sup>). **(B)** Inhibition of Akt phosphorylation during PV infection in



379 IMR5 cells treated with the PI3K inhibitor, wortmannin (Calbiochem, 100 nM and 500 nM).  
380 (Top) Cells were incubated or not incubated with the PI3K inhibitor for 2 h before PV  
381 infection, and the concentration of the inhibitor was maintained during the adsorption period  
382 and throughout PV infection. Levels of phospho (Ser473)-Akt in whole-cell lysates were  
383 determined by Western blotting, 30 min p.i.. Blots were then stripped and reprobed with an  
384 antibody recognizing all forms of Akt, to confirm equal protein loading. (Bottom) Mock- and  
385 PV-infected IMR5 cells (30 min p.i.), treated or not treated with wortmannin (100 nM), were  
386 stained for immunofluorescence with a specific antibody against phospho (Ser473)-Akt and a  
387 secondary, fluorescein isothiocyanate-conjugated antibody (green) (middle panel). Nuclei  
388 were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) (left panel). Merge, overlay of  
389 the DAPI image with the anti-phospho (Ser473)-Akt image (right panel).

390

391 **Fig. 2. UV-inactivated PV induces early Akt activation in IMR5 cells.** Akt activation was  
392 analyzed by Western blotting whole-cell lysates from cells infected with infectious or UV-  
393 inactivated PV (30 min p.i.) with specific anti-phospho (Ser473)-Akt antibody. Blots were  
394 then stripped and reprobed with an antibody recognizing all forms of Akt, to confirm equal  
395 protein loading.

396

397 **Fig. 3. Inhibition of the PI3K/Akt signaling pathway enhances PV-induced apoptosis in**  
398 **IMR5 cells**

399 **(A)** Enhancement of Bax activation in PV-infected cells treated with wortmannin. (Top) Cells  
400 were uninfected or infected with PV (8 h p.i.) in the presence or absence of wortmannin (100  
401 nM). Cells were lysed in immunoprecipitation buffer. Conformationally active Bax protein  
402 was immunoprecipitated (IP) with anti-Bax 6A7 antibody (Santa-Cruz) and precipitates were  
403 immunoblotted with anti-Bax antibody. The asterisk indicates immunoglobulin light chains.

404 (Bottom) Whole-cell lysates not incubated with 6A7 antibody were similarly tested for total  
405 Bax by immunoblotting with a specific antibody (Upstate) to check that the amounts of Bax  
406 protein in samples before immunoprecipitation were equivalent. Actin was used as a control  
407 for protein loading. **(B)** Greater cytochrome *c* (Cyt *c*) release in PV-infected cells treated with  
408 wortmannin. Cytochrome *c* release was analyzed in cytosolic fractions of mock-infected and  
409 PV-infected IMR5 cells (8 h p.i.) treated or not treated with wortmannin (100 nM) by  
410 Western blotting with a specific antibody (BD Pharmingen). Actin was used as a protein  
411 loading control. Protein levels were determined by densitometry and plotted as ratios relative  
412 to the actin levels. **(C)** Enhancement of apoptosis in PV-infected cells treated with  
413 wortmannin. Mock-infected and PV-infected IMR5 cells treated (black) or not treated (light  
414 gray) with wortmannin (100 nM) were analyzed at the indicated times p.i. by flow cytometry  
415 after Acridine Orange (AO, Molecular Probes) staining, and the increase (*n*-fold) in apoptosis  
416 was calculated as the ratio of the percentage of PV-infected IMR5 cells that were apoptotic to  
417 the percentage of mock-infected cells that were apoptotic. Data are means from three  
418 independent experiments. Error bars represent the standard errors of the means. \*,  $P < 0.05$  by  
419 Student's *t* test comparing untreated IMR5 cells to treated IMR5 cells. **(D)** Higher levels of  
420 apoptosis were observed after the knockdown of Akt expression in PV-infected cells. (Left)  
421 IMR5 cells were transfected with Akt siRNA (Cell Signaling) or nontargeted control siRNA  
422 (Cell Signaling) or left untreated. Akt protein was then assayed by immunoblotting with  
423 extracts from nontargeted control siRNA-transfected, Akt siRNA-transfected or untreated  
424 cells. Actin was used as a protein loading control. (Right) Cells were uninfected or were  
425 infected (8 h p.i.) with PV 72 h after transfection, and cells were analyzed by flow cytometry  
426 after AO staining and the increase (*n*-fold) in apoptosis was calculated as the ratio of the  
427 percentage of PV-infected IMR5 cells that were apoptotic to the percentage of mock-infected  
428 cells that were apoptotic. Data are means from three independent experiments. Error bars

429 represent the standard errors of the means. \*,  $P < 0.05$  by Student's *t* test comparing untreated  
430 IMR5 cells to treated IMR5 cells.

431

432 **Fig. 4. Effect of PI3K/Akt signaling inhibition on PV growth and externalization**

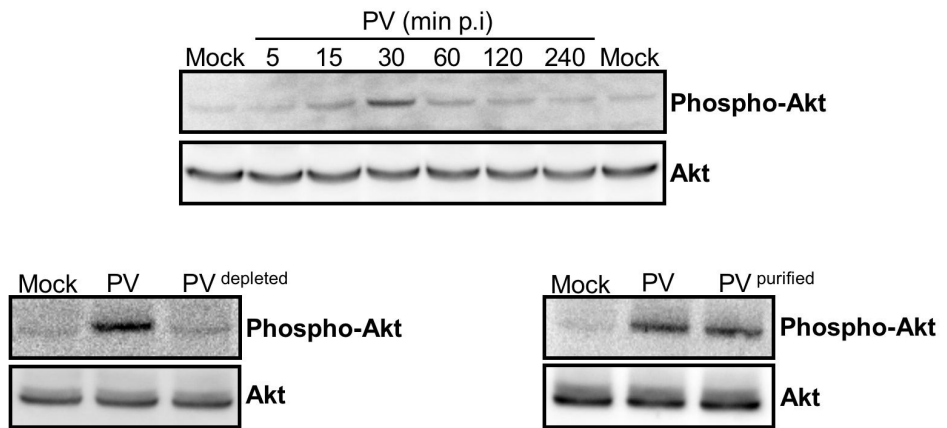
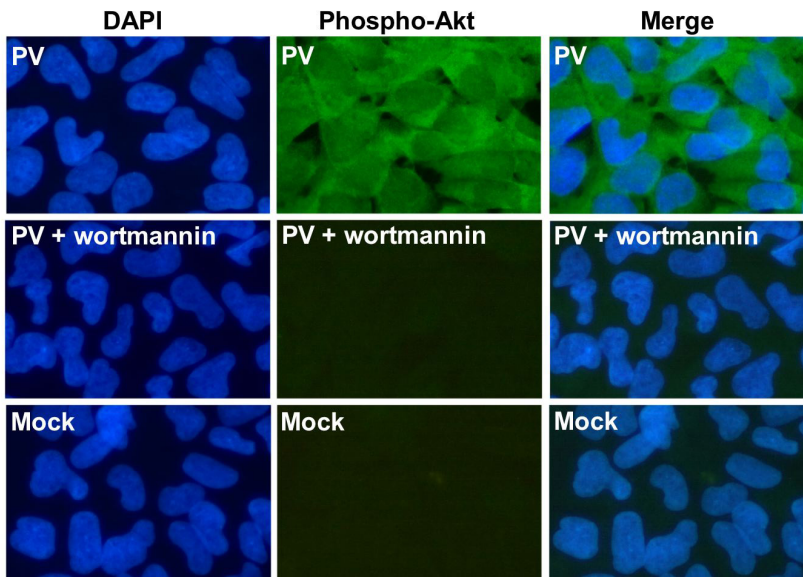
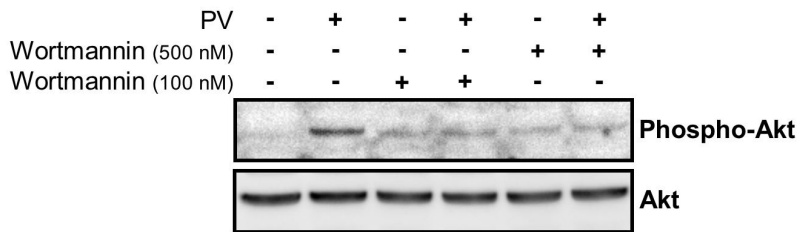
433 IMR5 cells were infected with PV in the presence or absence of wortmannin (100 nM). Total  
434 virus yield (extracellular and intracellular) was determined by TCID<sub>50</sub> assay at the indicated  
435 times after three cycles of freezing and thawing to release intracellular viruses. Extracellular  
436 virus titer was determined from the supernatant of PV-infected cells at the indicated times  
437 after the removal of detached cells by centrifugation. Each point represents the mean virus  
438 titers for two independent experiments. Standard errors of the mean are indicated, \* $P < 0.05$  by  
439 a Student *t* test comparing untreated to treated IMR5 cells.

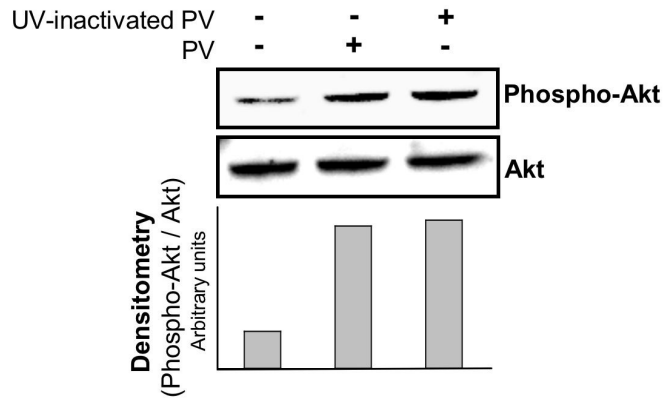
440

441 **Fig. 5. The PI3K/Akt signaling pathway limits JNK activation by promoting ASK1**  
442 **phosphorylation in PV-infected IMR5 cells**

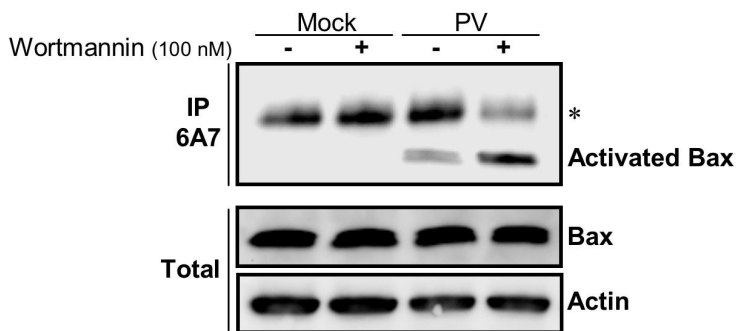
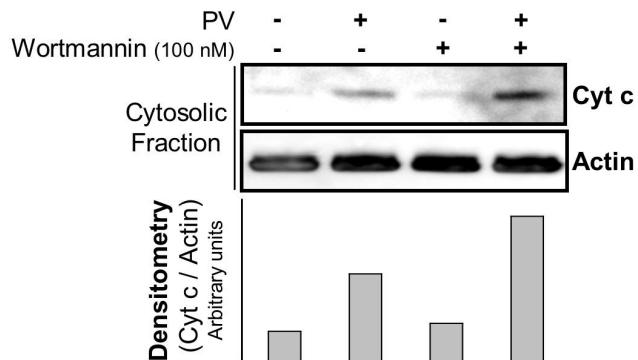
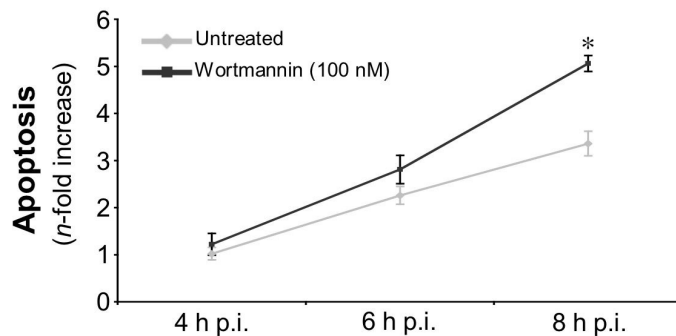
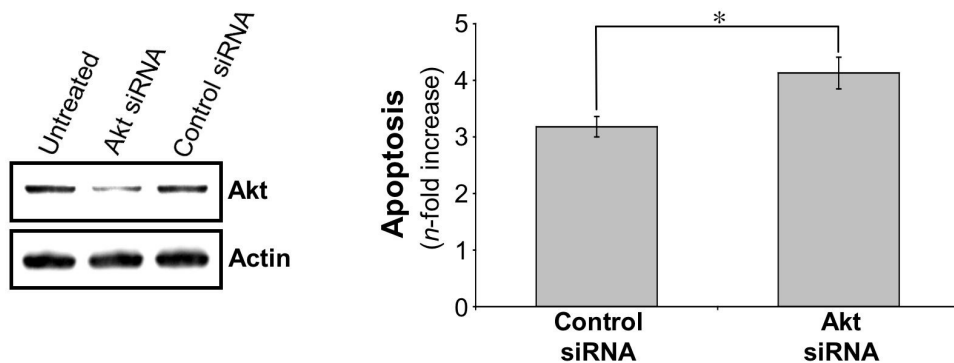
443 **(A)** JNK activation levels are higher in PV-infected cells treated with wortmannin. Cells were  
444 uninfected or infected with PV (30 min p.i.) in the presence or absence of wortmannin (100  
445 nM). JNK activation was analyzed in whole-cell lysates, by Western blotting with a specific  
446 anti-phospho (Thr183/Tyr185)-JNK (p46 [JNK1] and p54 [JNK2/3]) antibody, as previously  
447 described (6). Blots were then stripped and re probed with an antibody recognizing all forms  
448 of JNK, to confirm equal protein loading. **(B)** Inhibition of JNK activation after the  
449 knockdown of ASK1 expression in PV-infected IMR5 cells. (Left) IMR5 cells were  
450 transfected with ASK1 siRNA (37) or nontargeted control siRNA (Cell Signaling) or left  
451 untreated. ASK1 protein was then assayed by immunoblotting with extracts from nontargeted  
452 control siRNA-transfected, ASK1 siRNA-transfected or untreated cells. Actin was used as a  
453 protein loading control. (Right) Untreated, nontargeted control and ASK1 siRNA transfected

454 IMR5 cells were uninfected or infected with PV. JNK activation was analyzed (30 min p.i.) in  
455 whole-cell lysates, by Western blotting with a specific anti-phospho (Thr183/Tyr185)-JNK  
456 antibody. Blots were then stripped and reprobed with an antibody recognizing all forms of  
457 JNK, to confirm equal protein loading. Phosphorylated JNK protein levels were determined  
458 by densitometry and plotted as the ratios, relative to the levels of total JNK. Phosphorylated  
459 JNK levels following PV infection in untreated cells were taken as 100%. Data are means  
460 from three independent experiments. Error bars represent the standard errors of the means. \*,  
461  $P < 0.05$  by Student's *t* test comparing nontargeted control siRNA-transfected IMR5 cells to  
462 ASK1 transfected IMR5 cells. **(C)** Phosphorylation of ASK1 in PV-infected neuronal cells.  
463 ASK1 phosphorylation was analyzed in whole-cell lysates at the indicated times p.i., by  
464 Western blotting with a specific anti-phospho (Ser83)-ASK1 antibody (Cell Signaling). Blots  
465 were then stripped and reprobed with an antibody recognizing all forms of ASK1 (Cell  
466 Signaling), to confirm equal protein loading. **(D)** Inhibition of PV-induced ASK1  
467 phosphorylation by the PI3K/Akt pathway inhibitor wortmannin. Cells were uninfected or  
468 infected with PV in the presence or absence of wortmannin (100 nM). ASK1 phosphorylation  
469 was analyzed (30 min p.i.) in whole-cell lysates by Western blotting with a specific anti-  
470 phospho (Ser83)-ASK1 antibody. Blots were then stripped and reprobed with an antibody  
471 recognizing all forms of ASK1, to confirm equal protein loading. Phosphorylated ASK1  
472 protein levels were determined by densitometry, and plotted as the ratios relative to the levels  
473 of total ASK1.

**A****B****Figure 1**



**Figure 2**

**A****B****C****D****Figure 3**

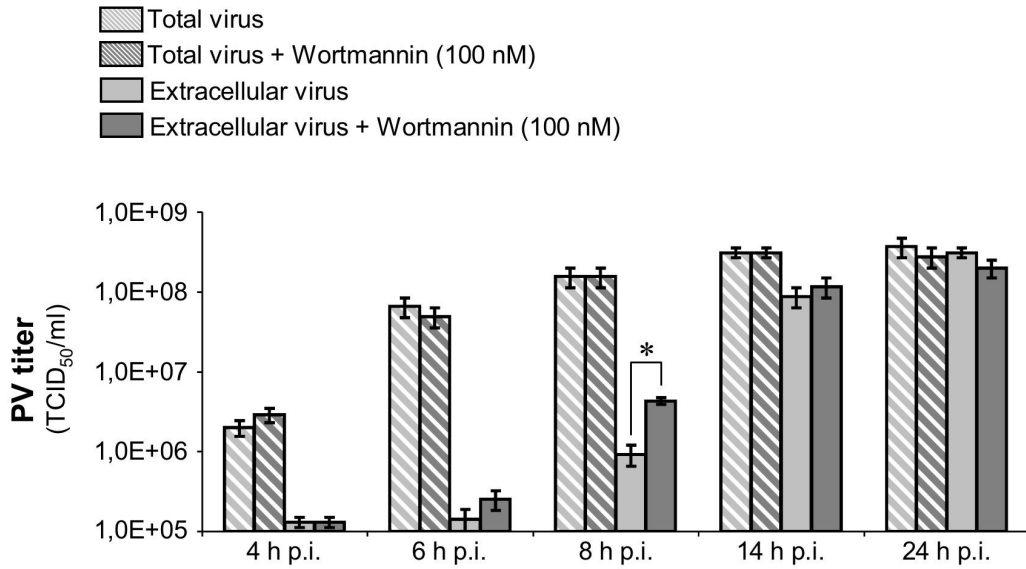
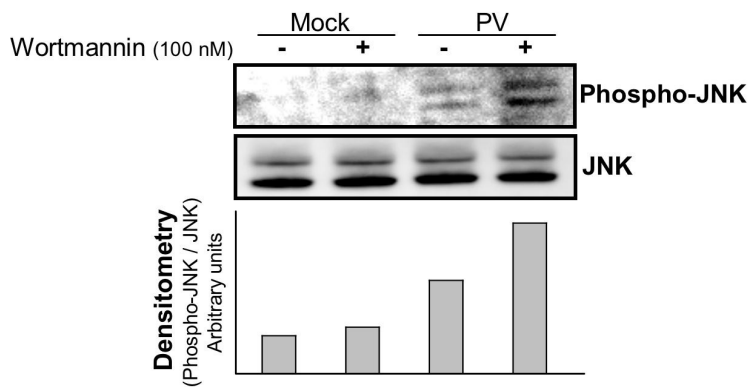
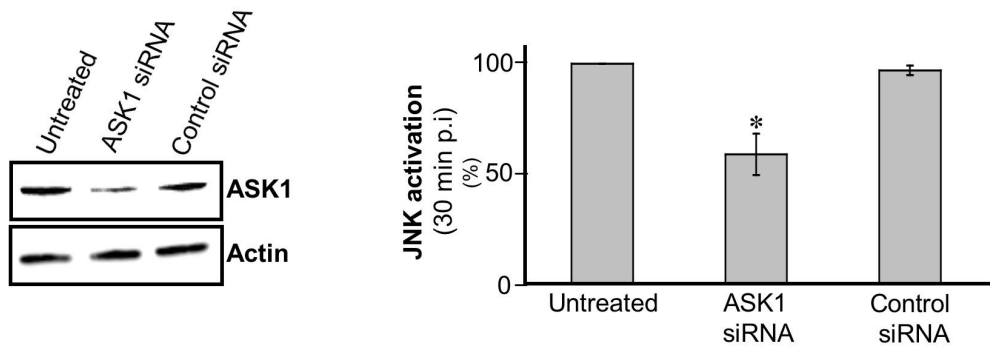
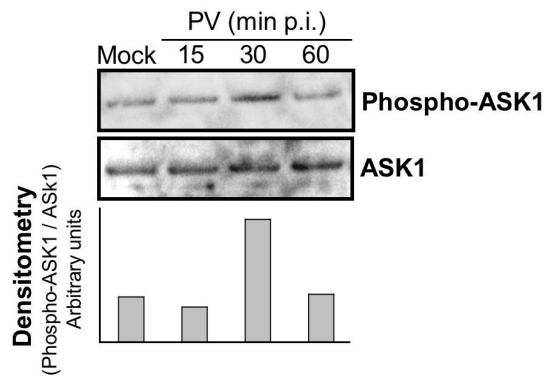
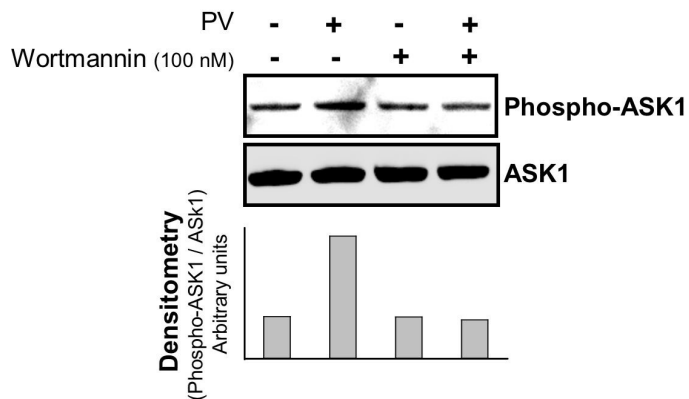


Figure 4



**A****B****C****D****Figure 5**