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Alphaherpesviral US3 Kinase Induces Cofilin Dephosphorylation To Reorganize the Actin Cytoskeleton

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The conserved alphaherpesviral serine/threonine kinase US3 causes dramatic actin rearrangements, associated with increased viral spread. Here, we show that US3 of pseudorabies virus (PRV) leads to activation (dephosphorylation) of the central actin regulator cofilin. A mutation that impairs US3 kinase activity and the group I p21-activated kinase inhibitor IPA-3 inhibited US3-mediated cofilin activation. Additionally, expression of phosphomimetic S3D cofilin significantly suppressed the ability of US3 to cause cell projections and cell rounding. In conclusion, the US3 kinase of PRV leads to activation (dephosphorylation) of cofilin, and cofilin contributes to US3-mediated actin rearrangements.

The US3 kinase is conserved among *Alphaherpesvirinae*. We and others have shown that this kinase induces dramatic rearrangements of the actin cytoskeleton, including disassembly of actin stress fibers and formation of cellular projections, which are associated with increased viral spread in cell culture (1–7). For the alphaherpesvirus pseudorabies virus (PRV), we previously reported that the US3-induced changes in the actin cytoskeleton are mediated through p21-activated kinases (PAKs), central regulators in RhoGTPase signaling (8). Apart from the involvement of PAKs, relatively little is known about the factors contributing to US3-mediated actin rearrangements.

Cofilin, a member of the actin depolymerizing factor (ADF)/cofilin family, is a central player in actin dynamics known to be activated through dephosphorylation on serine residue 3 (S3) (9). Phosphorylation and dephosphorylation of cofilin at S3 is complexly regulated by multiple kinases and phosphatases (10). Increasing evidence indicates that cofilin constitutes an important cellular target affected by both bacterial and viral infections (11–15). With regard to alphaherpesviruses, herpes simplex virus 1 (HSV-1) has been reported to induce a cell-type-dependent upregulation of cofilin levels and modulation of cofilin activity (16, 17). This may affect viral replication, although the underlying mechanism is unclear (15, 16). The best-characterized viral modulation of cofilin activity has been documented for HIV, which triggers cofilin S3 phosphorylation and thus inactivation through gp120-mediated activation of the Rac-PAK-LIMK pathway, which is involved in initiation of infection of CD4 T cells (17). HIV Nef also leads to cofilin inactivation through the activity of PAK2, thereby restricting migration of infected T lymphocytes (14). On the other hand, HIV-mediated activation of cofilin has also been described to affect initiation of infection (17–19).

In the current report, we investigated whether the US3 protein of the alphaherpesvirus PRV affects cofilin phosphorylation, and, if so, whether this contributes to the US3-mediated effects on the actin cytoskeleton.

US3 is required for PRV-mediated suppression of cofilin phosphorylation. We determined whether US3 modulates the activity of cofilin through altered phosphorylation at the critical S3 residue in cofilin. ST cells (seeded at 150,000 cells/ml, cultured as described in reference 20) were inoculated with the previously described isogenic NIA3 strains wild-type (WT) PRV or US3null PRV (containing a translational stop codon in US3) or a revertant virus of the latter (21). At 6 h postinoculation (hpi), cells were subjected to Western blotting (WB). Antibodies used were directed against S3 phospho-cofilin (Santa Cruz; sc-12912), total cofilin (Santa Cruz; sc-42824), US3 (kindly provided by LeighAnne Olsen and Lynn Enquist, Princeton University), and the viral membrane protein gE (18E8) (22). Band intensity was measured with the "Analyze gels" option in ImageJ, and phospho-S3 cofilin levels were normalized to mock levels. Figure 1 shows that, compared to mock-infected ST cells, WT and US3rescue PRV infection led to a strong decrease in S3 cofilin phosphorylation, in contrast to US3null PRV (Fig. 1A and B). Phospho-S3 cofilin levels in US3null PRV-infected cells were even increased, albeit not significantly, compared to those of mock-infected cells. In line with the early kinetics of US3 expression, the decrease in phospho-S3 cofilin could be observed already early in infection (from 4 hpi onward) (Fig. 1C). The ability of US3 to modulate cofilin activity levels is underscored by the fact that transfection of a WT US3-encoding construct in ST cells was sufficient to suppress phospho-S3 cofilin levels, as shown in Fig. 1D and E. Transfection with a control plasmid encoding red fluorescent protein DsRed (kindly provided by R. Y. Tsien, UCSD, La Jolla, CA) was used as a control (23). Hence, US3 leads to suppressed phospho-S3 cofilin levels in infected and transfected ST cells.

The kinase activity of US3 is required to suppress phosphorylation of cofilin. To assess the involvement of the kinase activity of US3 in suppressing cofilin phosphorylation in ST cells, cells were inoculated with a previously described PRV strain expressing a kinase-inactive US3 protein, containing a point mutation (D223A) in the catalytic base required for phosphotransfer (PRV-GS976) (24, 25). At 6 hpi, phospho-S3 cofilin, total cofilin, US3, and gE levels were evaluated by WB (Fig. 2A

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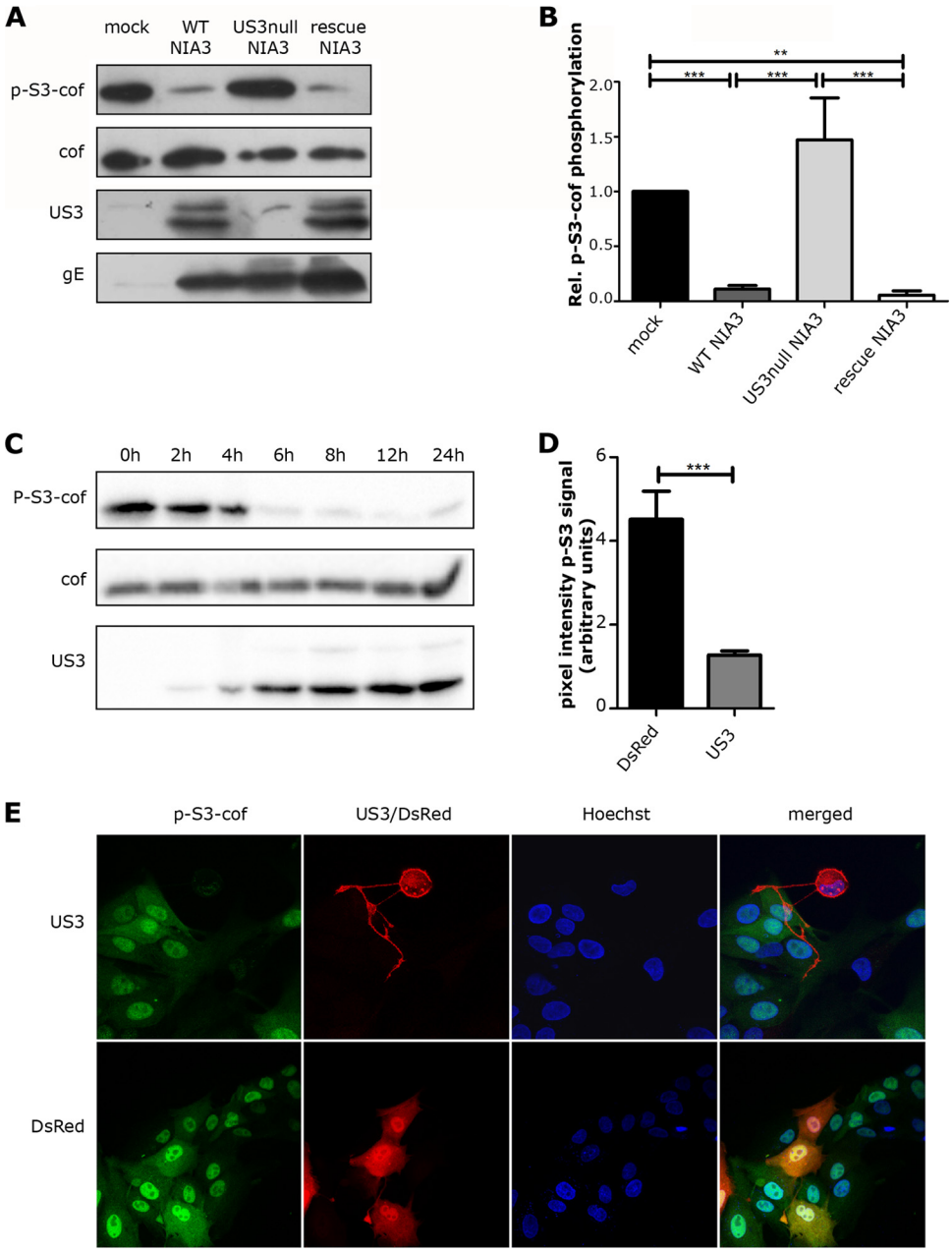

FIG 1 PRV infection leads to a US3-dependent suppression in S3 cofilin phosphorylation. (A) ST cells were mock inoculated or inoculated (MOI of 10) with WT PRV, US3null PRV, or US3rescue PRV. At 6 hpi, total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, US3, and gE. (B) Relative cofilin phosphorylation levels based on the phospho-S3 cofilin/cofilin ratio (with mock infection set to 1) are represented as means + standard errors of the means of data from three independent experiments, with ** indicating *P* values of <0.01 and *** indicating *P* values of <0.001. (C) ST cells were inoculated with WT PRV (MOI of 10) and lysed at 0 h, 2 h, 4 h, 6 h, 8 h, 12 h, or 24 h postinfection. Total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, and US3. (D and E) ST cells were transfected with US3 or with a control plasmid encoding DsRed (23) and stained for US3 and phospho-S3 cofilin. Panel D shows quantification of fluorescein isothiocyanate (FITC) (p-S3-cof) pixel intensities of 8 randomly chosen US3- or control plasmid-transfected cells, which were determined using ImageJ. Data shown represent means + standard errors of the means, with * indicating *P* values of <0.05.

and B). The PRV strain Becker expressing a kinase-inactive US3, unlike isogenic wild-type PRV (multiplicity of infection [MOI] of 10; PRV-GS847), did not suppress phospho-S3 levels of cofilin. A rescue strain in which the D223A mutation in US3 was restored (PRV-GS3000) (24, 25) acted like the WT virus and induced a strong suppression in cofilin phosphorylation. As observed for US3null PRV (Fig. 1), infection with PRV encoding kinase-inactive US3 resulted in increased phosphorylation of cofilin compared to that of mock-infected cells. Hence, the ability of US3 to suppress S3 phosphorylation of cofilin in ST cells relies on its kinase activity.

Interestingly, infection with US3null PRV or D223A US3 PRV

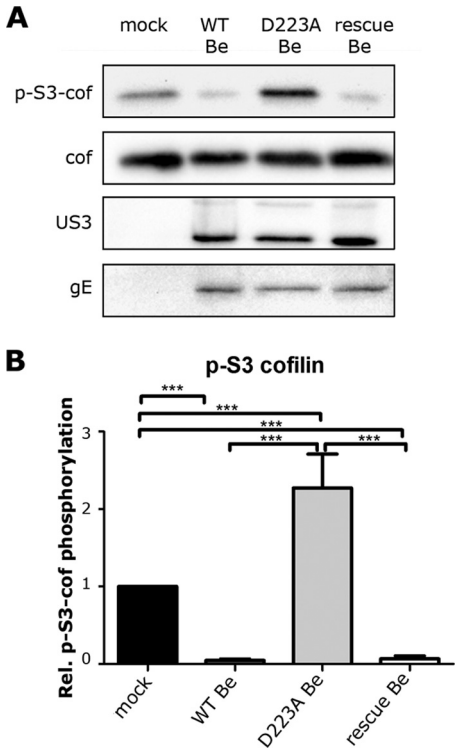

FIG 2 The kinase activity of US3 is required to suppress phosphorylation of cofilin. (A) ST cells were mock inoculated or inoculated (MOI of 10) with WT PRV, kinase-inactive D223A US3 PRV, or D223Arescue PRV. At 6 hpi, total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, US3, and gE. (B) Means + standard errors of relative cofilin phosphorylation levels from three independent experiments, with *** indicating P values of <0.001.

resulted in increased phospho-S3 cofilin levels compared to those of mock-infected cells (Fig. 1 and 2). One hypothetical way to explain this may be that infection leads to cofilin inactivation (S3 phosphorylation) and that US3 activity counteracts this and even reduces phospho-S3 cofilin levels below normal levels. Why would infection lead to increased phospho-S3 cofilin levels? Viral infection is known to lead to a stress response in cells (26–28), which may perhaps be involved in increased phosphorylation of cofilin. Indeed, other cellular stress stimuli have been reported to lead to increased S3 cofilin phosphorylation, including heat shock (29), fluid shear stress (30, 31), and scavenging of reactive oxygen species (32). It will be interesting to investigate the potential biological consequences of increased levels of phospho-S3 cofilin during US3null PRV infection for both virus and cell.

A constitutively inactive, S3D phosphomimetic cofilin variant interferes with US3-mediated cell rounding and cell projections. The experiments described above indicate that US3 leads to substantial S3 cofilin dephosphorylation, a hallmark of cofilin activation (9). If this cofilin activation is important for PRV US3-induced actin rearrangements, one would expect that overexpression of a constitutively inactive (phosphomimetic) S3D cofilin mutant will interfere with US3-mediated actin rearrangements, whereas overexpression of wild-type cofilin or a constitutively active S3A cofilin mutant should not. Likewise, overexpression of S3D (but not S3A) cofilin has been reported to suppress the formation of long actin-containing dendritic cell protrusions in hippocampal neurons (33).

To assess this, ST cells were cotransfected with US3 and constructs expressing previously described green fluorescent protein (GFP) fusions of wild-type cofilin, S3D cofilin, or S3A cofilin (34). At 24 h posttransfection, cells were stained with anti-US3 antibody and scored for US3-mediated effects on the actin cytoskeleton. In brief, 200 randomly chosen transfected cells per condition were scored for cell rounding (actin stress fiber disassembly) and cell projection formation. Phosphomimetic S3D cofilin, but not wild-type or S3A cofilin, significantly suppressed the ability of US3 to induce actin rearrangements in ST cells (Fig. 3). Overexpression of either WT or S3D cofilin on itself did not cause apparent changes in cell morphology. Overexpression of S3A cofilin on itself did not lead to obvious cell rounding but did induce cell projections that were shorter and less branched than observed upon transfection of US3 (data not shown). Notwithstanding the apparent colocalization of cofilin with US3 in some of the immunofluorescence images, immunoprecipitation experiments were not indicative for a direct interaction between US3 and cofilin (data not shown). In conclusion, expression of phosphomimetic S3D cofilin in ST cells interferes with the ability of US3 to induce actin rearrangements.

Group I PAKs are involved in the US3-mediated dephosphorylation of cofilin. The ability of PRV US3 to induce actin rearrangements has been shown to depend on the ability of US3 to phosphorylate and thereby activate group I PAKs (8). As a consequence, the group I PAK inhibitor IPA-3 is able to inhibit US3-mediated actin rearrangements in ST cells (25, 35). We investigated whether IPA-3 is also capable of reverting the observed US3-mediated suppression of S3 cofilin phosphorylation. To this end, ST cells were either mock inoculated or inoculated with WT PRV in the absence or presence of 33 μM IPA-3, used as described before (21). At 6 hpi, cells were lysed and phospho-S3 cofilin, total cofilin, and US3 levels were evaluated. The addition of IPA-3 restored the phospho-S3 cofilin signal in PRV-infected cells (Fig. 4B), while it did not influence phospho-S3 cofilin levels in mock-infected cells (Fig. 4A). Hence, the use of an inhibitor of group I PAK activity in ST cells interferes with the US3-mediated suppression of S3 cofilin phosphorylation.

This is in apparent contradiction with studies in HIV, where virus-induced activation of PAK2 (a group I PAK member) leads to S3 cofilin phosphorylation and thus inactivation in Jurkat cells (14, 17, 36). Nevertheless, ambiguity exists in the literature as to whether PAK activation leads to cofilin phosphorylation or dephosphorylation at S3. On the one hand, PAK activity has been associated with cofilin phosphorylation, mainly because LIM kinase isoforms are important downstream substrates of PAK that can lead to phosphorylated cofilin (37–40). On the other hand, more recently, increasing evidence indicates that group I PAK activity may also signal to several of the phosphatases, like PP2A, chronophin (CIN), and/or the slingshot (SSH) family, that are known to dephosphorylate and activate cofilin (41–44). Most likely, cell-type-specific or environmental factors may influence the outcome of PAK activation on cofilin activity (35, 45–48). Future research aimed at further dissecting the mechanistic details of US3-PAK-mediated cofilin dephosphorylation will further clarify the other molecular players in this pathway and may therefore generate important cell biological insights on PAK-mediated cofilin regulation.

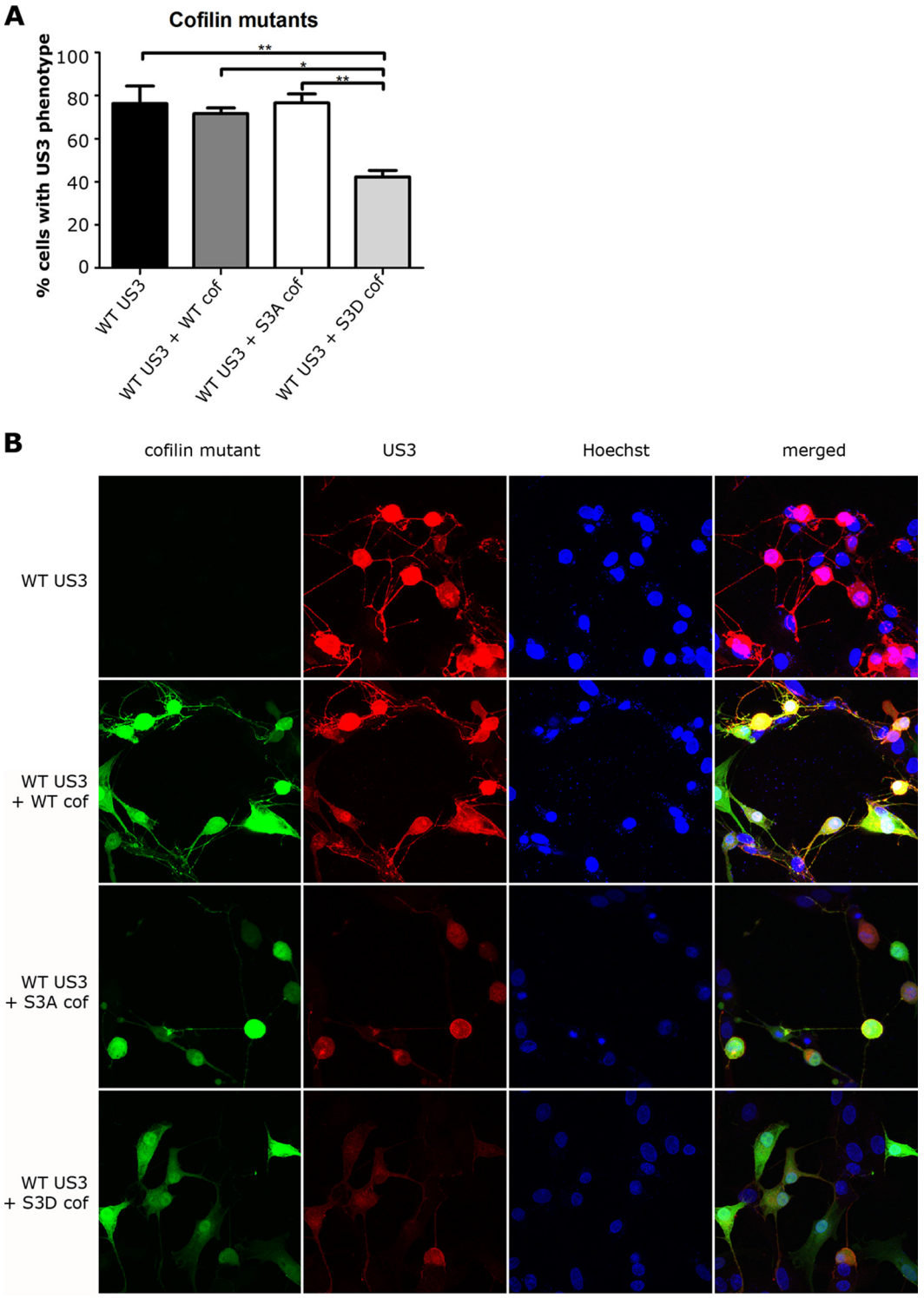

FIG 3 Overexpression of S3D phosphomimetic cofilin interferes with the ability of US3 to cause cell rounding and cell projections. (A and B) ST cells were transfected with US3 encoding plasmid or cotransfected with plasmids encoding US3 and GFP-tagged WT cofilin, S3A cofilin, or S3D cofilin. At 24 h posttransfection, cells were fixed and stained for US3 and nuclei and analyzed for expression of cofilin (GFP; green) and US3 (red). Panel A shows the percentage of transfected cells displaying actin rearrangements, as assessed by cell rounding and the formation of cell projections (means + standard errors of the means; data from three independent experiments), with * indicating P values of <0.05 and ** indicating P values of <0.01. Small blue dots in panel B represent leftover plasmid DNA-containing transfection reagent in cells and on the cover glass.

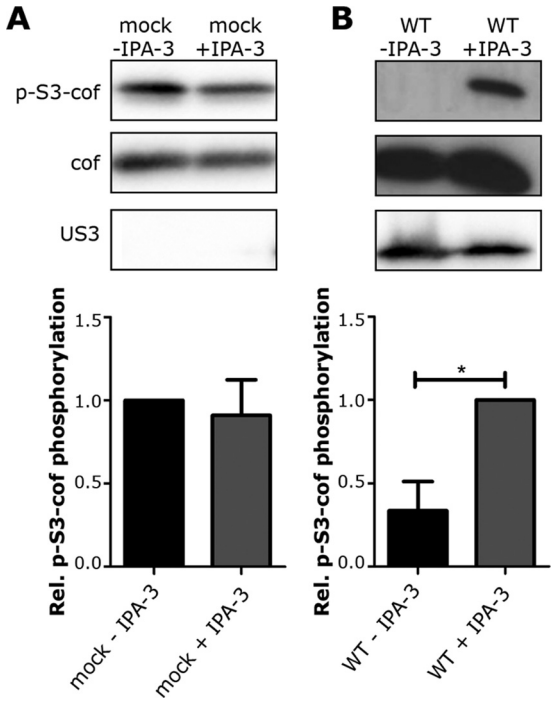

FIG 4 Group I PAKs are involved in US3-mediated suppression of S3 cofilin phosphorylation. (A and B) ST cells treated with or without 33 μM group I PAK inhibitor IPA-3 were either mock inoculated (A) or inoculated with WT PRV (B). At 6 hpi, total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, and US3. Values were normalized to mock (A) or to PRV and IPA-3 (B). The graphs represent the means + standard errors of the means from three independent experiments, with * indicating P values of <0.05.

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